



National Library
of Canada

Bibliothèque nationale
du Canada

Canadian Theses Service Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.



National Library
of Canada

Bibliothèque nationale
du Canada

Canadian Theses Service Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-56399-0

Canada

INTERACTION BETWEEN PROTEASE INHIBITORS AND PROTEASES

OF EUROPEAN CORN BORER LARVAE

***OSTRINIA NUBILALIS* HÜBNER
(LEPIDOPTERA: PYRALIDAE)**

ANGELA M. LAROCQUE

**Thesis submitted to the School of Graduate Studies and Research
in partial fulfillment of the requirements for the M.Sc.
degree in Biology**

University of Ottawa



Angela M. Larocque, Ottawa, Canada, 1989



UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ACKNOWLEDGEMENTS

Much thanks to my supervisor, Dr. J.G. Houseman, for suggesting this project and for providing assistance and continual support throughout the course of my study. This research was supported by the Ontario Ministry of Agriculture and Food and by the Natural Sciences and Engineering Research Council of Canada operating grants awarded to Drs. J.G. Houseman and B.J.R. Philogene. Thanks to B. Conilh de Beyssac for translation of the abstract.

ABSTRACT

The effects of ingested protease inhibitors on proteolytic activity and development of European corn borer larvae, *Ostrinia nubilalis* Hubner (Lepidoptera: Pyralidae) were examined. Inhibitors were chosen from the insects preferred host corn, soybeans and from a source not normally encountered, chicken egg white. Effects on proteolytic activity were examined both in a short term and long term exposure study. Enzyme assays used had been previously optimized for *O. nubilalis* high and low alkaline trypsin, chymotrypsin, aminopeptidase and total proteolysis.

When insects ingested a diet containing 1% (W/V) soybean trypsin inhibitor either in a short term or long term exposure study, larval weight and chymotryptic activity were significantly lower while protein content of the gut was significantly higher. The average number of days to reach the pupal and adult stages were not significantly different from control values.

Larvae that had ingested 2% (W/V) chicken ovomucoid trypsin inhibitor in a short term exposure study had significantly higher chymotryptic activity. In the long term exposure study, high and low alkaline tryptic activities were significantly higher in treated insects. When insects ingested a diet containing 1% (W/V) inhibitor the average number of days to the pupal and adult stages were not significantly different from control values.

Ingestion of 1% (W/V) corn trypsin inhibitor by larvae in a short term exposure study resulted in significantly higher chymotryptic activity, total proteolysis and protein content of the gut. High alkaline tryptic activity was significantly lower in treated insects. There was not a significant difference between treated and control insects for larval weight.

Ingestion of either the soybean trypsin inhibitor, chicken ovomucoid trypsin inhibitor or corn trypsin inhibitor were not detrimental to European corn borer larvae. The practical and theoretical implications of these results in relation to the current defense theory for protease inhibitors are discussed.

RESUME

Les effets inhibiteurs de protéases ingérés sur l'activité protéolytique et sur le développement des larves de la pyrale du maïs, *Ostrinia nubilalis* (Hübner), ont été étudiés. Ces inhibiteurs ont été extraits de l'hôte préféré de l'insecte le maïs, le soya, ainsi que d'une source inconnue, le blanc d'oeuf de poulet. Les effets sur l'activité protéolytique ont été examinés après des expositions de courte et de longue durées. Les titrages ont été optimisés au préalable pour les trypsine très et peu alcalines, la chymotrypsine, l'aminopeptidase d'*Ostrinia nubilalis* et pour la protéolyse totale.

Quand les insectes ont ingéré un repas contenant 1% (P/V) de l'inhibiteur provenant du soya, lors d'une exposition de courte ou de longue durée, le poids larvaire et l'activité chymotryptique étaient significativement plus bas tandis que la teneur en protéine de l'intestin était significativement plus élevée. Les nombres moyens de jours nécessaires pour atteindre les stades pupaire et adulte n'étaient pas significativement différents des valeurs témoins.

Les larves qu'ont ingéré 2% (P/V) de l'inhibiteur de la trypsine ovomucoïde du poulet lors d'une exposition de courte durée, ont montré une activité chymotryptique significativement plus élevée. Lors d'une longue exposition les activités tryptiques très et peu alcalines étaient significativement plus élevées chez les insectes traités. Chez les insectes ayant ingéré un repas contenant 1% (P/V) d'inhibiteur, les nombres moyens de jours nécessaires pour atteindre les stades pupaire et adulte n'étaient pas significativement différents des contrôles.

L'ingestion à court terme de 1% (P/V) d'inhibiteur de la trypsine provenant du maïs par des larves produisait des augmentations significatives de l'activité chymotryp-

tique, de la protéolyse totale et de la teneur en protéine de l'intestin. L'activité tryptique très alcaline était significativement plus basse chez les insectes traités. Il n'y avait pas de différences significatives entre les insectes traités et les témoins quant aux poids larvaires.

L'ingestion des inhibiteurs des trypsines du soya, ovomucoïde du poulet ou du maïs n'était pas nuisible aux larves de la pyrale du maïs. Les implications pratiques et théoriques de ces résultats, en relation avec la théorie courante de défense des inhibiteurs de protéases, sont discutées.

ABBREVIATIONS

1. **BAEE** benzoyl-arginine-ethyl ester
2. **BAPNA** benzoyl-arginine-p-nitroanilide
3. **BTEE** benzoyl-tyrosine-ethyl ester
4. **CTI** corn trypsin inhibitor
5. **LPNA** leucine-p-nitroanilide
6. **OVO** chicken ovomucoid trypsin inhibitor
7. **STI** soybean trypsin inhibitor
8. **TRIS** tris hydroxymethyl aminomethane

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	ii
ABSTRACT	iii
RESUME	v
ABBREVIATIONS	vii
 Chapter I: INTRODUCTION	 1
PROTEASE INHIBITORS: A GENERAL DISCUSSION	1
PLANT PROTEASE INHIBITORS	5
CORN TRYPSIN INHIBITOR	8
KUNTZ SOYBEAN TRYPSIN INHIBITOR	11
ANIMAL PROTEASE INHIBITORS	15
CHICKEN OVOMUCOID TRYPSIN INHIBITOR	16
EFFECT OF INGESTED PROTEASE INHIBITORS	18
INSECT PROTEASES: THE TARGET OF PROTEASE INHIBITORS	18
INHIBITORS INGESTED BY PHYTOPHAGOUS INSECTS	21
INHIBITORS INGESTED BY HAEMATOPHAGOUS INSECTS	24
TEST INSECT CHOSEN TO STUDY THE EFFECTS OF INGESTED INHIBITORS	25
CONCLUSIONS DRAWN FROM THE LITERATURE REVIEW	26
OBJECTIVES AND HYPOTHESIS	26
 Chapter II: MATERIALS AND METHODS	 28
MAINTENANCE OF INSECT COLONY	28
PREPARATION OF GUT SAMPLES AND ENZYME ASSAYS	28
SOURCE OF PROTEASE INHIBITORS	30
EFFECT OF TEST DIETS ON PROTEOLYTIC ACTIVITY	33
EXPERIMENTAL DESIGN	33
TEST DIETS	33
SOYBEAN TRYPSIN INHIBITOR	33
CHICKEN OVOMUCOID TRYPSIN INHIBITOR	34
CORN TRYPSIN INHIBITOR	34
CASEIN	34
DATA ANALYSIS	35
EFFECT OF TEST DIETS ON DEVELOPMENT	35

Chapter III: RESULTS	36
EFFECT OF TEST DIETS ON PROTEOLYTIC ACTIVITY	36
SOYBEAN TRYPSIN INHIBITOR	36
CHICKEN OVOMUCOID TRYPSIN INHIBITOR	42
CORN TRYPSIN INHIBITOR	52
CASEIN	52
EFFECT OF TEST DIETS ON DEVELOPMENT	66
 Chapter IV: DISCUSSION	 67
EFFECT OF TEST DIETS ON DIGESTION AND DEVELOPMENT	67
SOYBEAN TRYPSIN INHIBITOR	68
CHICKEN OVOMUCOID TRYPSIN INHIBITOR	72
CORN TRYPSIN INHIBITOR	74
PROTEASE INHIBITORS AND THE CORN BORER: A SUMMARY	77
SUGGESTED FUTURE WORK	80
GENERAL DISCUSSION	81
 Appendix A	 83
 Appendix B	 88
 Appendix C	 91
 Appendix D	 99
 Appendix E	 104
 BIBLIOGRAPHY	 107

LIST OF TABLES

Table 1.	Short term exposure study with 1% (W/V) soybean trypsin inhibitor.	41
Table 2.	Short term exposure study with 2% (W/V) chicken ovomucoid trypsin inhibitor.	51
Table 3.	Short term exposure study with 1% (W/V) corn trypsin inhibitor.	58
Table 4.	Short term exposure study with 1% (W/V) casein.	59
Table 5.	Short term exposure study with 2% (W/V) casein.	60

LIST OF FIGURES

Figure 1.	Purification of the corn trypsin inhibitor.	31
Figure 2a.	Preliminary study with 0.5, 1 and 1.5% (W/V) soybean trypsin inhibitor: Effect on chymotryptic activity of <i>O. nubilalis</i> larvae.	37
Figure 2b.	Preliminary study with 0.5, 1 and 1.5% (W/V) soybean trypsin inhibitor: Effect on gut protein content of <i>O. nubilalis</i> larvae.	38
Figure 2c.	Preliminary study with 0.5, 1 and 1.5% (W/V) soybean trypsin inhibitor: Effect on larval body weight of <i>O. nubilalis</i> .	38
Figure 2d.	Preliminary study with 0.5, 1 and 1.5% (W/V) soybean trypsin inhibitor: Effect on high alkaline tryptic activity of <i>O. nubilalis</i> larvae.	39
Figure 2e.	Preliminary study with 0.5, 1 and 1.5% (W/V) soybean trypsin inhibitor: Effect on low alkaline tryptic activity of <i>O. nubilalis</i> larvae.	39
Figure 2f.	Preliminary study with 0.5, 1, and 1.5% (W/V) soybean trypsin inhibitor: Effect on aminopeptidase activity of <i>O. nubilalis</i> larvae.	40
Figure 2g.	Preliminary study with 0.5, 1 and 1.5% (W/V) soybean trypsin inhibitor: Effect on total proteolytic activity of <i>O. nubilalis</i> larvae.	40
Figure 3a.	Long term exposure study with 1% (W/V) soybean trypsin inhibitor: Effect on chymotryptic activity of <i>O. nubilalis</i> larvae.	43
Figure 3b.	Long term exposure study with 1% (W/V) soybean trypsin inhibitor: Effect on aminopeptidase of <i>O. nubilalis</i> larvae.	44
Figure 3c.	Long term exposure study with 1% (W/V) soybean trypsin inhibitor: Effect on gut protein content of <i>O. nubilalis</i> larvae.	44
Figure 3d.	Long term exposure study with 1% (W/V) soybean trypsin inhibitor: Effect on larval body weight of <i>O. nubilalis</i> .	45
Figure 3e.	Long term exposure study with 1% (W/V) soybean trypsin inhibitor: Effect on high alkaline tryptic activity of <i>O. nubilalis</i> larvae.	45

Figure 3f.	Long term exposure study with 1% (W/V) soybean trypsin inhibitor: Effect on low alkaline tryptic activity of <i>O. nubilalis</i> larvae.	46
Figure 3g.	Long term exposure study with 1% (W/V) soybean trypsin inhibitor: Effect on total proteolytic activity of <i>O. nubilalis</i> larvae.	46
Figure 4a.	Preliminary study with 0.5, 1 and 1.5% (W/V) chicken ovomucoid trypsin inhibitor: Effect on high alkaline tryptic activity of <i>O. nubilalis</i> larvae.	47
Figure 4b.	Preliminary study with 0.5, 1 and 1.5% (W/V) chicken ovomucoid inhibitor: Effect on low alkaline tryptic activity of <i>O. nubilalis</i> larvae.	48
Figure 4c.	Preliminary study with 0.5, 1 and 1.5% (W/V) chicken ovomucoid trypsin inhibitor: Effect on total proteolytic activity of <i>O. nubilalis</i> larvae.	48
Figure 4d.	Preliminary study with 0.5, 1 and 1.5% (W/V) chicken ovomucoid trypsin inhibitor: Effect on larval body weight of <i>O. nubilalis</i> .	49
Figure 4e.	Preliminary study with 0.5, 1 and 1.5% (W/V) chicken ovomucoid trypsin inhibitor: Effect on aminopeptidase activity of <i>O. nubilalis</i> larvae.	49
Figure 4f.	Preliminary study with 0.5, 1 and 1.5% (W/V) chicken ovomucoid trypsin inhibitor: Effect on chymotryptic activity of <i>O. nubilalis</i> larvae.	50
Figure 4g.	Preliminary study with 0.5, 1 and 1.5% (W/V) chicken ovomucoid trypsin inhibitor: Effect on gut protein content of <i>O. nubilalis</i> larvae.	50
Figure 5a.	Long term exposure study with 2% (W/V) chicken ovomucoid trypsin inhibitor: Effect on high alkaline tryptic activity of <i>O. nubilalis</i> larvae.	53
Figure 5b.	Long term exposure study with 2% (W/V) chicken ovomucoid trypsin inhibitor: Effect on low alkaline tryptic activity of <i>O. nubilalis</i> larvae.	54

Figure 5c.	Long term exposure study with 2% (W/V) chicken ovomucoid trypsin inhibitor: Effect on chymotryptic activity of <i>O. nubilalis</i> larvae.	54
Figure 5d.	Long term exposure study with 2% (W/V) chicken ovomucoid trypsin inhibitor: Effect on chymotryptic activity of <i>O. nubilalis</i> larvae.	55
Figure 5e.	Long term exposure study with 2% (W/V) chicken ovomucoid trypsin inhibitor: Effect on gut protein content of <i>O. nubilalis</i> larvae.	55
Figure 5f.	Long term exposure study with 2% (W/V) chicken ovomucoid trypsin inhibitor: Effect on total proteolytic activity of <i>O. nubilalis</i> larvae.	56
Figure 5g.	Long term exposure study with 2% (W/V) chicken ovomucoid trypsin inhibitor: Effect on larval body weight of <i>O. nubilalis</i> .	56
Figure 6.	Molecular weight determination of the corn trypsin inhibitor.	57
Figure 7a.	Long term exposure study with 2% (W/V) casein: Effect on larval body weight of <i>O. nubilalis</i> .	62
Figure 7b.	Long term exposure study with 2% (W/V) casein : Effect on high alkaline tryptic activity of <i>O. nubilalis</i> larvae.	63
Figure 7c.	Long term exposure study with 2% (W/V) casein: Effect on low alkaline tryptic activity of <i>O. nubilalis</i> larvae.	63
Figure 7d.	Long term exposure study with 2% (W/V) casein: Effect on chymotryptic activity of <i>O. nubilalis</i> larvae.	64
Figure 7e.	Long term exposure study with 2% (W/V) casein: Effect on aminopeptidase activity of <i>O. nubilalis</i> larvae.	64
Figure 7f.	Long term exposure study with 2% (W/V) casein: Effect on total proteolytic activity of <i>O. nubilalis</i> larvae.	65
Figure 7g.	Long term exposure study with 2% (W/V) casein: Effect on gut protein content of <i>O. nubilalis</i> larvae.	65

Chapter I
INTRODUCTION

1.1 PROTEASE INHIBITORS: A GENERAL DISCUSSION

Ryan and Walker-Simmons (1981) define the role of proteolytic enzymes "as part of an overall process of protein turnover". The nomenclature used here for proteolytic enzymes is that recommended by the nomenclature committee of the International Union of Biochemistry (1984). The term protease is used to describe all enzymes that break peptide bonds. Proteases are divided into proteinases, formerly called endopeptidases and peptidases, formerly called exopeptidases. Proteinases cleave peptide bonds in the central region of the polypeptide chain while peptidases act at the N- or C- terminal positions of the polypeptide chain.

Proteinases are classified according to the catalytic mechanism of their active centers. Serine proteinases have a serine and histidine in their active center and a pH optimum of 7-9, metalloproteinases contain a metal ion as an essential part of their structure and have a pH optimum of 7-9, cysteine proteinases have a cysteine in their active center and a pH optimum of 3-7, and aspartic proteinases have a pH optimum below 5 and an acidic residue is involved in the catalytic process. Specificity is used only to identify individual enzymes within these 4 groups. For example, trypsin prefers to cleave a peptide bond adjacent to an arginyl or lysyl residue while chymotrypsin prefers to cleave a peptide bond adjacent to hydrophobic amino acid residues.

Peptidases are divided into 5 groups according to specificity which include those which hydrolyze single amino acids from the N-terminus of the polypeptide chain, those hydrolyzing single residues from the C-terminus, those specific for dipeptide substrates

and those splitting off dipeptide units either from the N-terminus or the C-terminus. Those which hydrolyze single residues from the C-terminus are subdivided into 3 classes which include the serine carboxypeptidases, metallo-carboxypeptidases and the cysteine carboxypeptidases.

Both synthetic and whole protein substrates are used to measure proteolytic activity. The structure of a synthetic substrate is based upon the specificity of a protease towards a protein substrate. For example, substrates for trypsin can be described by the formula R-ARG-X and R-LYS-X where R could be acetyl, benzoyl, carbobenzyloxy or tosyl residue and the leaving group X can as an example be an ethyl or methylester in addition to a nitroanilide, naphthylamine, thiobenzyl alcohol or methylcoumarin group. Alternatively, total proteolytic activity can be measured using whole protein substrates such as casein or haemoglobin. Peptide bond hydrolysis can be determined by measuring the decrease of the intact protein used as a substrate, the increase of amino or carboxylic groups or the increase of defined peptide products.

Laskowski and Sealock (1971) define a protein proteinase inhibitor as "a protein which may associate reversibly with one or more proteinases to form complexes of discrete stoichiometry in which all the catalytic functions of the proteinases are competitively inhibited". A competitive inhibitor "competes with the substrate for binding to the active site but once bound cannot be transformed by the enzyme but can be reversed or relieved by increasing the substrate concentration" (Lehninger 1982). The mechanism of interaction between protease inhibitors and proteases is based on the inhibition of serine proteinases and is referred to as the "standard mechanism" (Laskowski and Kato 1980). On the surface of each inhibitor molecule there is at least one (more in multihead inhibitors) peptide bond encompassed in a disulfide loop called the reactive site. The reactive site specifically interacts with the active site of the related enzyme. The amino acid at the reactive site (P1) on the inhibitor determines specific-

ty of the interaction. If P1 is LYS or ARG the inhibitor is specific for trypsin. If P1 is TYR, PHE, TRP, LEU, or MET the inhibitor is specific for chymotrypsin. If P1 is ALA or SER the inhibitor is specific for elastase-like enzymes. The equilibrium constant for the association between enzyme and inhibitor is extremely high (10^7 - $10^{13}M^{-1}$) and hydrolysis of the inhibitor is extremely slow at neutral pH. Hydrolysis of the inhibitory reactive sites does not proceed to virtual completion meaning that at neutral pH the equilibrium constant between modified inhibitor (reactive site peptide bond hydrolyzed) and virgin inhibitor (reactive site peptide bond intact) is near unity. Both virgin and modified forms of the inhibitor are equally strong inhibitors of the cognate enzyme; however, the rate of complex formation from modified inhibitor and the enzyme is much lower. The complex between inhibitor and enzyme is in the form of a partial tetrahedron resulting from the attraction between the carbonyl oxygen of the inhibitor's reactive site peptide bond and the oxyanion binding site at the reactive site of the enzyme.

One of the most commonly used methods in the isolation and purification of protease inhibitors is column chromatography. A solution containing a mixture of proteins is passed through a column containing a porous solid matrix (Alberts *et al.* 1983). Matrices can separate proteins by charge (ion-exchange chromatography), size (gel filtration), or by the ability to bind a particular substance on the matrix (affinity chromatography). Ion-exchange columns are packed with tiny beads that carry either a positive or a negative charge. Fractionation is according to the arrangement of charge on the surface of the protein. Gel filtration columns are packed with small porous beads that only small molecules can enter so that large molecules must move between the beads. Thus large molecules move much more quickly through the column and emerge first. Separation using an affinity column is based on binding interactions between a protein and a substance coupled to an inert matrix, as an example, polysaccharide

beads. For example, an enzyme specific for a particular inhibitor can be coupled to a matrix that will almost exclusively bind only that particular inhibitor. The inhibitor can then be eluted from the column by addition of a denaturing agent. Due to its high specificity affinity chromatography yields a higher purification than either ion-exchange or gel filtration chromatography. Protease inhibitory activity by a specific inhibitor is commonly quantified by measuring the reduction in the activity of an inhibited protease towards a synthetic substrate.

The number of well characterized inhibitors for the serine proteinases is much greater than for those inhibitors of the metallo, cysteine, or aspartic proteinases and peptidases (Laskowski and Kato 1980). Serine proteinase inhibitors are grouped into units called families (Laskowski and Kato 1980). For each family at least one amino acid sequence is determined ; the reactive site is assigned and the standard mechanism for inhibition is obeyed. Members of a particular family show extensive sequence homology. The molecular weight range for protease inhibitors is 5,000 - 60,000 daltons, but most have a molecular weight of less than 20,000 daltons (Laskowski and Sealock 1971). Most contain cysteine in disulfide linkage, proline and no tryptophan (Laskowski and Sealock 1971). Protease inhibitors are very stable proteins and are resistant to denaturing conditions (Laskowski and Sealock 1971). Many animal protease inhibitors are glycoproteins (Laskowski *et al.* 1978); however, no plant protease inhibitor has yet been identified as a glycoprotein (Ryan 1981). Gene duplication and elongation has occurred in some cases resulting in inhibitors with two halves, each with an active site. Such inhibitors are referred to as being "double-headed". Double-headed inhibitors are found in some plant inhibitors (Ryan 1981) but several animal protease inhibitors are double and triple headed (Laskowski *et al.* 1978).

1.1.1 PLANT PROTEASE INHIBITORS

Within the plant kingdom there are over 90 species of food plants that are known to contain protease inhibitors (Liener and Kakade 1980). Despite their extensive distribution, plant protease inhibitors have been most intensively studied in the Leguminosae, Graminae, and Solanaceae.

Inhibitors are mostly located in storage organs such as seeds or tubers. For example, seeds of the Leguminosae contain inhibitors of trypsin and chymotrypsin (Birk 1976, Baumgartner and Chrispeels 1976, Warsy *et al.* 1974 and Smirnov *et al.* 1979). Potato tubers contain 6 different protease inhibitors (Ryan and Hass 1981). Mikola and Kirsi (1972) have shown that several different inhibitors are present in high concentrations (5-10% of water soluble protein) in the seeds of barley, wheat, oats and rye.

Green and Ryan (1972) were the first to demonstrate that tomato or potato leaf tissue damaged by insects or by mechanical means causes induction of protease inhibitors. More recently the wound induction of trypsin inhibitors has been demonstrated in alfalfa leaves (Brown *et al.* 1985) and soybean leaves (Kraemer *et al.* 1987).

The intracellular location of only a few protease inhibitors is known. For both mung bean cotyledons (Baumgartner and Chrispeels 1976) and pea seeds (Hobday *et al.* 1973), inhibitors are located in the cytosol. Walker-Simmons and Ryan (1977) found that inhibitors are located in vacuoles of leaf cells from wounded tomato leaves, and electron microscopy shows that these inhibitors are in the form of membraneless protein bodies. Similarly, Hollander-Czytko *et al.* (1985) found that wound-induced carboxypeptidase inhibitor in potato leaves is localized in the central vacuoles of the cells. In general, the majority of plant protease inhibitors have been screened for using bovine trypsin and chymotrypsin. Ryan (1981) reports on over 100 serine proteinase inhibitors of plant origin.

In contrast to the serine proteinases, few inhibitors of the other 3 groups of proteinases and of the peptidases have been isolated to date. Inhibitors of cysteine proteinases are found in pineapple stems (Reddy *et al.* 1975), potato tubers (Hoff *et al.* 1972), corn (Abe *et al.* 1980), *Bauhinia* seeds (Goldstein *et al.* 1973) and mung beans (Baumgartner and Chrispeels 1976). An inhibitor of aspartic proteinases (cathepsin D) is present in potatoes (Keilova and Tomasek 1976). Inhibitors of metallo-carboxypeptidases are found in extracts of potato tubers (Hass *et al.* 1981) and in extracts of tomato fruit (Hass and Ryan 1980).

Regulation of the wound-induced accumulation of protease inhibitors has been most extensively studied in Solanaceae plants, particularly the potato and the tomato. Wounded leaf tissue releases a hormone-like substance, protease inhibitor inducing factor (PIIF) that is translocated from the wound to the other tissues of the plant where it induces a light-dependent accumulation of inhibitor (Ryan 1974). Protease inhibitor inducer factor activity is not restricted to the Solanaceae family. McFarland and Ryan (1974) report that 37 species representing 20 families from 4 major plant subdivisions exhibit PIIF-like activities. Considerable attention has been devoted to the chemical nature of PIIF and to the construction of a model which explains how it functions.

In 1978 Ryan tentatively identified PIIF activity as due to a pectin-like molecule. Ryan *et al.* (1981) tested 3 polysaccharides from sycamore cells for PIIF activity and found that rhamnogalacturonan is effective at inducing inhibitor accumulation in excised tomato leaves. Both PIIF and rhamnogalacturonan have galacturonic acid as the major glycosyl residue. Bishop *et al.* (1984), after purifying and characterizing a series of galacturonic acid oligomers derived from tomato leaf PIIF by enzymatic and chemical hydrolyses, found that the 4- α -D-galacturonisyl-D-galacturonic acid moiety is sufficient for PIIF activity. They suggest that small oligosaccharides are the systemic signals that activate inhibitor genes whereas fragments of degree of polymerization (DP) equal to 10 or longer are signals of localized responses.

A model for wound induction of protease inhibitors is presented by Ryan (1978) and Ryan *et al.* (1986). The proposed scheme for the production and transport of cell wall fragments during attack by insects is as follows. The chewing action of insects releases plant endopolygalacturonases from compartments within plant cells. These enzymes generate cell wall fragments which are transported throughout the plant where the message is received by leaf cells and relayed to the nucleus. New mRNA is produced and is translated to make inhibitor proteins which are sequestered in the central vacuole of the cell. Zuroske *et al.* (1980) suggest that PIIF moves via the phloem. Baydoun and Fry (1985) tested the hypothesis that pectic substances are mobile within the plant by using infiltration of radioactive pectic fragments into wounds on tomato leaves. They found that less than 1ng of pectic polysaccharide is exported from leaves treated with 1-10 μ g of pectic fragments and that pectic oligosaccharides of DP 6-14 are also not translocated. They conclude that the long distance wound hormone is not pectic in nature and suggest that the pectic polysaccharides act locally at their site of production (injured tissue) where they induce the release of a second long distance messenger.

Three roles have been proposed for plant protease inhibitors: regulation of endogenous plant proteases, a storage depot for protein and protection for the plant against invading insect pests. As potential regulators of endogenous plant proteases, inhibitors are thought to prevent degradation of storage protein during seed maturation. In cowpeas (Royer *et al.* 1974), rice (Horiguchi and Kitagishi 1971) and lettuce (Shain and Mayer 1968) protease inhibitors are thought to regulate endogenous proteases before germination. Protease inhibitors which occur particularly in storage tissues, such as seeds and tubers, are thought to function as storage depots of protein which are immune to digestion until required during germination or sprouting. A storage role is proposed for a protease inhibitor found in potato tubers where its level in the tubers

declines during sprouting while there is a corresponding accumulation in young growing sprouts. This continues until the mature plants begin to set tubers, at which time the inhibitor disappears from the vegetative tissue and starts to build up in new tubers (Ryan 1973). The third role for plant protease inhibitors is one of defense against invading insects. Several reviews deal with this role and supporting evidence (Ryan 1973, 1981, Richardson 1977, 1980-81).

The remainder of this section will be devoted to the corn trypsin inhibitor and the Kunitz soybean trypsin inhibitor. For each inhibitor a detailed description of its physiochemical properties and its role in the plant, excluding defense (see section 1.2.2.), will be discussed.

1.1.1.1 CORN TRYPSIN INHIBITOR

Melville and Scandalios (1972) assayed whole corn seedlings and various tissues which included the scutella, root, leaf, pericarp, endosperm, and embryo for trypsin inhibitory activity. All tissues tested had the capacity to inhibit trypsin; however, inhibitory activity is highest in the starchy endosperm of the dry seed and very low in scutellar extracts. Prior to germination trypsin inhibitory activity declines in the seedlings.

Two methods have been employed in the purification of trypsin inhibitor from corn seeds. One approach utilizes chromatography on immobilized trypsin and is referred to as a trypsin affinity column (Hochstrasser *et al.* 1967, 1970, Swartz *et al.* 1977, Johnson *et al.* 1980, Mahoney *et al.* 1984). When a crude extract is passed through this column the inhibitor selectively binds to trypsin and can be eluted by addition of a denaturing agent. An alternative method is chromatography on immobilized antibodies against the inhibitor, called the immunoabsorbent technique (Corfman and Reeck 1982). Here, when the crude corn extract is passed through the column, the inhibitor binds to the antibodies and can be eluted by addition of a denaturing agent. One drawback of affinity chromatography is that the method yields a mixture of roughly

half virgin and half modified corn trypsin inhibitor (Swartz *et al.* 1977) while isolation using the immunoabsorbent technique yields almost exclusively virgin inhibitor (Corfman and Reeck 1982).

Two primary structures have been proposed for the corn trypsin inhibitor. Hochstrasser *et al.* (1967), using affinity chromatography, isolated a trypsin inhibitor from the seeds of an unspecified strain of corn and report that the intact inhibitor has a molecular weight of approximately 19,000 daltons. The amino acid sequence consists of 65 residues and lacks tryptophan (Hochstrasser *et al.* 1970). Since the total number of amino acid residues represents only 1/3 of the total amino acids indicated by the molecular weight (Hochstrasser *et al.* 1967), Hochstrasser *et al.* (1970) state that the native trypsin inhibitor from these corn seeds is a polymer of 3 subunits each of which consists of 65 amino acid residues. Swartz *et al.* (1977) isolated a trypsin inhibitor from opaque-2 corn seeds using affinity chromatography and report a primary structure different from Hochstrasser *et al.* (1970). Two forms of the inhibitor, virgin and modified, were eluted from the trypsin affinity column and then separated using ion-exchange (DEAE-cellulose) chromatography. The first form is the single chain virgin inhibitor. The second form consists of 2 polypeptide chains and is thought to have been produced by the single chain inhibitor's exposure to trypsin on the affinity column. Both forms of the inhibitor are identical in amino acid composition and molecular weight. The molecular weight of the trypsin inhibitor from opaque-2 corn is reported to be 12,500 daltons by Swartz *et al.* (1977) and 12,028 daltons by Mahoney *et al.* (1984). One unusual feature of the amino acid composition of the trypsin inhibitor isolated from opaque-2 corn seeds is that it contains tryptophan residues. Swartz *et al.* (1977) reported the presence of 4 tryptophan residues while Mahoney *et al.* (1984) included 3 tryptophan residues in the amino acid composition. Tryptophan is usually not present in the amino acid composition of plant protease inhibitors that have been

sequenced (Mahoney *et al.* 1984). The secondary structure of the trypsin inhibitor isolated from opaque-2 corn, based on a theoretical prediction, consists of 40% α -helix and 20% β -pleated sheet (Mahoney *et al.* 1984). The authors point out that the higher helix content differs from the secondary structure of those protease inhibitors whose structures have been elucidated by X-ray crystallography, where the content of β -pleated sheet is greater than the content of α -helix. The presence of a second trypsin inhibitor is suggested by the work of Richardson *et al.* (1987). They describe the sequence of a maize protein which is a potent inhibitor of trypsin and of the α -amylase from *Tribolium castaneum* beetles. The authors point out that this protein is not similar in sequence to any of the other enzyme inhibitors but is highly similar to the sweet protein thaumatin and to a protein induced in tobacco following infestation with the tobacco mosaic virus. It consists of 206 amino acids and has a relative molecular mass of 22,077 daltons. Gel filtration and reverse phase HPLC were used in the extraction process.

A possible explanation for the difference in primary structure between the work of Hochstrasser *et al.* (1967,1970) and Swartz *et al.* (1977) is the source material. Opaque-2 corn seeds exhibit about two times the inhibitory activity of normal corn seeds (Halim *et al.* 1973). To test this idea trypsin inhibitors were isolated from 3 different sources of corn which included floury-2 corn, dent corn, and popcorn (Johnson *et al.* 1980) and from *Zea mexicana* (Corfman and Reeck 1982). The amino acid composition for each inhibitor was determined and each was allowed to react immunologically with antibodies against inhibitor from opaque-2 corn. The inhibitors from the 3 different sources of corn and from *Z. mexicana*, compared to the inhibitor from opaque-2 corn, are similar in amino acid composition and in immunological properties. Source material is therefore not considered to be an explanation for the differences seen in the primary structure. The amino acid composition proposed by Hochstrasser

et al. (1970) differs in its lack of tryptophan. Swartz *et al.* (1977) state that the difference in molecular weight between the trypsin inhibitor from opaque-2 corn and that proposed by Hochstrasser *et al.* (1967) might be due to the apparent tendency of the inhibitor to self-associate.

Several physiochemical properties of the corn trypsin inhibitor have been determined. Swartz *et al.* (1977) report that it inhibits trypsin in a 1:1 stoichiometry but it does not inhibit chymotrypsin. However, Chen and Mitchell (1973) report that the inhibitor isolated from sweet corn is moderately active against chymotrypsin. In its interaction with trypsin, the corn trypsin inhibitor obeys the "standard mechanism" for serine proteinases (Mahoney *et al.* 1984) and the reactive site peptide bond is ARG³⁶-LEU³⁷. When heated at 100°C for 6 hours the corn inhibitor retained 82% of its inhibitory activity which illustrates its heat stability (Chen and Mitchell 1973). The inhibitor has no significant carbohydrate content and no free sulfhydryl groups (Swartz *et al.* 1977).

The precise physiological role of the corn trypsin inhibitor is unknown. A regulatory role for endogenous proteases is unlikely since the corn trypsin inhibitor does not inhibit an endogenous protease of corn (Melville and Scandalios 1972). It is possible that they serve as storage for protein to be used by the plant during germination, since inhibitor activity declines prior to germination (Melville and Scandalios 1972).

1.1.1.2 KUNITZ SOYBEAN TRYPSIN INHIBITOR

Inhibitors are concentrated in the seeds of soybeans where they account for approximately 6% of total seed protein (Rackis and Anderson 1964). Low levels of trypsin inhibitory activity are found in pre-bloom foliage indicating that distribution is not restricted only to the seed (Kraemer *et al.* 1987). Intracellularly, Kunitz inhibitory activity is associated with the soybean storage proteins which are located in protein bodies of the cotyledons (Koshiyama *et al.* 1981).

Protease inhibitors that have been isolated from soybeans fall into two groups (Liener and Kakade 1980). Inhibitors with a molecular weight of 20,000-25,000 daltons, relatively few disulfide bonds and a specificity directed primarily toward trypsin are referred to as Kunitz inhibitors. Those with a molecular weight of 6,000-10,000 daltons, a high proportion of disulfide bonds and a capacity to inhibit trypsin and chymotrypsin at independent binding sites are referred to as Bowman-Birk inhibitors. There are isoinhibitors in each of these categories that differ slightly from the Kunitz and Bowman-Birk inhibitors (Liener and Kakade 1980).

The procedure used to isolate and purify the Kunitz inhibitor varies. One of the most commonly used methods is that developed by Yamamoto and Ikenaka (1967). This method consists of 4 steps which include in chronological order extraction, salting-out, ion-exchange chromatography on DEAE-cellulose and zone electrophoresis.

The primary structure of the Kunitz inhibitor is based on the work of Koide and Ikenaka (1973a), Koide *et al.* (1973) and Koide and Ikenaka (1973b). It has a molecular weight of 20,100 daltons and consists of 181 amino acid residues (Koide and Ikenaka 1973a). The complete amino acid sequence was reported by Koide and Ikenaka (1973b) and was later amended by Kim *et al.* (1985).

The reactive site peptide bond for the Kunitz inhibitor's interaction with trypsin is located at ARG⁶³-ILE⁶⁴ (Koide and Ikenaka 1973a). It is the nature of the amino acid located at position 63 that determines the specificity of the Kunitz inhibitor (Liener and Kakade 1980). If Arg⁶³ is replaced by tryptophan, inhibitor specificity is changed towards chymotrypsin (Leary and Laskowski 1973) and if ARG⁶³ is substituted with lysine specificity towards trypsin is not changed (Sealock and Laskowski 1969). Replacement of the amino acid at position 64 with alanine, leucine, or glycine does not change specificity toward trypsin (Kowalski and Laskowski 1976a) but addition of an amino acid to the N-terminal of ILE⁶⁴ produces an inactive derivative (Kowalski and

Laskowski 1976b). These results indicate that the distance separating ARG⁶³ from ILE⁶⁴ is important.

De Vonis Bidlingmeyer *et al.* (1972) have shown that the reactive site peptide bond located at ARG⁶³-ILE⁶⁴ is also hydrolyzed by chymotrypsin. According to the authors, this shows the importance of the proper reactive-site conformation over the type of residue contributing the carbonyl group to the reactive-site peptide bond. Quast and Steffen (1975) found a second binding site for chymotrypsin by blockage of the trypsin binding site, and the kinetics of complex formation at either binding site have been determined by Bosterling and Quast (1981). The inhibitor binds two molecules of chymotrypsin with comparable affinity but the contributions of the two different sites are hard to separate (Bosterling and Quast 1981). Location of the second binding site on the inhibitor molecule is not yet known but is thought to be in the region around the MET⁸⁴-LEU bond (Bosterling and Quast 1981). Quast and Steffen (1975) state that the Kunitz inhibitor can be considered as double-headed, but it is unusual in that most double-headed inhibitors arise by partial gene duplication or elongation.

There are 3 genetic variants of the Kunitz inhibitor which can be distinguished after polyacrylamide gel electrophoresis, and these are identified as Ti^a, Ti^b and Ti^c (Orf and Hymowitz 1979). These electrophoretically distinct forms of the Kunitz inhibitor are inherited at codominant multiple alleles (Hymowitz and Hadley 1972). The Ti^a form is the most common and is present in 88.8% of 3,039 tested soybean accessions while Ti^b and Ti^c alleles are found in 10.9 and 0.3% of the tested accessions respectively (Hymowitz *et al.* 1978). Kim *et al.* (1985) determined the amino acid sequences of the 3 variants and compared the results to the sequence for the soybean trypsin inhibitor determined by Koide and Ikenaka (1973b). All 3 variants are composed of 181 amino acid residues and have the reactive site peptide bond at the same position, ARG⁶³-ILE⁶⁴. The sequences of Ti^a and Ti^c differ only at one position. The other 180 amino acid residues are identical.

Koide and Ikenaka (1973b) predicted that the secondary structure of the Kunitz inhibitor, based on amino acid sequence, would contain little α -helix and be in the form of a random coil. This was later confirmed by X-ray crystallographic data (Blow *et al.* 1974). Kunitz (1947) was the first to characterize the specificities of the soybean trypsin inhibitor. The inhibitor combines with trypsin in a 1:1 stoichiometric fashion but is a weak inhibitor of chymotrypsin (Kunitz 1947). The soybean trypsin inhibitor is capable of inhibiting trypsin from a variety of sources including cow, pig, and salmon (Kassel 1970). In its interaction with trypsin, the soybean trypsin inhibitor follows the "standard mechanism" for serine proteinases (Laskowski and Kato 1980). The structure of the complex between porcine trypsin and the soybean trypsin inhibitor was determined using X-ray crystallography (Sweet *et al.* 1974). Only 12 amino acids out of 181 which make up the inhibitor come into contact with the trypsin molecule (Sweet *et al.* 1974). The complex is in the form of a tetrahedral adduct between the active site serine residue and the carbonyl group of ARG⁶³ (Sweet *et al.* 1974).

Freed and Ryan (1980) characterized the interaction between trypsin and the 3 genetic variants of the soybean trypsin inhibitor (Ti^a, Ti^b, Ti^c) by calculation of association equilibrium constants and hydrolysis equilibrium constants. The Ti^b variant interacts weakly with trypsin based on a low association equilibrium constant value and is hydrolyzed by trypsin at a high rate. The Ti^a and Ti^c variants form stronger associations with trypsin and each is hydrolyzed at a much slower rate than for the Ti^b variant. The Ti^b variant is less active against trypsin based on these results (Freed and Ryan 1980).

The soybean trypsin inhibitor is resistant to thermal and chemical denaturation. Edelhoch and Steiner (1963) found that the structure of the inhibitor is only slightly changed in 9M urea and Wu and Scheraga (1962) found that denaturation does not occur until the temperature exceeds 60°C. Sanderson *et al.* (1982) point out that there

are no known unusual structural features of the inhibitor molecule to explain this stability; however, Liener and Kakade (1980) attribute this stability in part to its secondary structure which is in the form of a random coil. According to Sanderson *et al.* (1982) "thermal denaturation of the Kunitz inhibitor is not a simple two-state process but consists of a low-temperature readily reversible transition followed by a higher-temperature non-readily reversible transition". The thermal stability of the Ti^c variant is significantly lower compared to the Ti^b and Ti^a variants (Sanderson *et al.* 1982).

The precise physiological role for the Kunitz soybean trypsin inhibitor is unknown. Using an immunological assay specific for the Kunitz inhibitor, Freed and Ryan (1978) found that its concentration is decreased by 13% on a dry weight basis during the first 9 days of germination. Similarly Collins and Sanders (1976) and Bates *et al.* (1977) using non-specific enzyme assays found that total protease inhibitor content does not decrease appreciably during germination. Freed and Ryan (1978) point out that the magnitude of the changes which take place in the Kunitz inhibitor are considerably less than those which might be expected for storage protein and conclude that it is improbable that the inhibitor functions as a storage protein. Birk (1968) found that the soybean trypsin inhibitor does not inhibit soybean proteases when assayed using casein. This suggests that a regulatory role for endogenous proteases is unlikely. Kraemer *et al.* (1987) found that infestation of soybean plants by the Mexican bean beetle, *Epilachna varivestis mulsant*, increases trypsin inhibitory activity in foliage from 4-23 fold and is suggestive of a defensive role.

1.1.2 ANIMAL PROTEASE INHIBITORS

The main sources of animal protease inhibitors are the pancreas, colostrum, blood plasma and avian egg whites (Whitaker 1981). Laskowski *et al.* (1978) estimate that a single mammal or bird contains at least 100 different inhibitors. Consequently, only those inhibitors relevant to this work will be discussed in the following section.

1.1.2.1 CHICKEN OVOMUCOID TRYPSIN INHIBITOR

Four protease inhibitors have been characterized from avian egg white: ovomucoid, ovomucoid, cystatin and ovostatin (Nagase *et al.* 1983). Ovomucoid (Rhodes *et al.* 1960) and ovomucoid (Tomimatsu *et al.* 1966) are serine proteinase inhibitors. Cystatin inhibits cysteine proteinases (Barret 1981, Fossum and Whitaker 1968, Keilova and Tomasek 1974) and ovostatin is an inhibitor of collagenase (Nagase *et al.* 1983).

Rhodes *et al.* (1960) isolated ovomucoids from 11 different avian species and examined some of their properties. They divided ovomucoids into 4 classes based on inhibitory activity : one group that primarily inhibits trypsin, a second group which primarily inhibits chymotrypsin, a third group which inhibits equal molar amounts of trypsin and chymotrypsin separately or simultaneously and a fourth group which inhibits twice as much trypsin as chymotrypsin separately or simultaneously.

Ovomucoid accounts for approximately 11% of the total protein present in chicken egg white (Rhodes *et al.* 1960). Frequently the acid precipitation technique developed by Lineweaver and Murray (1947) is used as an initial step in the purification process and ovomucoid is then further purified by the use of ion-exchange chromatography (Stevens and Feeney 1963) or by the use of gel-exclusion chromatography (Kato *et al.* 1987).

Rhodes *et al.* (1960) first reported the amino acid sequence for chicken ovomucoid and it was later confirmed by Kato *et al.* (1987). Its amino acid composition contains no tryptophan, little methionine and large amounts of cysteine (Osuga and Feeney 1968). The molecular weight for the inhibitor is 28,000 daltons (Osuga and Feeney 1968).

It is possible to divide the chicken ovomucoid molecule into 3 intact domains where each domain is a native protein (Kato *et al.* 1987). The reactive site for bovine

trypsin is the ARG⁸⁹-ALA peptide bond in the second domain (Kato *et al.* 1987). Neither the third or first domain is an effective inhibitor of bovine trypsin (Kato *et al.* 1987). However, Nagata and Yoshida (1984) show that the first domain inhibits a trypsin-like enzyme from *Streptomyces erythraeus* at the LYS²⁴-ASP reactive site. The chicken ovomucoid molecule is not known to inhibit serine proteinases with chymotrypsin-like specificity (Kato *et al.* 1987), although Kato *et al.* (1987) refer to a study which shows the third domain of the chicken ovomucoid molecule to be a weak inhibitor of chymotrypsin where the association constant between the enzyme and the inhibitor is low.

In its interaction with trypsin, chicken ovomucoid obeys the "standard mechanism" proposed for serine proteinase inhibitors (Laskowski and Kato 1980). It inhibits bovine (Feeney *et al.* 1969) and porcine (Vithayathil *et al.* 1961) trypsin but not human trypsin (Feeney *et al.* 1969).

Chicken ovomucoid is a glycoprotein where carbohydrate accounts for approximately 25% (W/W) of the inhibitor; however, the role of the carbohydrate moiety is unknown (Tranter and Board 1982). The inhibitor is stable to heating at 80°C at pH < 9, but heating to 100°C at pH 6 or to 80°C at pH 9 results in a rapid loss of inhibitory activity (Stevens and Feeney 1963). It is also resistant to treatment with trichloroacetic acid-acetone and 9M urea at 80°C (Stevens and Feeney 1963). The complex formed between chicken ovomucoid and bovine trypsin is unaffected by addition of 3M urea (Stevens and Feeney 1963).

The physiological role of chicken ovomucoid is unknown. Tranter and Board (1982) suggest that the inhibitor is part of a chemical defense system for the egg against microorganisms; however, there is no direct evidence to support this theory.

1.2 EFFECT OF INGESTED PROTEASE INHIBITORS

Green and Ryan (1972) suggest that inducible protease inhibitors may provide the plant with a defense mechanism against insects. This work sparked interest in the effects of ingested protease inhibitors on insects.

Growth studies and growth studies combined with proteolytic studies are the methods used to study the effects of ingested protease inhibitors. To ascertain effects on growth the inhibitor is added to an artificial diet or the insect is fed on a plant containing the inhibitor(s) in question. Proteolytic activity is measured either by addition of the inhibitor to the protease(s) assay or the inhibitor is added to an artificial diet and guts are later dissected. Before the effects of ingested inhibitors are dealt with, a discussion of insect proteases is essential.

1.2.1 INSECT PROTEASES: THE TARGET OF PROTEASE INHIBITORS

Proteinases and peptidases are used by insects to digest protein, but a large majority of the insect proteinases that have been isolated and characterized to date are serine-like in nature (Applebaum 1985). Since the current work deals with the effects of ingested protease inhibitors on the digestive physiology of a lepidopteran insect, attention will now be focussed on proteases of insects from this order.

The digestive fluid of lepidopteran larvae is highly alkaline and contains proteolytic enzymes which are active at alkaline pH (Pritchett *et al.* 1981). Proteases from lepidopteran larvae have been characterized using whole protein substrates combined with synthetic substrates that are specific for a particular protease, whole protein substrates alone or only synthetic substrates.

Eguchi and Iwamoto (1976) found proteolytic activity in the midgut tissue and digestive fluid from silkworm larvae, *Bombyx mori*. Hydrolysis of casein is optimal at pH 11.2 for proteolytic activity from either source. Using gel filtration chromatography, proteolytic activity in the digestive fluid is separated into 3 proteinases which are

named 6B1, 6B2, and 6B3 (Eguchi and Iwamoto 1982). Each differs in its specificity towards the trypsin substrates BAPNA (benzoyl-arginine-p-nitroanilide) and TAME (tosyl-arginine methyl ester), effect of inhibitors and the influence of Mn^{2+} . Using casein as a substrate, Ahmad *et al.* (1976) found that optimal proteolytic activity is at pH 11.0 in the larval gut of the army worm, *Spodoptera litura*. Activity was resolved into 3 proteinases that were purified by gel filtration and ion-exchange chromatography (Ahmad *et al.* 1980). Optimal activities using casein as substrate are at pH values of 11.0, 10.5 and 9.0 respectively. All 3 proteinases hydrolyzed the trypsin substrates BAPNA and BAEE (benzoyl-arginine-ethyl ester) but not the chymotrypsin substrate BTEE (benzoyl-tyrosine-ethyl ester). The pH optimum for hydrolysis of each synthetic substrate by the 3 proteinases was not determined. Larval midgut homogenates of the corn borer, *Ostrinia nubilalis*, have maximal activity against azocasein at a pH value of 10.0 or higher (Houseman *et al.* 1989). Based on the pH optimum for hydrolysis of synthetic substrates and the effects of calcium or magnesium on hydrolysis, 2 trypsin-like proteinases are identified. The high alkaline trypsin hydrolyses BAPNA maximally at pH values greater than 10, and hydrolysis is increased 30% in the presence of calcium or magnesium. Low alkaline trypsin has maximal activity against BAEE at pH 9.0 and hydrolysis is not increased by the presence of calcium or magnesium. Also present in larval midgut homogenates is a chymotrypsin-like proteinase that hydrolyzes BTEE optimally at pH 7.5-8.0 (Houseman *et al.* 1989) and a N-terminal peptidase that hydrolyzes LPNA (leucine-p-nitroanilide) maximally at pH 7.5 (Houseman and Larocque 1987). Proteolytic activity in the digestive fluid of larvae of *Trichoplusia ni* is optimal against casein at pH 9.8 (Pritchett *et al.* 1981). Both tryptic and chymotryptic activities are present within the digestive fluid as seen by the hydrolysis of TAME and BTEE; however, the pH optimums for hydrolysis of these synthetic substrates were not determined.

Using whole body extracts from larvae of *Galleria mellonella*, two proteinases (P-1, P-2) were separated by ion-exchange and gel filtration chromatography (Hamed and Attias 1987). The optimal pH using azocoll as a general protease substrate is 10.5 for P-1 and 11.2 for P-2. Larval midguts from *Heliothis zea* have optimal proteolytic activity against casein at pH 11.0 (Klocke and Chan 1982).

When larval midgut homogenates of *Erinnyis ello* were centrifuged, both the supernatant and pellet were assayed for proteolytic activity (Santos and Terra 1986). Soluble trypsin (measured from the supernatant) hydrolyzes BAPNA maximally at pH 9.5 and membrane bound trypsin (measured from detergent treated pellets) hydrolyses BAPNA maximally at pH 10.0. Carboxypeptidase A activity was optimal at pH 9.0 against the synthetic substrate ZGlyPhe (A/N-carbobenzoxy-glycyl-L-phenylalanine) and a membrane bound aminopeptidase has optimal activity against LPNA at pH 8.0. Broadway and Duffey (1986a) tested for carboxypeptidase A, carboxypeptidase B, chymotryptic and tryptic activity in larval guts of *H. zea* and *Spodoptera exigua* using the synthetic substrates but the pH optimum of hydrolysis for each case was not determined. Using HPLA (hippuryl DL-phenyllactic acid) and HP (hippuryl phenylalanine) as substrates no carboxypeptidase A activity is detected at pH 7.5 and no carboxypeptidase B activity using HA (hippuryl-arginine) as substrate is detected at pH 7.5. Chymotryptic activity was detected using BTEE at pH 8.1 but was so low that it was difficult to measure accurately. Tryptic activity was measured using TAME at pH 8.1 and is considered to be the primary protease present in the gut. Miller *et al.* (1974) isolated a trypsin-like proteinase from the larval midgut of *Manduca sexta*. Tryptic activity was measured using BAEE at pH 8.0 but it was not stated if this was the pH at which optimal hydrolysis occurs.

In 6 different lepidopteran species optimal hydrolysis of whole protein substrate occurs in the pH range of 9.8-11.2 (Eguchi and Iwamoto 1976, Ahmad *et al.* 1976,

Houseman *et al.* 1989, Pritchett *et al.* 1981, Hamed and Attias 1987, Klocke and Chan 1982). Multiple trypsins are seen in larvae of the silkworm (Eguchi and Iwamoto 1982), army worm (Ahmad *et al.* 1980) and the corn borer (Houseman *et al.* 1989). A high pH optimum for proteolytic activity is an unique feature of lepidopteran larvae. This contrasts with vertebrate serine proteinases where assay conditions are at pH 7.8 for chymotrypsin and pH 8.1 for trypsin (Hummel 1959).

1.2.2 INHIBITORS INGESTED BY PHYTOPHAGOUS INSECTS

Work done using phytophagous insects can be divided by the most commonly studied insect orders, Lepidoptera and Coleoptera.

The effect of ingested protease inhibitors on lepidopteran larvae has been most studied with the beet armyworm, *S. exigua*, a pest of many agricultural crops including soybean, tomato and potato. A statistically significant reduction of larval weight gain is observed when larvae feed on reconstituted leaf material from tomato plants in which the induction of inhibitors is initiated (Broadway *et al.* 1986). A mode of action for protease inhibitors is proposed in an elaborate study by Broadway and Duffey (1986b) where the effects of the soybean trypsin inhibitor and potato inhibitor II (trypsin and chymotrypsin) were examined on *S. exigua* larval growth, *in vitro* tryptic activity, *in vivo* proteinase activity (trypsin and chymotrypsin) and *in vivo* digestion. Using concentrations of inhibitors comparable to those found in tomato plants (0-0.18% wet weight), a significant reduction of larval growth is seen, but neither inhibitor has an effect on the *in vivo* digestion of protein. Tryptic activity is reduced *in vitro* but when either inhibitor is chronically ingested there is a significant elevation of tryptic activity. The authors conclude that the mode of action for protease inhibitors "is to cause the pernicious hyperproduction of trypsin and this coupled with insufficient dietary availability of sulphur containing amino acids (methionine) needed for enzyme synthesis results in inhibition of growth". Subsequently, the authors found that protein quality

significantly changes the toxicity of the soybean trypsin inhibitor towards larvae of *S. exigua* where the inhibitor is less toxic when a nutritious protein source is ingested (Broadway and Duffey 1988).

The remainder of the studies with lepidopteran larvae examine the effects of ingested trypsin inhibitors on growth using inhibitors in or out of the insect's normal plant host range. Shukle and Murdock (1983) found that when larvae of *M. sexta*, whose preferred host is tobacco, consumes a diet containing 5% soybean trypsin inhibitor a significant reduction in body weight of treated larvae compared to control is seen. When *O. nubilalis* larvae, whose preferred host is corn, consume a diet containing 3% soybean trypsin inhibitor weight is reduced and pupation is delayed to 30 days compared to 24 for control (Steffens *et al.* 1978). However, when larvae ingest a diet containing 3% corn trypsin inhibitor no effect is seen on weight and pupation takes 23 days. Insect survival on and damage to tobacco plants genetically transformed with the cowpea trypsin inhibitor gene, by larvae of *Heliothis virescens* (tobacco is its preferred host), is decreased compared to controls (Hilder *et al.* 1987). These three studies point out that inhibitors within the host range of an insect appear not to affect growth while those inhibitors outside the host range have a negative effect upon growth.

The bruchid beetle (a pest of cowpeas), *Callosobruchus maculatus*, is the most studied insect within the Coleoptera with respect to the effects of ingested protease inhibitors. Gatehouse and Boulter (1983) found the cowpea trypsin inhibitor to be an effective antimetabolite against *C. maculatus* when added to either a susceptible variety of cowpeas or chickpea, where adult emergence takes approximately 20% longer. The soybean trypsin inhibitor (at concentrations of 2, 5, or 10%) and limabean trypsin inhibitor (at concentrations of 5 or 10%) were ineffective. Gatehouse *et al.* (1985) attempt to relate these results to proteolytic activity within larval gut homogen-

ates. The antimetabolic effects observed cannot be attributed to the cowpea trypsin inhibitor since larval gut homogenates do not hydrolyze a synthetic trypsin substrate. Proteolytic activity has a pH optimum of 5.4, is not inhibited by synthetic or natural (soybean trypsin inhibitor, lima bean trypsin inhibitor) serine proteinase inhibitors, but is inhibited by reagents reactive against thiol groups (Gatehouse *et al.* 1985). Papain inhibitors partially purified from cowpea seeds are effective inhibitors of total proteolysis and the authors state that the presence of cysteine inhibitory activity in the cowpea trypsin inhibitor may account for its inhibitory activity towards larval proteases (Gatehouse *et al.* 1985). These 2 studies point out the importance of doing growth studies in combination with proteolytic studies, otherwise effects observed may be attributed to the incorrect factor.

Baker *et al.* (1984) examined the effects of extracts from 5 sweet potato cultivars that had a 20-fold difference in trypsin inhibitor concentration on *in vitro* proteolytic activity of sweet potato larvae *Cylas formicarius elegantulus*. The cultivar with the highest trypsin inhibitory activity against *C. f. elegantulus* gut homogenates is more susceptible to attack by *C. f. elegantulus* in field tests (Mullen *et al.* 1980). This study points out that effects observed with protease inhibitors in *in vitro* protease assays may not reflect what occurs *in vivo*.

Benz *et al.* (1985) examined the implications of artificial wounds on the leaves of *Solanum tuberosum* for larval development of 2 beetles adapted to feed on Solanaceous plants, *Leptinotarsa decemlineata* and *L. haldemani*. Experiments with either beetle on control or wounded potato plants showed that development of both insects was only slightly delayed on wounded plants and no difference in mortality or pupal weights was observed. This study does not uphold the Green and Ryan (1972) theory that inducible inhibitors provide protection to plants against insect pests.

Early within the literature a soybean fraction is described that inhibits larval growth and digestive processes *in vitro* of the flour beetles *Tribolium confusum* (Lipke and Fraenkel 1954) and *T. castaneum* (Birk and Applebaum 1960). This effect however cannot be duplicated with either the Kunitz or Bowman-Birk inhibitor (Birk *et al.* 1962).

1.2.3 INHIBITORS INGESTED BY HAEMATOPHAGOUS INSECTS

Protease production is regulated by a secretagogue mechanism in blood feeding insects (Gooding 1975, Applebaum 1985). A "factor" in the blood meal stimulates the midgut cells resulting in secretion of proteases. A secretagogue mechanism is characterized by a statistically significant correlation between protease activity and the amount of protein in the gut (Gooding 1975). Serum and plasma contain protease inhibitors, including those of trypsin and chymotrypsin (Vogel 1968, Heimberger 1975). Gooding (1977) remarked that it may be advantageous for haematophagous insects to use protease inhibitors as the stimulants for protease production. Using the stable fly, *Stomoxys calcitrans*, evidence suggesting the inhibitors as the secretagogue for protease production will be described as well as the effects of an inhibitor out of its host range, soybean trypsin inhibitor.

A secretagogue mechanism controls protease production in the stable fly where trypsin, chymotrypsin, carboxypeptidase A and carboxypeptidase B activities all correlate with protein content of the gut (Schneider *et al.* 1987). In an attempt to further characterize the secretagogue for trypsin production, Houseman *et al.* (1988) show that it is contained within the non-cellular components of the ingested blood meal. In flies fed either whole blood, serum or plasma tryptic activity in the posterior midgut increases more than 10-fold and protein content of the posterior midgut correlates significantly and positively with tryptic activity. However, there is only a 2-fold increase in tryptic activity in the posterior midgut when intact or lysed red blood cells are ingested and

there is no correlation between protein content of the posterior midgut and tryptic activity. When the stable fly ingests a diet of whole blood supplemented with the soybean trypsin inhibitor (3 mg/ml), fecundity is reduced 71% and there is a reduction in proteolytic digestion of the blood meal (Spates 1979).

Based on the work using the stable fly, the role of the ingested protease inhibitor appears to differ depending on its source. Inhibitors within serum or plasma (within the host range) appear to be the stimulus for protease production while inhibitors outside of its host range (soybean trypsin inhibitor) act as disruptive agents of proteolysis.

1.2.4 TEST INSECT CHOSEN TO STUDY THE EFFECTS OF INGESTED INHIBITORS

The European corn borer, *Ostrinia nubilalis* Hubner (Lepidoptera: Pyralidae) is a world-wide pest of economic importance in corn growing regions (Hudon and Le Roux 1986). The corn borer is a polyphagous insect but is primarily considered to be a pest of corn.

The corn borer's lifecycle consists of 5 larval instars, a resting pupal stage and the adult stage (Hudon and LeRoux 1986). Eggs are deposited in masses on the underside of leaves and usually complete development in 5-7 days. Damage to corn is caused by the early larval instars chewing the leaves, resulting in destruction of the leaf surface and midrib breakage. Later instars tunnel all parts of the stalks and ears, resulting in broken stalks and tassles, poor ear development and ear drop. The borers pass the winter in diapause as fully grown larvae concealed in parts of the plants on which they have been feeding. Pupation takes place in the spring, with the adult moth appearing in May and June.

1.3 CONCLUSIONS DRAWN FROM THE LITERATURE REVIEW

1. Protease inhibitors are abundant within the tissues of both plants and animals.
2. Although much is known concerning the structure and physiochemical properties of protease inhibitors, less is known of their precise physiological role.
3. Host specificity is an important factor when assessing the effects of ingested protease inhibitors. Inhibitors within an insect's host range appear not to be detrimental to the insect while those inhibitors outside this range are detrimental.
4. The effect of ingested plant protease inhibitors is mainly based on growth and less on proteolytic studies.
5. Lepidopteran proteolytic activity is unique in that optimal activity is in the high alkaline range.
6. The role for serum and plasma inhibitors as the secretagogue for protease production in haematophagous insects contrasts with the defensive role for plant protease inhibitors.

1.4 OBJECTIVES AND HYPOTHESIS

The objective of the current research was to look at the effects of ingested protease inhibitors on proteolytic activity and development of European corn borer larvae. Inhibitors were chosen from the corn borer's preferred host corn, from soybeans and from a source not normally encountered, chicken egg white. Potential effects on proteolytic activity were examined using enzyme assays that had been previously optimized for corn borer proteases (Houseman *et al.* 1989).

It was hypothesized that inhibitors within the host plant range of corn borer larvae would not affect proteolytic activity or development while those inhibitors out of this range would have a negative effect on these two factors, and that there would be an

inverse relationship between host plant preference and the ability of ingested protease inhibitors to disrupt digestive processes.

Chapter II

MATERIALS AND METHODS

2.1 MAINTENANCE OF INSECT COLONY

The colony of *Ostrinia nubilalis* (Hubner) originated from animals obtained from M.Hudon (Agriculture Canada, St.Jean, Quebec) and from G.Mcleod (Agriculture Canada, London, Ontario). The insects were maintained in the laboratory under a 16h light (26.5°C) and 8h dark (19.0°C) photoperiod. The relative humidity of the Conviron incubator was set at 80%. Light sources were either cool white or vita lite fluorescent tubes combined with incandescent bulbs.

Insects were reared according to the methods of Guthrie *et al.* (1985) except that corn cob grits were added to the larval diet. Adults and larvae were maintained in separate incubators. Adults oviposited on sheets of wax paper that were suspended in the cage. Egg masses were cut out, pinned to diet cubes within plastic containers with screened tops. Before pupation a corrugated cardboard ring was placed in each container so that larvae could pupate within compartments of the ring. Rings with pupae were placed in the adult cage where emergence occurred.

2.2 PREPARATION OF GUT SAMPLES AND ENZYME ASSAYS

The entire midgut and contents were dissected in 0.15M NaCl, individually placed in 1.5ml microcentrifuge tubes and frozen (-15°C) until required. When needed, samples were thawed and homogenized in 190µl of 0.15M NaCl using a microhomogenizer made from epoxy that was molded to fit within the microcentrifuge tube. The microhomogenizer was then rinsed with 190µl of 0.15M NaCl to remove any residual gut

material. The tubes were centrifuged at 13,500g at 4°C for 10 minutes using a Beckman microfuge. The supernatant was removed and taken up to either 0.5 or 1ml with 0.15M NaCl, and this was referred to as the gut homogenate.

All enzyme assays, both timed and continuous, were carried out at 30°C. Spectrophotometric measurements were made using a Beckman DU-65 spectrophotometer equipped with a thermoregulated six-cell transport. All substrates used were purchased from Sigma. Assay conditions used were those optimized for *O. nubilalis* proteases by Houseman *et al.* (1989) and Houseman and Larocque (1987).

High alkaline tryptic activity and aminopeptidase activity were measured using a stop assay procedure. Gut homogenate, 10 μ l for high alkaline trypsin and 15 μ l for aminopeptidase, was added to 1.5ml of 0.1M glycine 5mM MgCl₂ pH 10.0 for high alkaline trypsin and to 1.5ml of 0.1M tris pH 7.5 at 30°C for aminopeptidase. The substrates used to measure high alkaline tryptic activity and aminopeptidase activity were 40 μ l of BAPNA, 20mg/ml in dimethylsulfoxide, and 40 μ l of LPNA, 40mg/ml in dimethylsulfoxide, respectively. Assays were run for 45 minutes for high alkaline trypsin and 60 minutes for aminopeptidase. The reaction was stopped by addition of 0.5ml of 30%(V/V) acetic acid. To account for spontaneous breakdown of substrate and any absorbance due to the gut homogenate, the same procedure as above was carried out except that gut homogenate, 10 μ l for high alkaline trypsin and 15 μ l for aminopeptidase, was added after addition of acetic acid. A molar extinction coefficient of 8,800 for BAPNA and for LPNA (Erlanger *et al.* 1961) was used to determine micromoles of substrate hydrolyzed.

Low alkaline tryptic activity and chymotryptic activity were measured using a continuous assay procedure where readings were taken over a 20 minute period. Gut homogenate, 15 μ l for low alkaline trypsin and 25 μ l for chymotrypsin, was added to 0.5ml of 0.1M bis tris propane pH 9.0 at 30°C for low alkaline trypsin and to 0.5ml of 0.2M tris

pH 8.0 at 30°C for chymotrypsin. The reaction was started by addition of substrate, 0.5ml of 1mM BAEE in 0.15M NaCl for low alkaline trypsin and 0.5ml of 1mM BTEE in 0.15M NaCl containing 10% (V/V) methanol for chymotrypsin. Molar extinction coefficients of 808 for BAEE (Kezdy *et al.*1965) and 964 for BTEE (Hummel 1959) were used to calculate micromoles of substrate hydrolyzed.

Total proteolysis was measured using a stop assay procedure. Gut homogenate, 25 μ l, was added to 1.5ml microcentrifuge tubes containing 0.3ml of 0.2M glycine 10mM MgCl₂ pH 10.0. The reaction was started by addition of 0.3ml of azocasein as substrate, 20mg/ml in 0.15M NaCl. The reaction was stopped after 3 hours by addition of 0.6ml of 20%(W/V) trichloroacetic acid. To account for spontaneous breakdown of substrate and any absorbance due to the gut homogenate, the same procedure was carried out except that 25 μ l gut homogenate was added after addition of trichloroacetic acid. Tubes were kept at 4°C for at least 2 hours and then centrifuged at 13,500g, 4°C for 10 minutes. The supernatants were read at 366nm. One azocasein unit was the change of one absorbance unit per minute per ml of final reaction mixture.

Gut protein content was determined by the method of Bramhall *et al.* (1969) using bovine serum albumin (Fraction V, Sigma Chemical Co.) as the standard. For each gut sample, 30 μ l of gut homogenate was spotted in duplicate.

2.3 SOURCE OF PROTEASE INHIBITORS

The soybean trypsin inhibitor (type II-S, crude) and chicken ovomucoid (type III-O, purified) were purchased from Sigma Chemical Co.

The procedure used for isolation and purification of the corn trypsin inhibitor was a modification of the methods of Chen and Mitchell (1973) , Hochstrasser *et al.* (1967) and Swartz *et al.* (1977) (Fig 1).

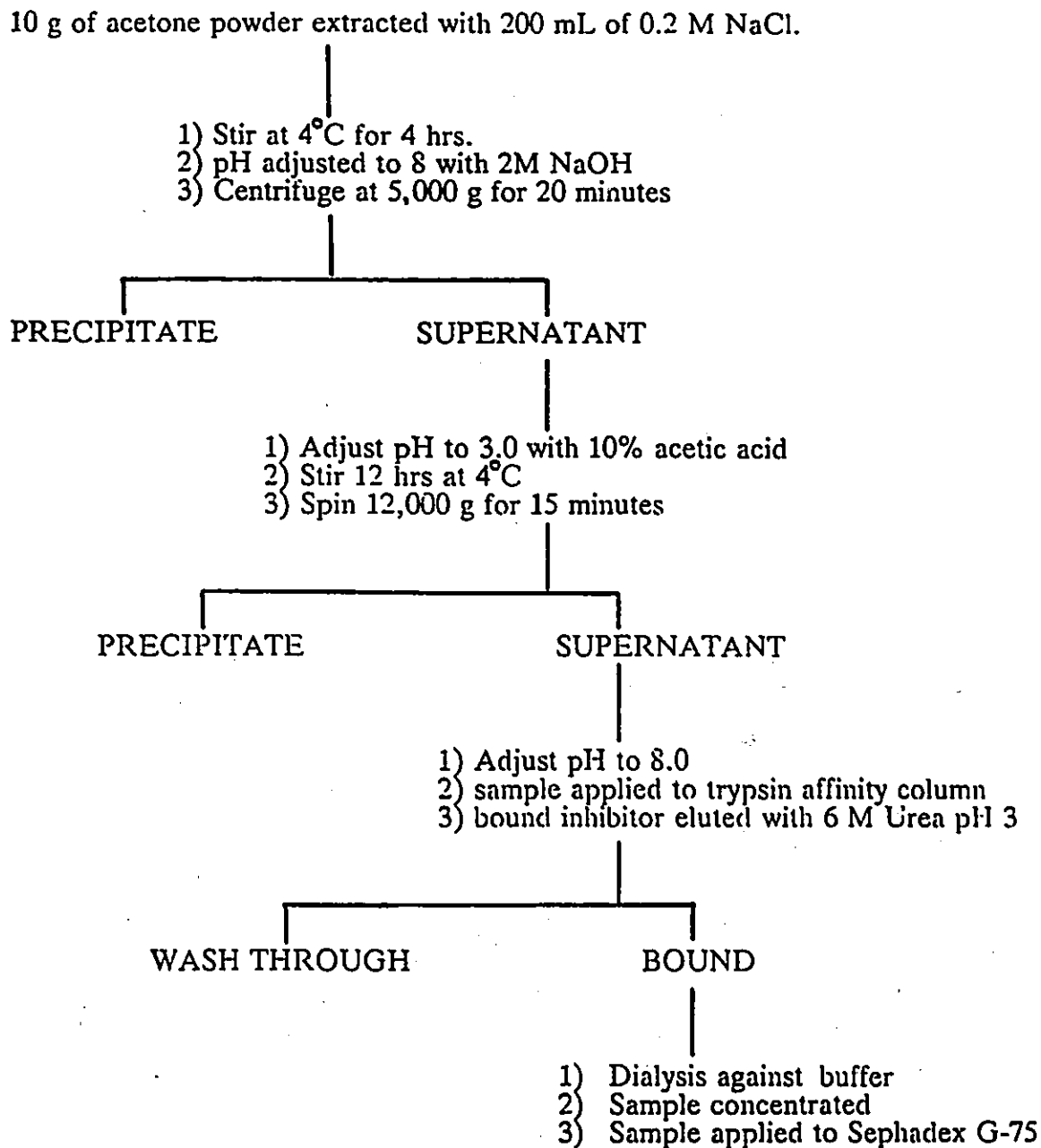


Figure 1. Purification of the corn trypsin inhibitor.

Kernels from fresh sweet corn cobs were ground in a Waring blender with cold (-15°C) acetone. This mixture was filtered using a Buchner funnel with Whatman no. 1 filter paper and the filter cake formed was rinsed several times with acetone until its yellow color was removed. Once the filter cake had dried it was ground into a fine powder, the corn acetone powder, using a mortar and pestle.

Ten grams of acetone powder were extracted with 200ml of 0.2M NaCl at 4°C by stirring. The pH of this mixture was adjusted to 8.0 with NaOH and then centrifuged at 5,000g, 4°C, for 20 minutes. The supernatant was adjusted to pH 3 by addition of 10%(V/V) acetic acid. This solution was stirred at 4°C for 12 hours to allow precipitation formation and was then centrifuged at 12,000g at 4°C for 15 minutes. The supernatant was removed and adjusted to pH 8.0 with 5M NaOH. This extract was applied to the trypsin affinity column prepared according to the instruction pamphlet provided by Pharmacia. The affinity column was washed with buffer (50mM tris 0.1M NaCl 10mM CaCl₂ pH 8.0 at 25°C) until absorbance of the wash through fractions at 280nm was zero. Bound inhibitor was eluted with 6M urea pH 3.0. Fractions were tested for inhibitory activity using bovine pancreatic trypsin and the same buffer applied to the affinity column. Fractions containing inhibitor were pooled, dialyzed against the same buffer supplied to the affinity column and then concentrated using an Amicon YM5 ultrafiltration membrane. The resulting concentrate was lyophilized for incorporation into the diet.

The molecular weight of the corn trypsin inhibitor was determined by gel filtration chromatography using Sephadex G75 (Pharmacia) which fractionates proteins in the molecular weight range of 6,500-66,000 daltons. The column was equilibrated with the same buffer as for the trypsin affinity column. A molecular weight marker kit, MW-GF-70 Sigma was used, and consisted of 4 proteins: bovine long aprotinin MW 6,500, horse heart cytochrome c MW 12,400, bovine erythrocytes carbonic anhydrase

MW 29,000 and bovine serum albumin MW 66,000. Also included in the kit was blue dextran MW 2,000,000 for determining the void volume (V_0) of the column. A calibration curve was generated by plotting molecular weight versus the V_e/V_0 ratio for each protein standard.

2.4 EFFECT OF TEST DIETS ON PROTEOLYTIC ACTIVITY

2.4.1 EXPERIMENTAL DESIGN

Two different time periods were chosen to study the effects of ingested protease inhibitors on proteolytic activity and these were referred to as "short term exposure", which looked at effects after two days on the diet and "long term exposure", which examined effects throughout the larval life cycle of the insect. On day 0, for the short term exposure studies, 35-40 mg larvae were individually placed in glass vials with one diet cube and the vials were plugged with absorbent cotton. Insects were allowed to feed for 48 hours and were then weighed using a Mettler AE163 analytical balance. Total midgut and contents were collected as previously described.

On day 0, for the long term exposure 7-9 mg larvae were placed in glass vials as previously described. Insects were taken off the diet between days 5-9, weighed, and total midgut and contents were dissected as previously described.

2.4.2 TEST DIETS

2.4.2.1 SOYBEAN TRYPSIN INHIBITOR

Preliminary studies with the soybean trypsin inhibitor were conducted in order to determine what concentrations to use. The inhibitor was incorporated into the diet at 0.5, 1 and 1.5% (W/V) which were used in short term exposure studies. Based on these preliminary studies 1% (W/V) inhibitor was subsequently used. The short term exposure experiments were repeated 3 times with a sample size of 4-20 animals. The

long term exposure experiments were repeated 4 times with a sample size of 2-10 animals with samples taken on days 5, 6, 7, 8, and 9.

2.4.2.2 CHICKEN OVOMUCOID TRYPSIN INHIBITOR

As was described for the soybean trypsin inhibitor, preliminary studies were carried out to determine at what concentrations to conduct the experiments with chicken ovomucoid. Based on the results of these preliminary studies, possible stimulation of proteolytic activity was tested for by incorporating the inhibitor at 2% (W/V) into the diet for the short and long term exposure studies. The short term exposure study was done once with N=20 for control and inhibitor treated insects. The long term exposure study was run once with a sample size of N=8-10 for control and inhibitor treated insects with samples taken on days 6,7 ,8 and 9.

2.4.2.3 CORN TRYPSIN INHIBITOR

The corn trypsin inhibitor was incorporated into the diet at the 1% (W/V) level for use in a short term exposure experiment with a sample size of N=20-24 for control and inhibitor treated insects.

2.4.2.4 CASEIN

Casein was added to the diet at a concentration of 1% (W/V) and 2% (W/V) to insure that effects with the inhibitors were not due to the fact that extra protein was added to the diet but were due to the properties of the respective inhibitor. It should be noted that there are two protein sources in the diet for rearing of corn borer larvae. Casein is at a concentration of 2.9% (W/V) while wheat germ is at a concentration of 3.4% (W/V). Therefore 1 or 2% (W/V) casein refers to the extra experimental casein added to the diet. The short term exposure experiment was repeated 3 times for 1% (W/V) casein with a sample size of 9-25 for control and treated insects and once with 2% (W/V) casein with N= 20 for control and treated insects. The long term exposure

study was run once with 2% (W/V) casein and a sample size of N=7-10 for control and casein treated insects with samples taken on days 6,7,8 and 9.

2.4.2.5 DATA ANALYSIS

Differences between control and treated insects for each variable were tested for using an independent-samples t-test at the 95% confidence interval. In cases where multiple replicates were run the data were combined for statistical analysis. Experiments were initially run with a small sample size, 4-7 insects for the short term exposure study and approximately 7 insects for the long term exposure study. In such small sample sizes any variability tended to bias the results making reproducibility between replicates difficult. However, when the data of replicate runs were combined the variability decreased. In cases where multiple replicates were run results were the combined data but individual replicates were included in an appendix.

2.5 EFFECT OF TEST DIETS ON DEVELOPMENT

This study began with 7-9mg larvae. Each larva was placed in a glass vial containing one food cube. Each day the number of pupae and the number of adults observed was recorded.

Test diets consisted of control, 1% (W/V) soybean trypsin inhibitor, 1% (W/V) chicken ovomucoid and 1% (W/V) casein. The experiment was repeated 4 times with a sample size of N=15-35 for control and treated insects. In the first replicate only the number of pupae was recorded.

Chapter III

RESULTS

3.1 EFFECT OF TEST DIETS ON PROTEOLYTIC ACTIVITY

3.1.1 SOYBEAN TRYPSIN INHIBITOR

In the preliminary study with soybean trypsin inhibitor chymotryptic activity was significantly lower in treated insects when 0.5, 1, or 1.5% (W/V) inhibitor was ingested (Fig. 2a). When either 1 or 1.5% (W/V) inhibitor was ingested, protein content of the gut was significantly higher in treated insects (Fig 2b). Larval weight was only significantly lower in treated insects when 1.5% (W/V) inhibitor was ingested (Fig 2c). No significant effects were seen for high or low alkaline tryptic activity, aminopeptidase or total proteolysis when 0.5, 1, or 1.5% (W/V) soybean trypsin inhibitor was ingested (Fig. 2d, 2e, 2f, 2g). Results of individual runs are included in Appendix A. Since an increase in inhibitor concentration did not appreciably change the significant effects seen at the 1% (W/V) level for chymotryptic activity or protein content of the gut, the remainder of the studies were conducted at the 1% (W/V) level.

In the short term exposure study when larvae ingested a diet containing 1% (W/V) soybean trypsin inhibitor chymotryptic activity was significantly lower but low alkaline tryptic activity was significantly higher (Table 1). Larval weight was significantly lower and protein content of the gut was significantly higher (Table 1). There were no significant differences between control and treated insects for high alkaline tryptic activity, aminopeptidase activity or total proteolysis (Table 1). Results of individual runs are included in Appendix B.

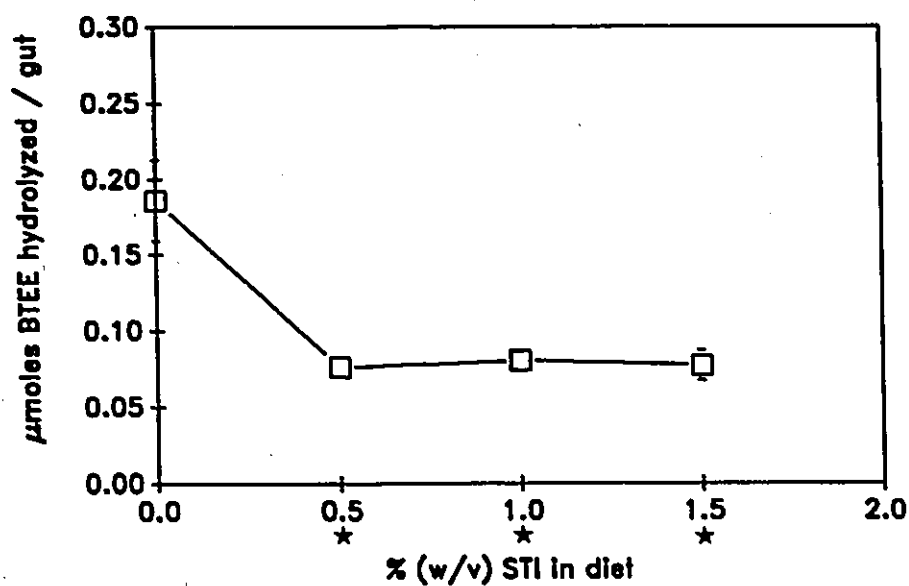


Figure 2a. Preliminary study with 0.5, 1 and 1.5% (W/V) soybean trypsin inhibitor: Effect on chymotryptic activity of *O. nubilalis* larvae.

Animals were placed on diet that contained either 0, 0.5, 1 or 1.5% (W/V) soybean trypsin inhibitor as 35-40 mg larvae and were allowed to feed for two days. Enzyme activity as μ moles substrate hydrolyzed/gut. Experiment was repeated twice and results were combined to generate a total sample size of N=14-16 for control and treated insects. Values are the mean \pm S.E.M and * indicates significant differences between treated and control insects, $p \leq 0.05$. For results of individual runs see Appendix A.

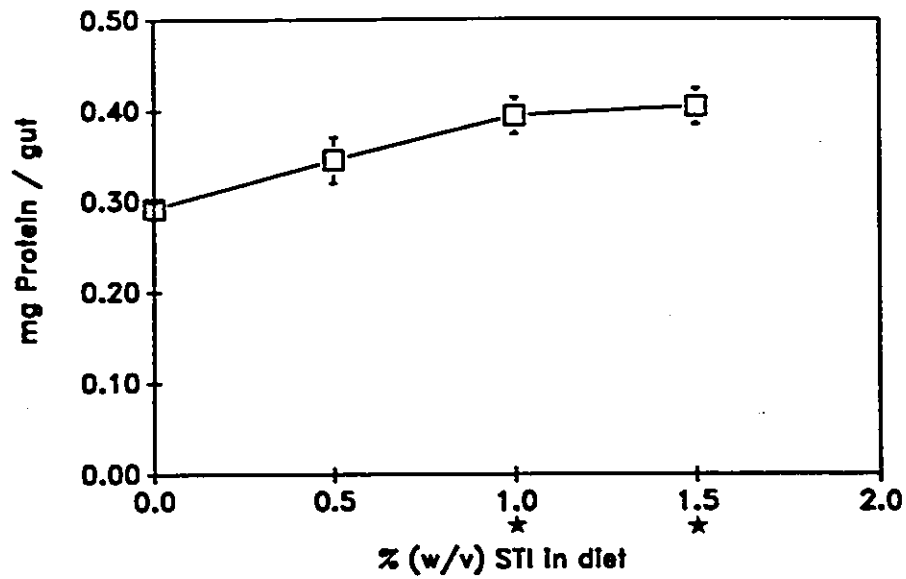


Figure 2b. Preliminary study with 0.5, 1 and 1.5% (W/V) soybean trypsin inhibitor: Effect on gut protein content of *O. nubilalis* larvae.

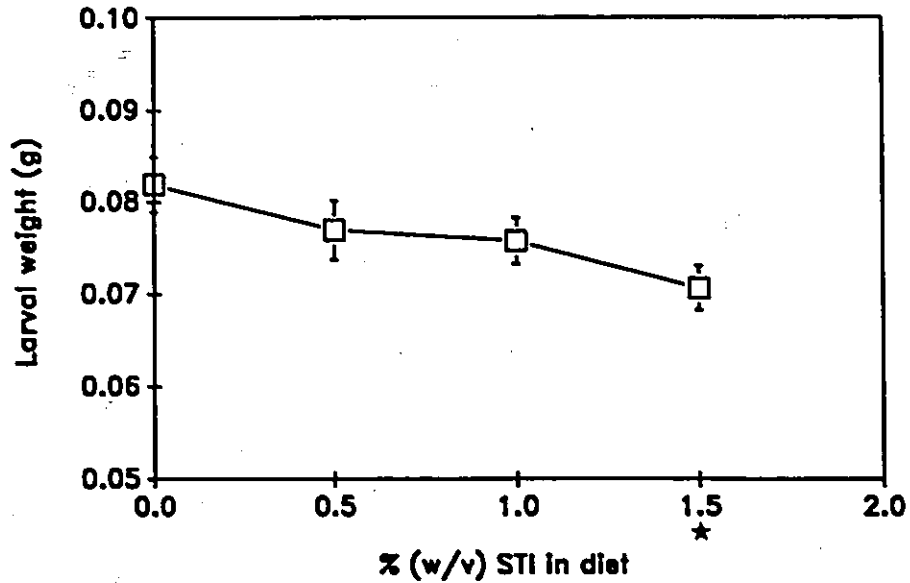


Figure 2c. Preliminary study with 0.5, 1 and 1.5% (W/V) soybean trypsin inhibitor: Effect on larval body weight of *O. nubilalis*.

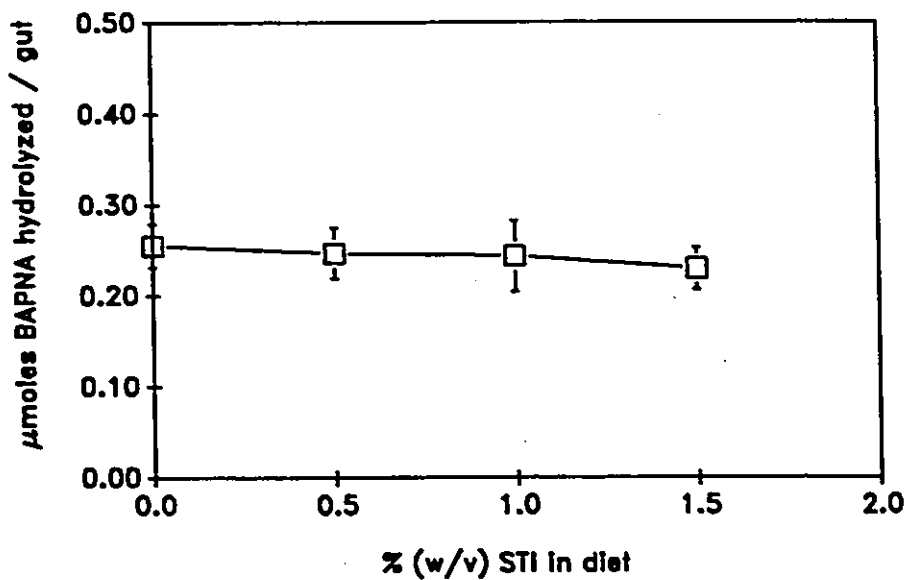


Figure 2d. Preliminary study with 0.5, 1 and 1.5% (W/V) soybean trypsin inhibitor: Effect on high alkaline tryptic activity of *O. nubilalis* larvae.

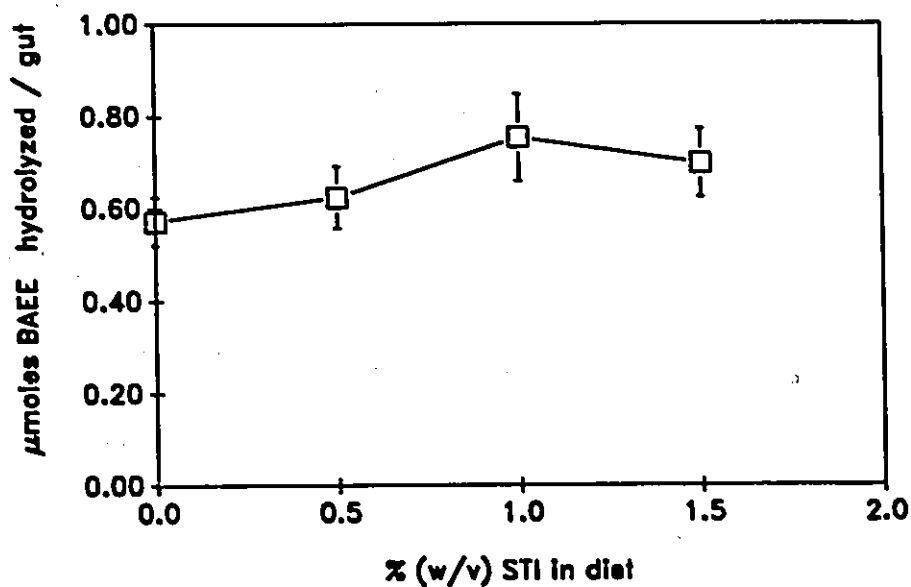


Figure 2e. Preliminary study with 0.5, 1 and 1.5% (W/V) soybean trypsin inhibitor: Effect on low alkaline tryptic activity of *O. nubilalis* larvae.

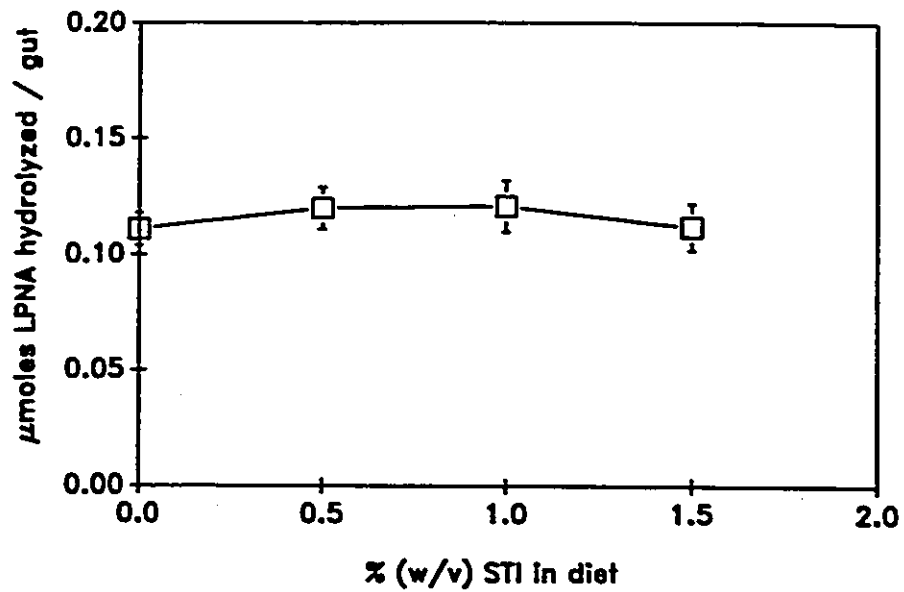


Figure 2f. Preliminary study with 0.5, 1 and 1.5% (W/V) soybean trypsin inhibitor: Effect on aminopeptidase activity of *O. nubilalis* larvae.

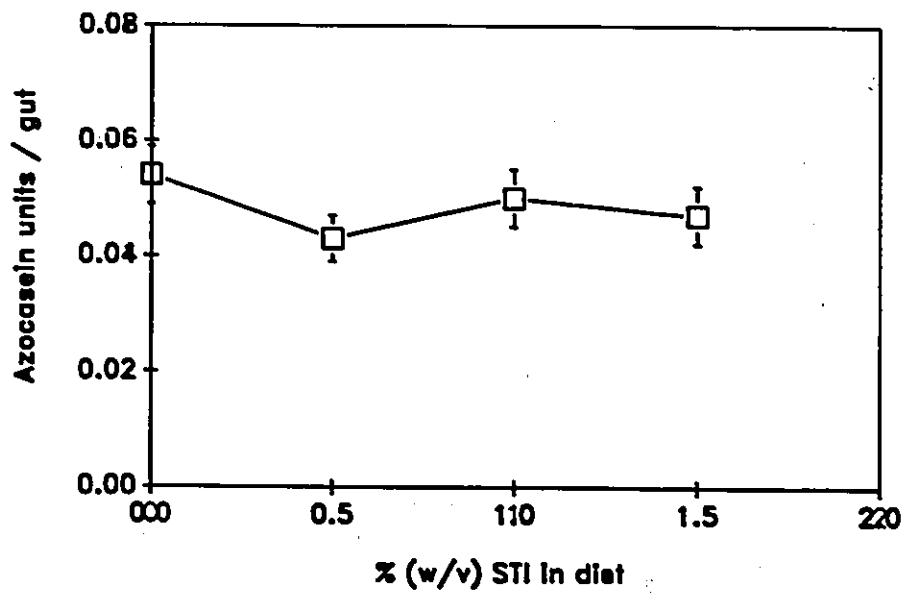


Figure 2g. Preliminary study with 0.5, 1 and 1.5% (W/V) soybean trypsin inhibitor: Effect on total proteolytic activity of *O. nubilalis* larvae.

Table 1. Short term exposure study with 1% (W/V) soybean trypsin inhibitor.

	Control	1% STI
High Alkaline Trypsin (BAPNA)	0.180 ± 0.014	0.177 ± 0.016
Low Alkaline Trypsin (BAEE)	0.407 ± 0.031	0.551 ± 0.038*
Chymotrypsin (BTEE)	0.159 ± 0.013	0.058 ± 0.006*
Aminoamidase (LPNA)	0.086 ± 0.004	0.078 ± 0.004
Total Proteolysis (Azocasein)	0.025 ± 0.003	0.020 ± 0.002
mg Protein/gut	0.310 ± 0.012	0.372 ± 0.015*
Larval Weight (g)	0.0739 ± 0.0015	0.0663 ± 0.0017*

The effect of ingested soybean trypsin inhibitor (STI), 1% (W/V) in diet, on proteolytic activity, gut protein content and larval weight of *O. nubilalis*. Animals were placed on diet as 35-40 mg larvae and were allowed to feed for two days. Enzyme activity as μ moles substrate hydrolyzed/gut. Experiment was repeated three times to generate a total sample size of N=34 for control insects and N=36 for treated insects. Values are the mean \pm S.E.M. and * indicates significant differences between treated and control insects, $p \leq 0.05$. For results of individual runs see Appendix B.

In the long term exposure study chymotryptic activity was significantly lower on days 6, 7, and 8 when insects ingested a diet containing 1% (W/V) soybean trypsin inhibitor (Fig. 3a). Aminopeptidase activity was significantly higher in treated insects on days 5 and 8 (Fig. 3b). On days 7 and 8 in treated insects protein content of the gut was significantly higher (Fig. 3c) and on day 7 larval weight was significantly lower in treated insects (Fig. 3d). In control insects, high and low alkaline tryptic activity, chymotryptic activity and total proteolysis levels dropped sharply between days 8 and 9 (Fig. 3e, 3f, 3a, 3g). This sharp decline was not seen with inhibitor treated insects (Fig. 3e, 3f, 3a, 3g). Results of individual runs are included in Appendix C.

3.1.2 CHICKEN OVOMUCOID TRYPSIN INHIBITOR

In the preliminary study with 0.5, 1, and 1.5% (W/V) chicken ovomucoid trypsin inhibitor, high and low alkaline tryptic activity and total proteolysis levels were significantly higher for insects that consumed a diet containing 1.5% (W/V) inhibitor (Fig. 4a, 4b, 4c). There were no significant differences from control insects for larval weight, aminopeptidase or chymotryptic activity when larvae ingested a diet containing 0.5, 1, or 1.5% (W/V) inhibitor (Fig. 4d, 4e, 4f). When larvae ingested a diet containing 0.5% (W/V) inhibitor protein content of the gut was significantly higher (Fig. 4g). Results of individual runs are included in Appendix D. To test for possible stimulation of proteolytic activity the short term exposure and long term exposure studies were conducted at the 2% (W/V) level.

When insects ingested a diet containing 2% (W/V) ovomucoid in the short term exposure study chymotryptic activity was significantly higher in treated insects compared to control (Table 2). There were no significant differences between control and treated insects for larval weight, high or low alkaline tryptic activity, aminopeptidase activity, total proteolysis or protein content of the gut (Table 2).

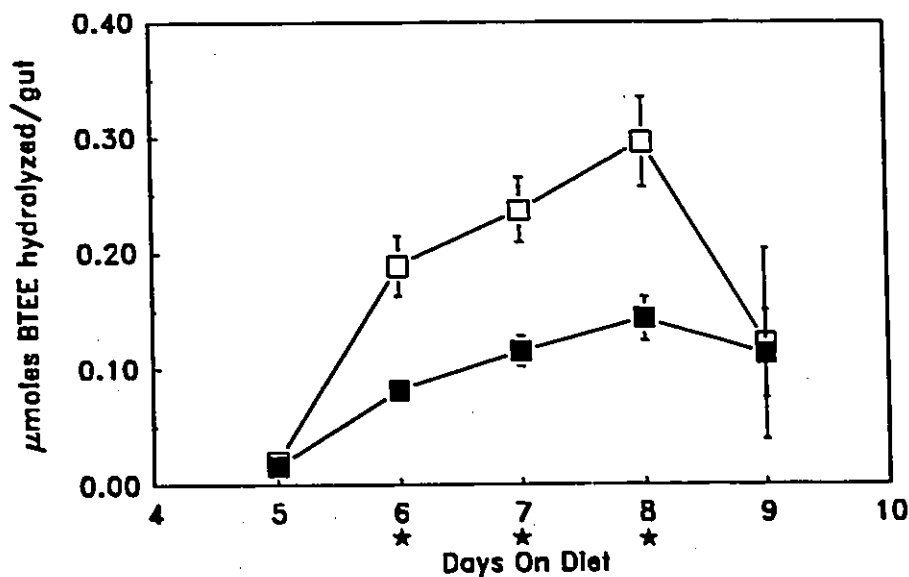


Figure 3a. Long term exposure study with 1% (W/V) soybean trypsin inhibitor: Effect on chymotryptic activity of *O. nubilalis* larvae.

Animals were placed on diet containing 1% (W/V) soybean trypsin inhibitor as 7-9 mg larvae and were allowed to feed for 5-9 days. Enzyme activity as μmoles of substrate hydrolyzed/gut. Experiment was repeated four times and results were combined to generate a total sample size of $N=17-31$ for control (□) and treated insects (■). Values are the mean \pm S.E.M. and * indicates significant differences between treated and control insects, $p \leq 0.05$. For results of individual runs see Appendix C.

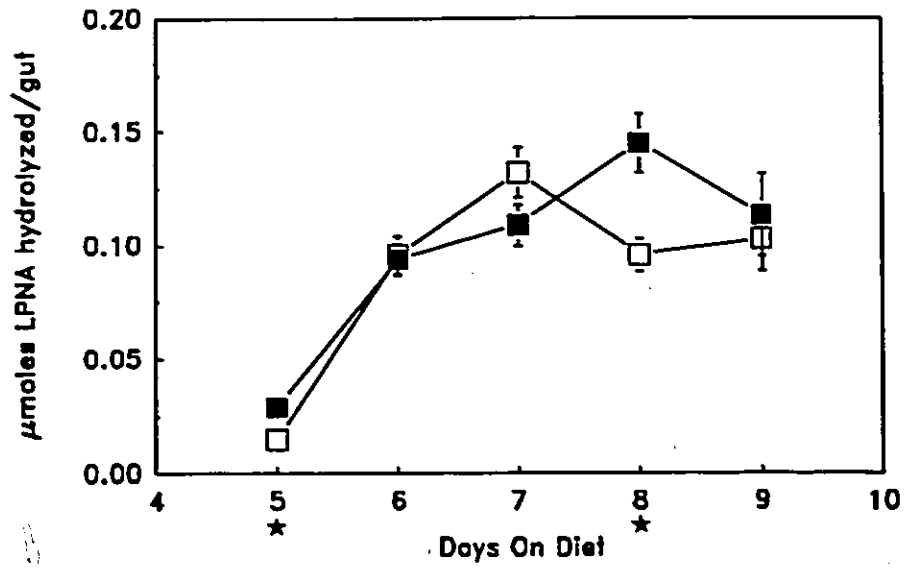


Figure 3b. Long term exposure study with 1% (W/V) soybean trypsin inhibitor: Effect on aminopeptidase activity of *O. nubilalis* larvae.

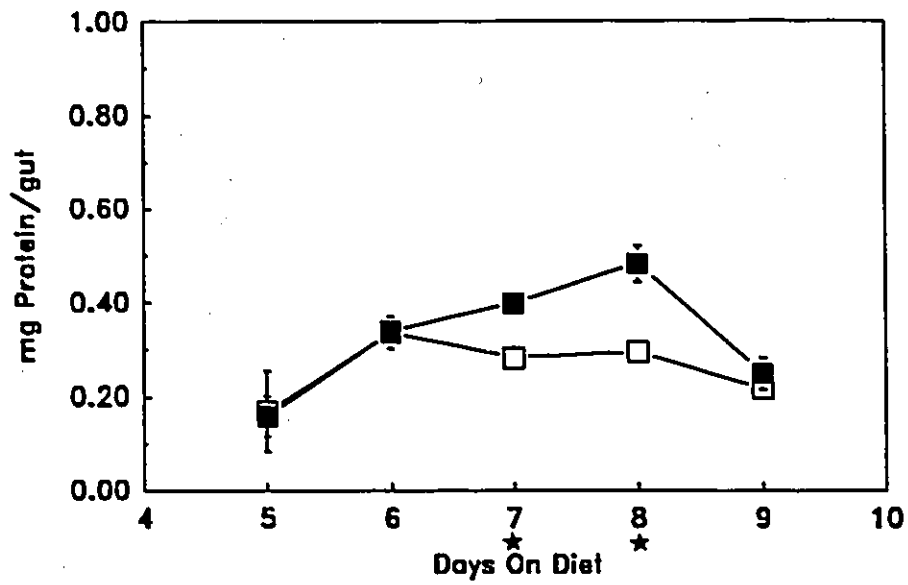


Figure 3c. Long term exposure study with 1% (W/V) soybean trypsin inhibitor: Effect on gut protein content of *O. nubilalis* larvae.

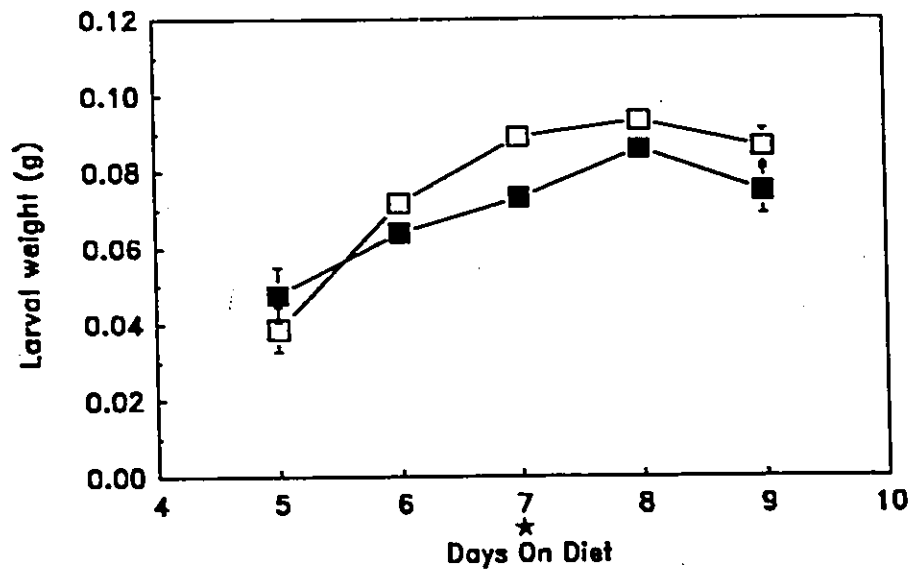


Figure 3d. Long term exposure study with 1% (W/V) soybean trypsin inhibitor: Effect on larval body weight of *O. nubilalis*.

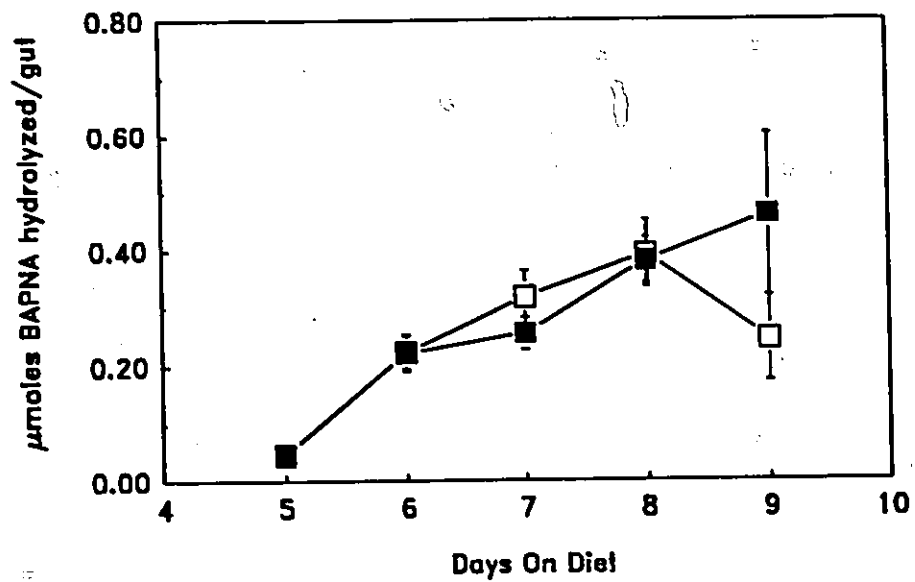


Figure 3e. Long term exposure study with 1% (W/V) soybean trypsin inhibitor: Effect on high alkaline tryptic activity of *O. nubilalis* larvae.

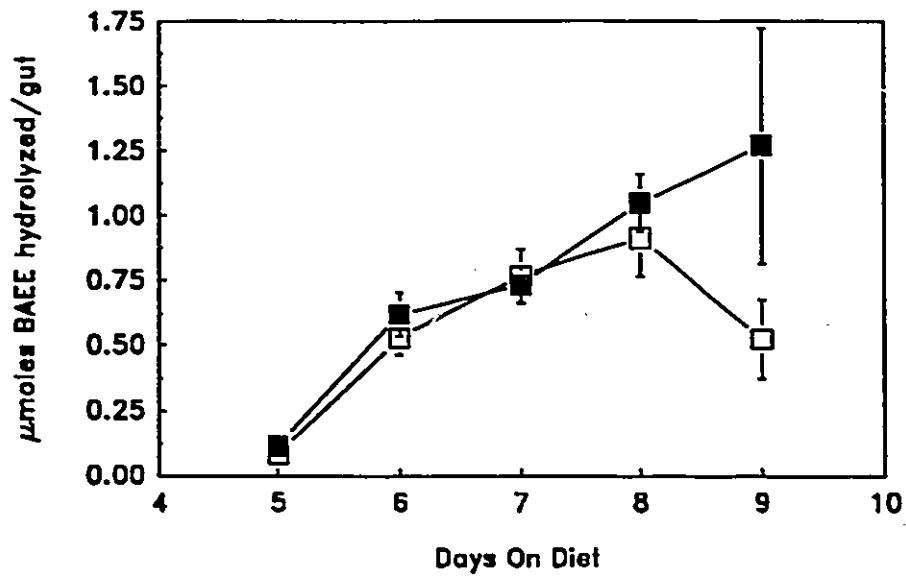


Figure 3f. Long term exposure study with 1% (W/V) soybean trypsin inhibitor: Effect on low-alkaline tryptic activity of *O. nubilalis* larvae.

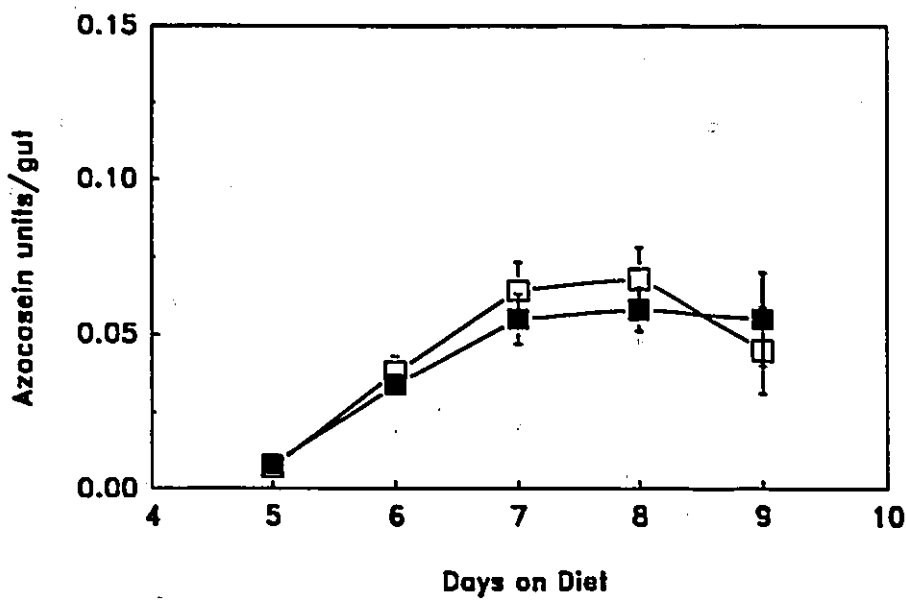


Figure 3g. Long term exposure study with 1% (W/V) soybean trypsin inhibitor: Effect on total proteolytic activity of *O. nubilalis* larvae.

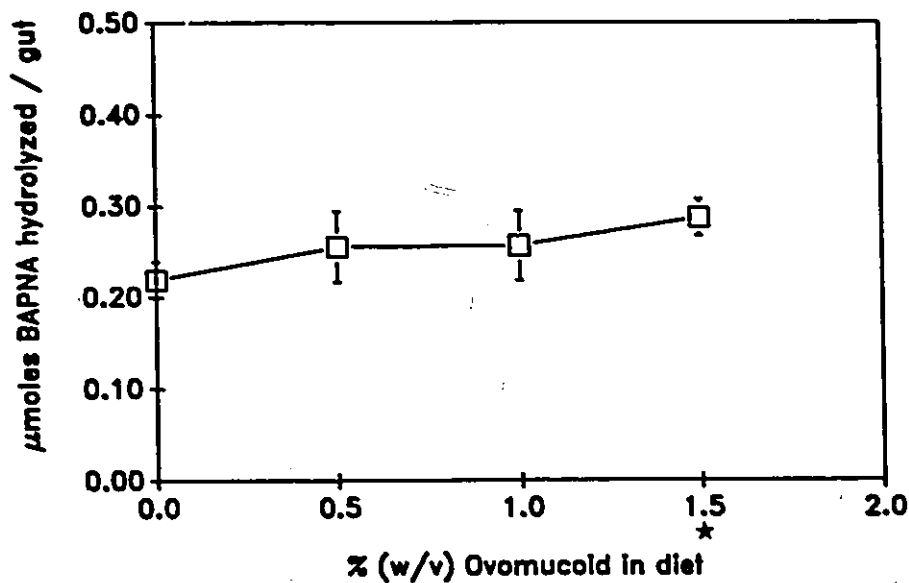


Figure 4a. Preliminary study with 0.5, 1 and 1.5% (W/V) chicken ovomucoid trypsin inhibitor: Effect on high alkaline tryptic activity of *O. nubilalis* larvae.

Animals were placed on diet containing either 0, 0.5, 1 or 1.5% (W/V) chicken ovomucoid trypsin inhibitor as 35-40 mg larvae and were allowed to feed for two days. Enzyme activity as μ moles substrate hydrolyzed/gut. Experiment was repeated twice and results were combined to generate a total sample size of $N=14$ for control and treated insects. Values are the mean \pm S.E.M. and \star indicates significant differences between treated and control insects, $p \leq 0.05$. For results of individual runs see Appendix D.

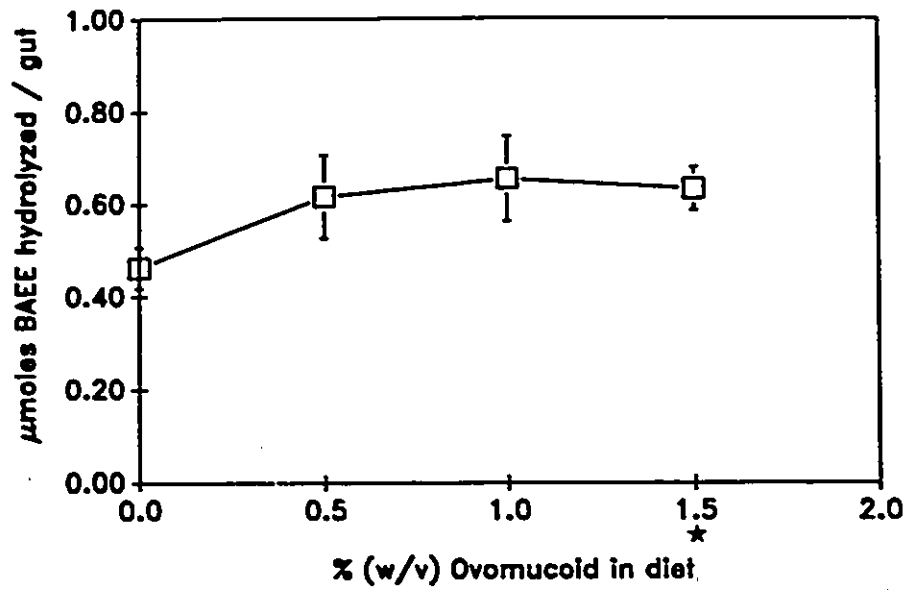


Figure 4b. Preliminary study with 0.5, 1 and 1.5% (W/V) chicken ovomucoid trypsin inhibitor: Effect on low alkaline tryptic activity of *O. nubilalis* larvae.

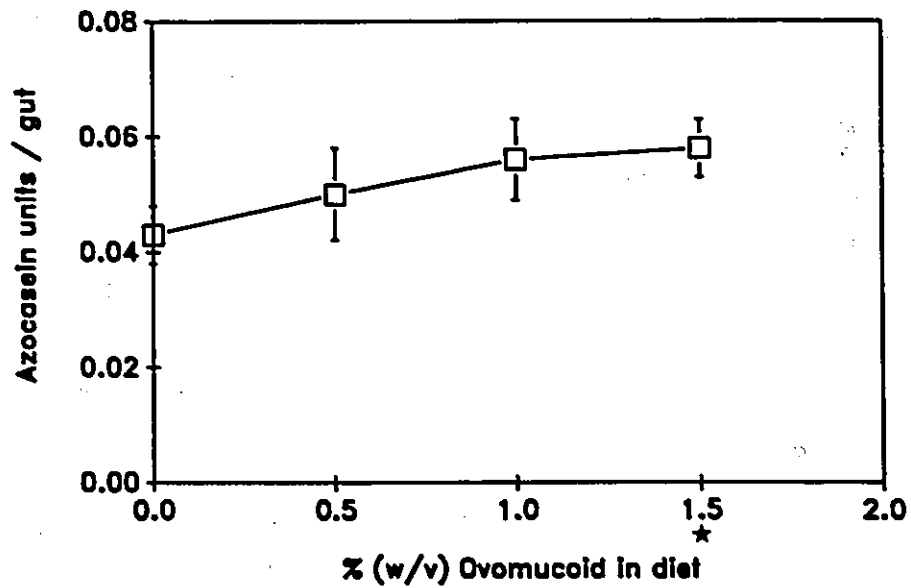


Figure 4c. Preliminary study with 0.5, 1 and 1.5% (W/V) chicken ovomucoid trypsin inhibitor: Effect on total proteolytic activity of *O. nubilalis* larvae.

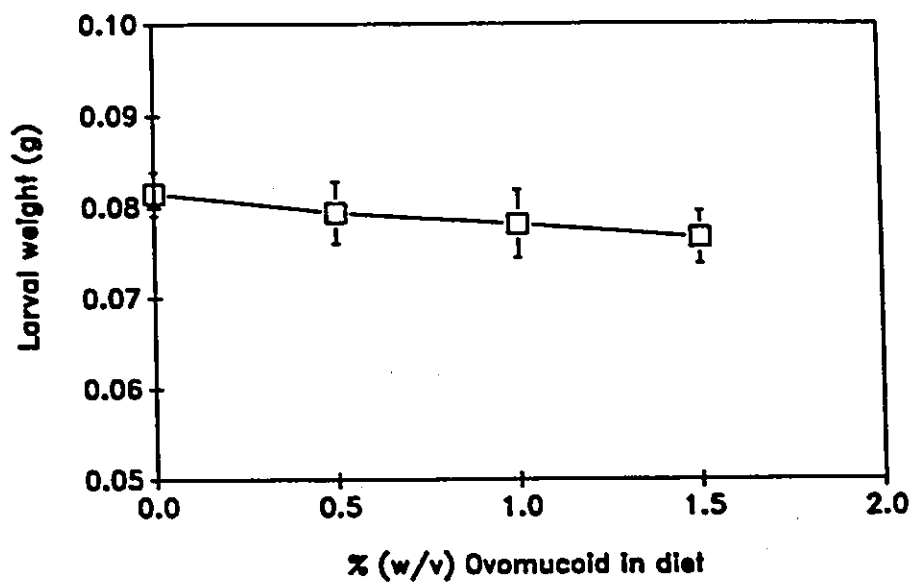


Figure 4d. Preliminary study with 0.5, 1 and 1.5% (W/V) chicken ovomucoid trypsin inhibitor: Effect on larval body weight of *O. nubilalis*.

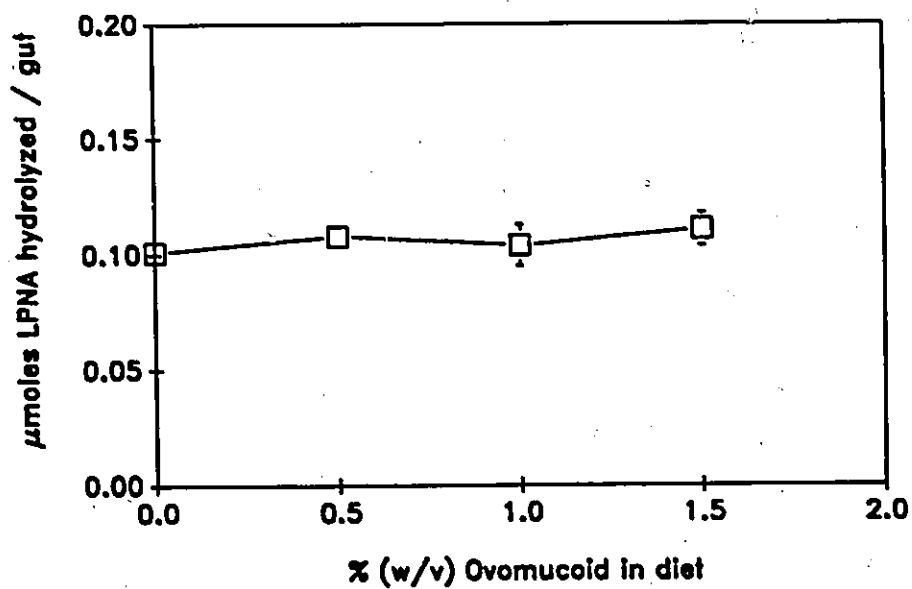


Figure 4e. Preliminary study with 0.5, 1 and 1.5% (W/V) chicken ovomucoid trypsin inhibitor: Effect on aminopeptidase activity of *O. nubilalis* larvae.

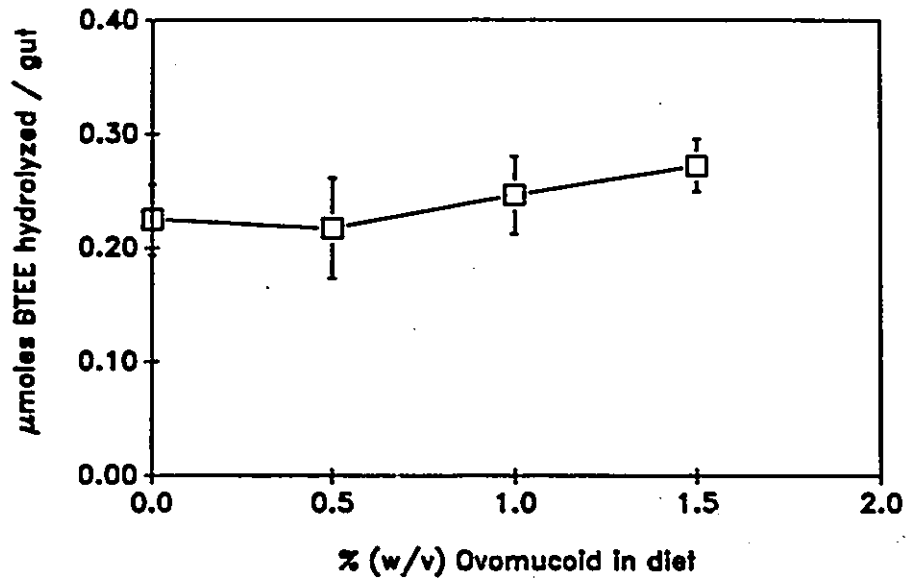


Figure 4f. Preliminary study with 0.5, 1 and 1.5% (W/V) chicken ovomucoid trypsin inhibitor: Effect on chymotryptic activity of *O. nubilalis* larvae.

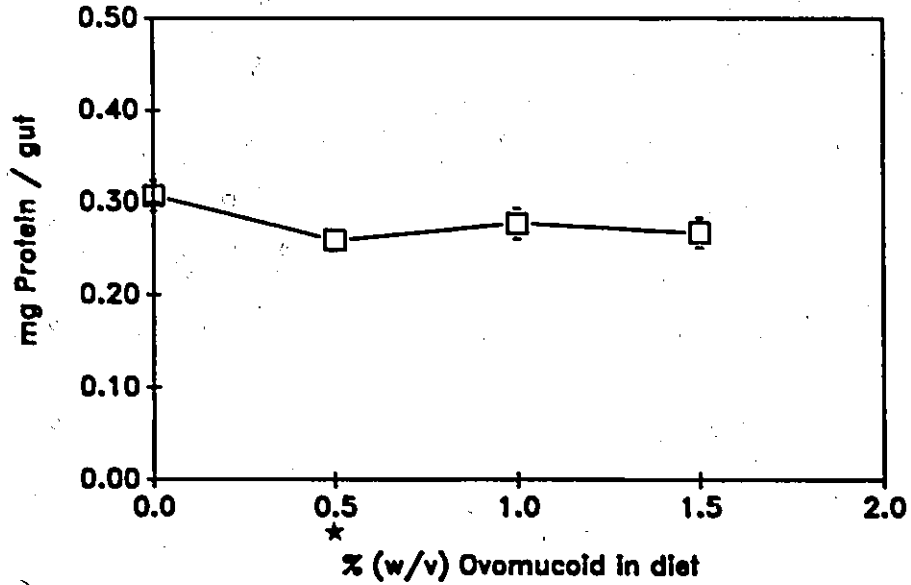


Figure 4g. Preliminary study with 0.5, 1 and 1.5% (W/V) chicken ovomucoid trypsin inhibitor: Effect on gut protein content of *O. nubilalis* larvae.

Table 2. Short term exposure study with 2% (W/V) chicken ovomucoid trypsin inhibitor.

	Control	2% Ovo
High Alkaline Trypsin (BAPNA)	0.203 ± 0.024	0.194 ± 0.022
Low Alkaline Trypsin (BAEE)	0.441 ± 0.052	0.521 ± 0.051
Chymotrypsin (BTEE)	0.151 ± 0.016	0.231 ± 0.027*
Aminopeptidase (LPNA)	0.072 ± 0.004	0.083 ± 0.007
Total Proteolysis (Azocasein)	0.017 ± 0.001	0.020 ± 0.002
mg Protein / gut	0.294 ± 0.021	0.275 ± 0.013
Larval Weight (g)	0.0658± 0.0021	0.0681± 0.0022

The effect of ingested chicken ovomucoid trypsin inhibitor (Ovo), 2% (W/V) in diet, on proteolytic activity, gut protein content and larval weight of *O. nubilalis*. Animals were placed on diet as 35-40 mg larvae and were allowed to feed for two days. Enzyme activity as μ moles substrate hydrolyzed/gut. N=20 for control and treated insects. Values are mean \pm S.E.M. * Indicates significant differences between treated and control insects, $p \leq 0.05$.

In the long term exposure study with 2% (W/V) chicken ovomucoid trypsin inhibitor high and low alkaline tryptic activities were found to be significantly higher in treated insects on days 7 and 9 (Fig. 5a, 5b). Chymotryptic activity was significantly higher in treated insects on days 6 and 7 (Fig. 5c). On day 6 aminopeptidase activity and protein content of the gut were significantly higher in treated insects (Fig. 5d, 5e). There were no significant differences between control and treated insects for total proteolysis or larval weight (Fig. 5f, 5g).

3.1.3 CORN TRYPSIN INHIBITOR

Affinity chromatography was used as the major step in the purification of the corn trypsin inhibitor. The molecular weight of the inhibitor using gel filtration chromatography was found to be 11,500 daltons (Fig. 6).

When insects ingested a diet containing 1% (W/V) corn trypsin inhibitor in the short term exposure study chymotryptic activity, total proteolysis and protein content of the gut were significantly higher compared to control values (Table 3). There were no significant differences between control and treated insects for low alkaline tryptic activity, aminopeptidase activity or larval weight (Table 3). High alkaline tryptic activity was significantly lower in treated insects (Table 3).

3.1.4 CASEIN

In the short term exposure study with 1% (W/V) casein treated insects had significantly higher aminopeptidase activity compared to control insects (Table 4). There were no significant differences between control and treated insects for high or low alkaline tryptic activity, chymotryptic activity, total proteolysis, protein content of the gut or larval weight (Table 4). Results of individual runs are included in Appendix E. When insects ingested a diet containing 2% (W/V) casein chymotryptic activity, gut protein content and larval weight were significantly higher in treated insects (Table 5).

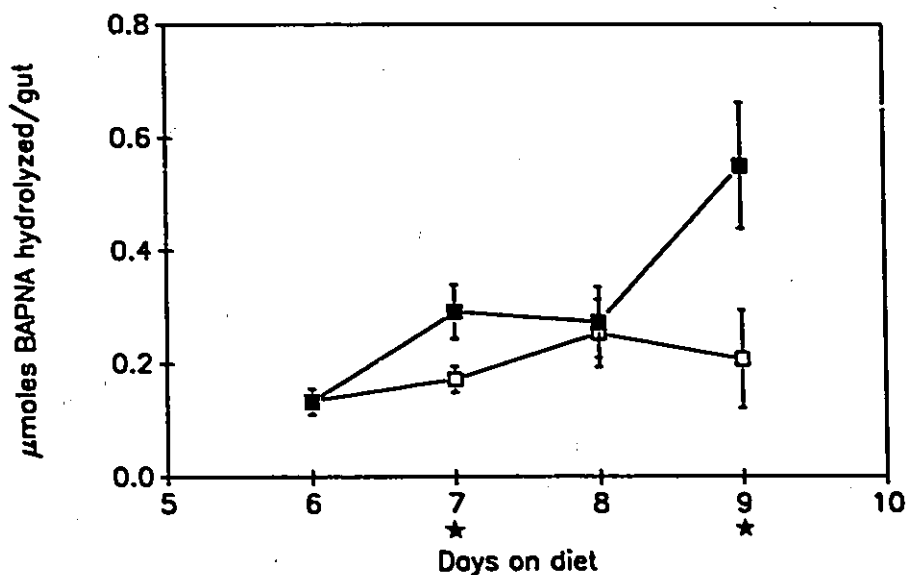


Figure 5a. Long term exposure study with 2% (W/V) chicken ovomucoid trypsin inhibitor: Effect on high alkaline tryptic activity of *O. nubilalis* larvae.

Animals were placed on diet containing 2% (W/V) chicken ovomucoid trypsin inhibitor as 7-9 mg larvae and were allowed to feed for 6-9 days. Enzyme activity as μmoles of substrate hydrolyzed/gut. Experiment was done once with $N=8-10$ for control (□) and inhibitor treated insects (■) with samples taken on days 6-9. Values are mean \pm S.E.M. and \star indicates significant differences between treated and control insects, $p \leq 0.05$.

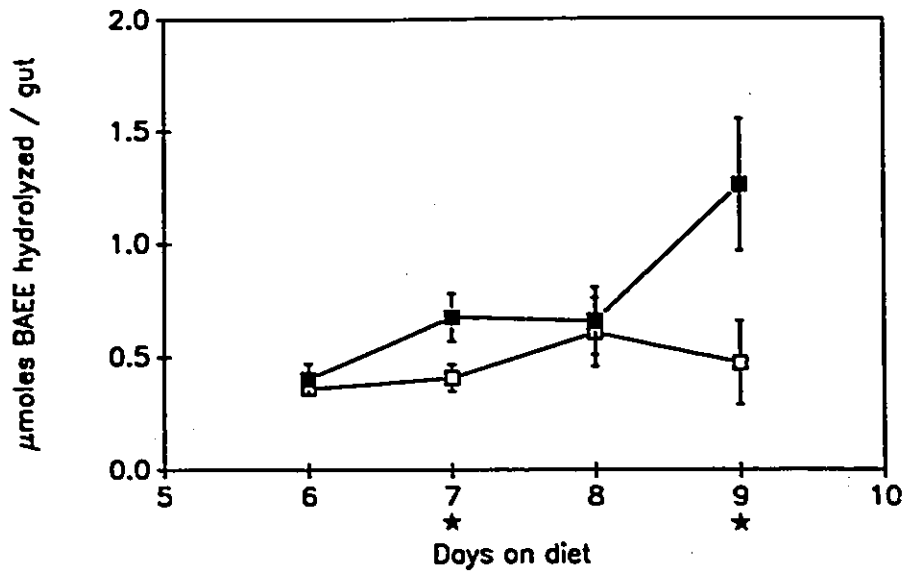


Figure 5b. Long term exposure study with 2% (W/V) chicken ovomucoid trypsin inhibitor: Effect on low alkaline tryptic activity of *O. nubilalis* larvae.

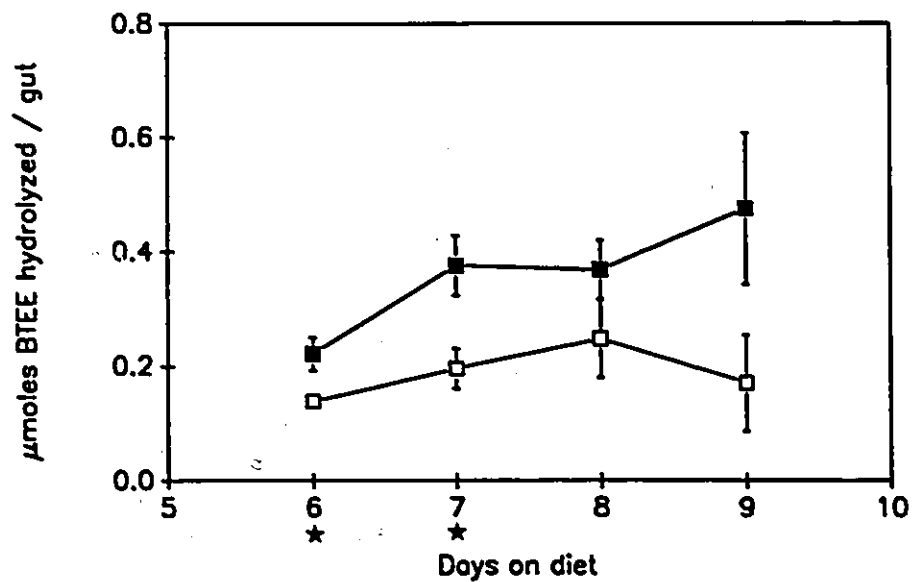


Figure 5c. Long term exposure study with 2% (W/V) chicken ovomucoid trypsin inhibitor: Effect on chymotryptic activity of *O. nubilalis* larvae.

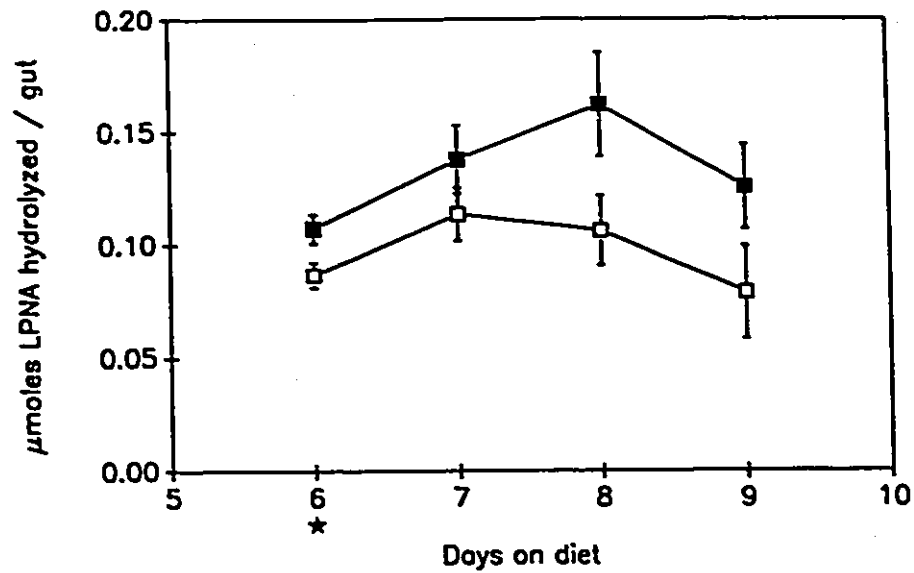


Figure 5d. Long term exposure study with 2% (W/V) chicken ovomucoid trypsin inhibitor: Effect on aminopeptidase activity of *O. nubilalis* larvae.

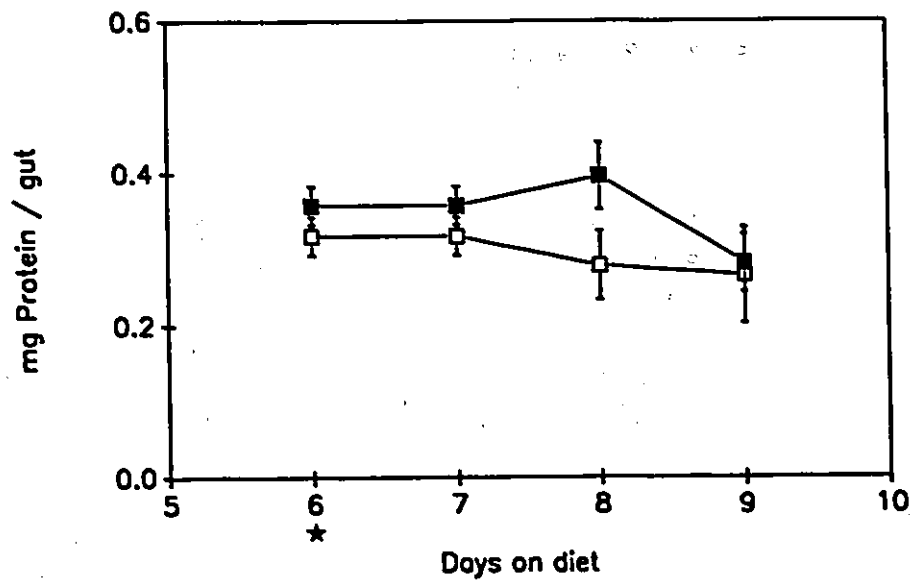


Figure 5e. Long term exposure study with 2% (W/V) chicken ovomucoid trypsin inhibitor: Effect on gut protein content of *O. nubilalis* larvae.

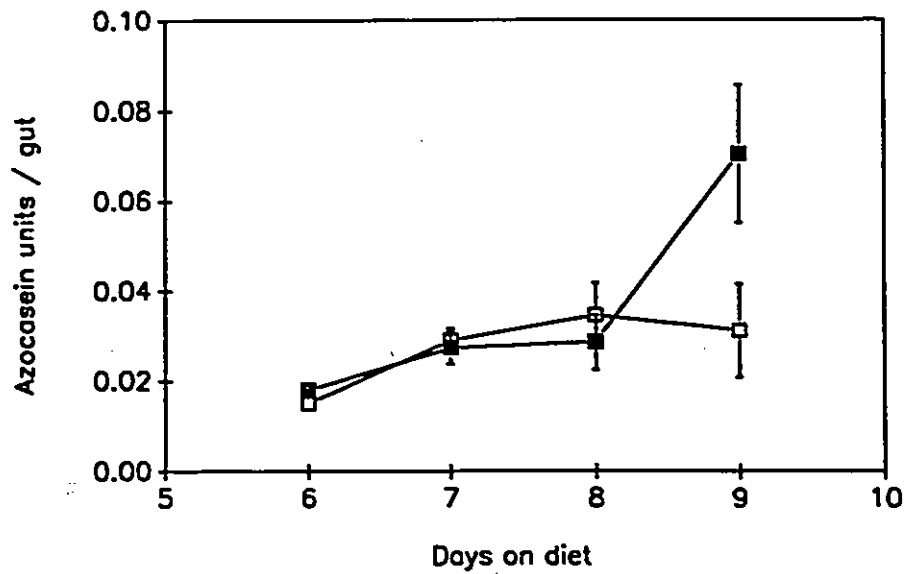


Figure 5f. Long term exposure study with 2% (W/V) chicken ovomucoid trypsin inhibitor: Effect on total proteolytic activity of *O. nubilalis* larvae.

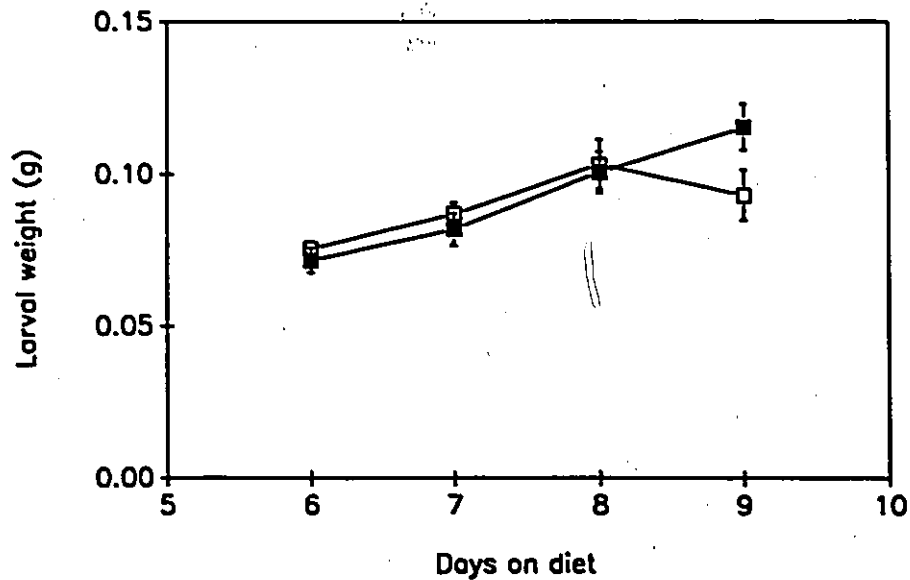


Figure 5g. Long term exposure study with 2% (W/V) chicken ovomucoid trypsin inhibitor: Effect on larval body weight of *O. nubilalis*.

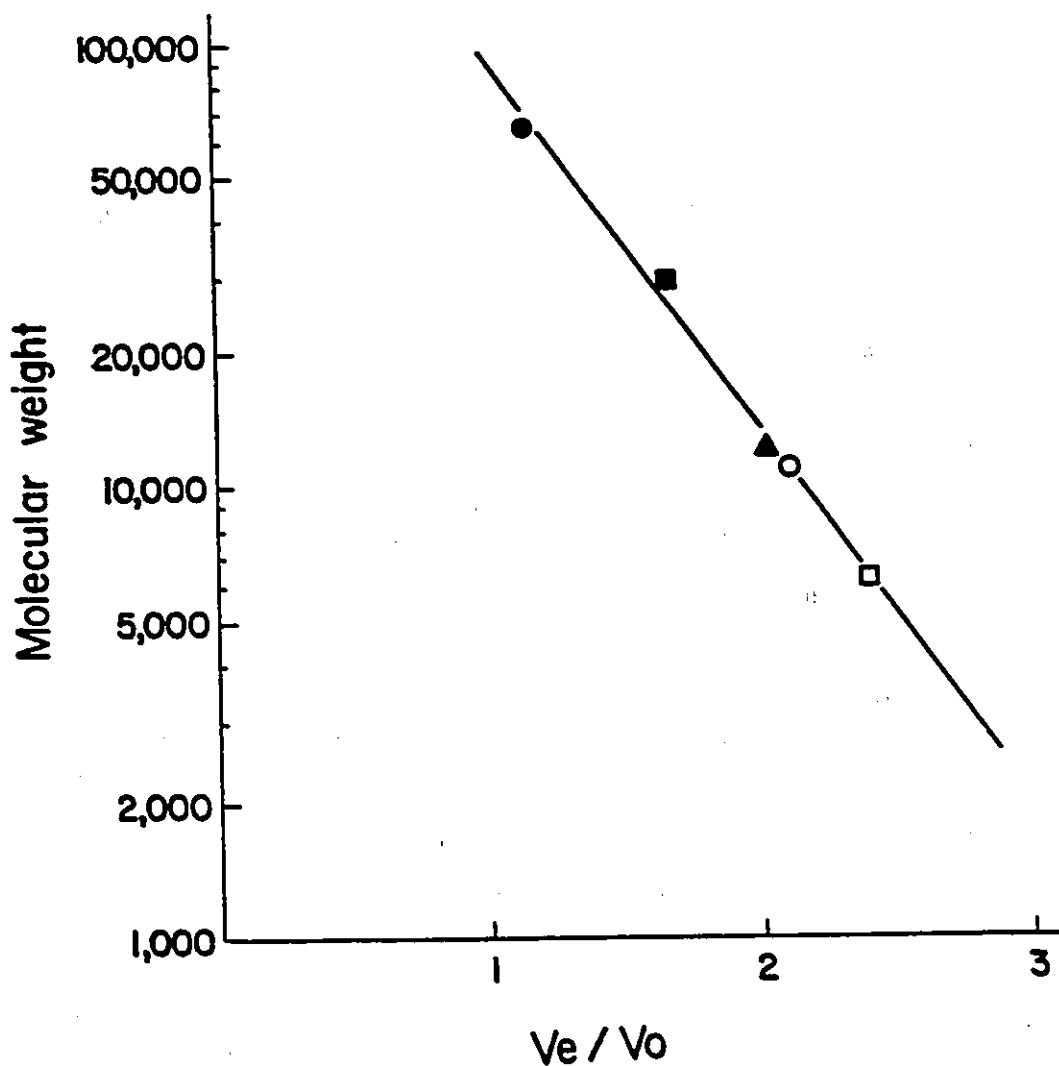


Figure 6. Molecular weight determination of the corn trypsin inhibitor.

A G-75 column (16x85cm) was equilibrated with 50 mM Tris 0.1M NaCl 10 mM CaCl_2 pH 8.0 at 25°C. The column was calibrated with aprotinin (□), cytochrome c (▲), carbonic anhydrase (■) and bovine serum albumin (●). The corn trypsin inhibitor is indicated by the (○) symbol.

Table 3. Short term exposure study with 1% (W/V) corn trypsin inhibitor.

	Control	1% CTI
High Alkaline Trypsin (BAPNA)	0.246 ± 0.024	0.174 ± 0.021*
Low Alkaline Trypsin (BAEE)	0.529 ± 0.050	0.653 ± 0.072
Chymotrypsin (BTEE)	0.182 ± 0.019	0.297 ± 0.031*
Aminopeptidase (LPNA)	0.082 ± 0.005	0.090 ± 0.007
Total Proteolysis (Azocasein)	0.021 ± 0.001	0.029 ± 0.003*
mg Protein / gut	0.260 ± 0.013	0.320 ± 0.016*
Larval Weight (g)	0.0766 ± 0.0022	0.0749 ± 0.0025

The effect of ingested corn trypsin inhibitor (CTI), 1% (W/V) in diet, on proteolytic activity, gut protein content and larval weight of *O. nubilalis*. Animals were placed on diet as 35-40 mg larvae and were allowed to feed for two days. Enzyme activity as μ moles substrate hydrolyzed/gut. Values are mean \pm S.E.M. N=24 for control insects and N=20 for treated insects. * Indicates significant differences between treated and control insects, $p \leq 0.05$.

Table 4. Short term exposure study with 1% (W/V) casein.

	Control	1% Casein
High Alkaline Trypsin (BAPNA)	0.198 ± 0.014	0.184 ± 0.012
Low Alkaline Trypsin (BAEE)	0.436 ± 0.028	0.426 ± 0.026
Chymotrypsin (BTEE)	0.169 ± 0.011	0.167 ± 0.009
Aminopeptidase (LPNA)	0.082 ± 0.003	0.094 ± 0.004*
Total Proteolysis (Azocasein)	0.019 ± 0.001	0.023 ± 0.002
mg Protein/gut	0.296 ± 0.010	0.309 ± 0.009
Larval Weight (g)	0.0750 ± 0.0012	0.0765 ± 0.0014

The effect of ingested casein 1% (W/V) in diet on proteolytic activity, gut protein content and larval weight of *O. nubilalis*. Animals were placed on diet as 35-40 mg larvae and were allowed to feed for two days. Enzyme activity as μ moles substrate hydrolyzed/gut. Experiment was repeated three times to generate a total sample size of N=53 for control insects and N=55 for treated insects. Values are the mean \pm S.E.M and * indicates significant differences between treated and control insects, $p \leq 0.05$. For results of individual runs see Appendix E.

Table 5. Short term exposure study with 2% (W/V) casein.

	Control	2% Casein
High Alkaline Trypsin (BAPNA)	0.203 ± 0.024	0.181 ± 0.018
Low Alkaline Trypsin (BAEE)	0.441 ± 0.052	0.447 ± 0.040
Chymotrypsin (BTEE)	0.151 ± 0.016	0.208 ± 0.021*
Aminopeptidase (LPNA)	0.072 ± 0.004	0.075 ± 0.005
Total Proteolysis (Azocasein)	0.017 ± 0.001	0.016 ± 0.001
mg Protein / gut	0.294 ± 0.021	0.342 ± 0.009*
Larval Weight (g)	0.0658 ± 0.0021	0.0774 ± 0.0014*

The effect of ingested casein, 2% (W/V) in diet, on proteolytic activity, gut protein content and larval weight of *O. nubilalis*. Animals were placed on diet as 35-40 mg larvae and were allowed to feed for two days. Enzyme activity as μ moles substrate hydrolyzed/gut. Values are mean \pm S.E.M. N=20 for control insects and N=18 for casein treated insects. * Indicates significant differences between treated and control insects, $p \leq 0.05$.

There were no significant differences between control and treated insects for high or low alkaline tryptic activity, aminopeptidase activity or total proteolysis (Table 5). In the long term exposure study with 2% (W/V) casein larval weight of treated insects was significantly lower on day 6 (Fig 7a). There were no significant differences between control and treated insects for weight on days 7, 8, or 9 and no significant differences for high or low alkaline tryptic activity, chymotryptic activity, aminopeptidase activity, total proteolysis, or protein content of the gut on days 6, 7, 8, or 9 (Fig. 7a, 7b, 7c, 7d, 7e, 7f, 7g).

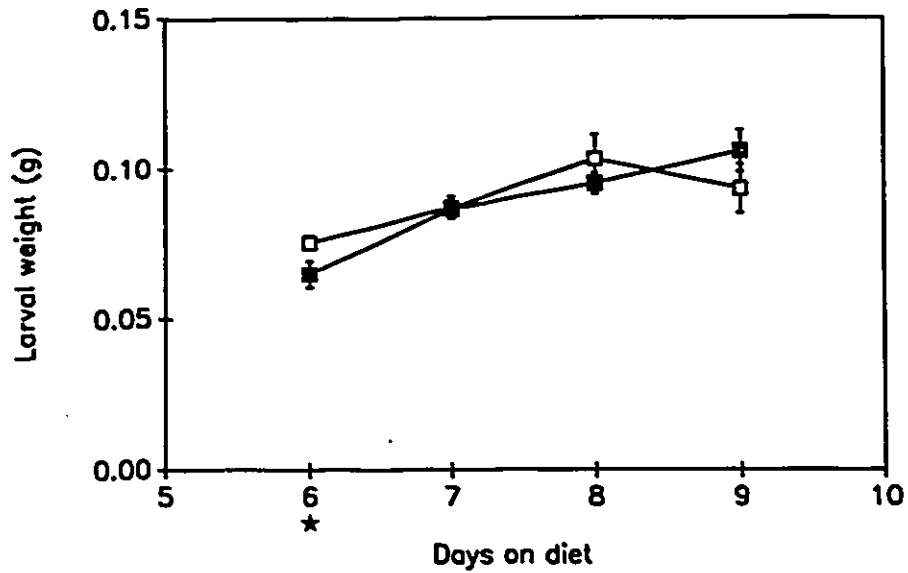


Figure 7a. Long term exposure study with 2% (W/V) casein: Effect on larval body weight of *O. nubilalis*.

Animals were placed on diet containing 2% (W/V) casein as 7-9 mg larvae and were allowed to feed for 6-9 days. Enzyme activity as μ moles substrate hydrolyzed/gut. N=7-10 for control (\square) and inhibitor treated (\blacksquare) insects with samples taken on days 6-9. Values are mean \pm S.E.M. and \star indicates significant differences between treated and control insects, $p \leq 0.05$.

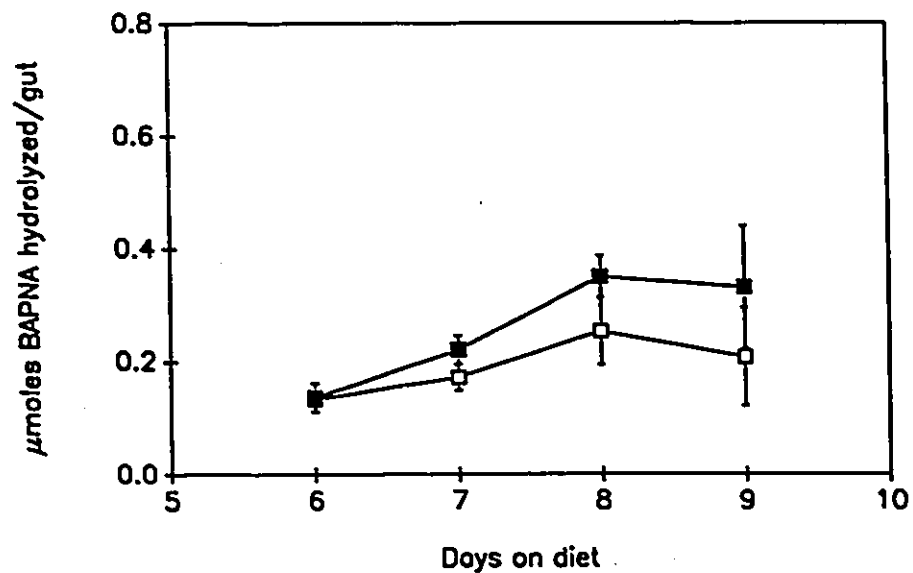


Figure 7b. Long term exposure study with 2% (W/V) casein: Effect on high alkaline tryptic activity of *O. nubilalis* larvae.

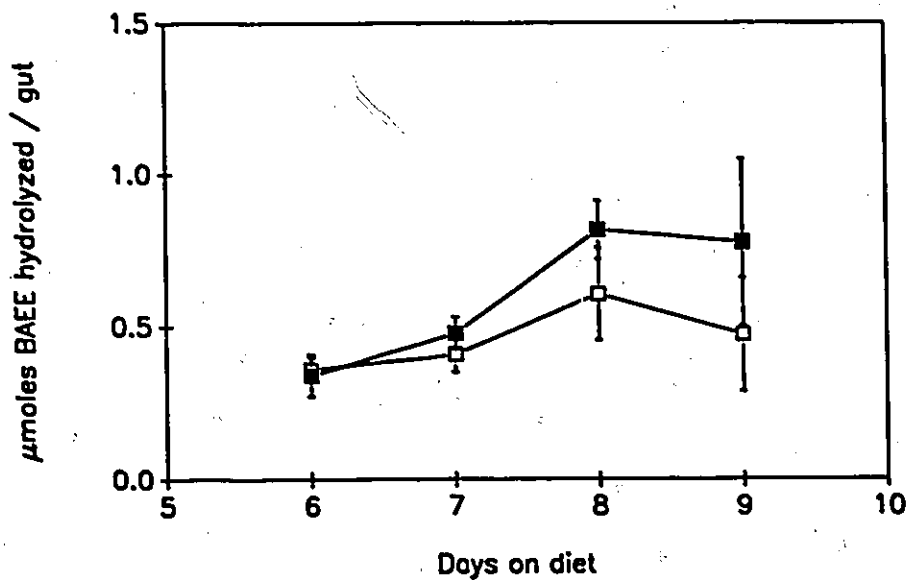


Figure 7c. Long term exposure study with 2% (W/V) casein: Effect on low alkaline tryptic activity of *O. nubilalis* larvae.

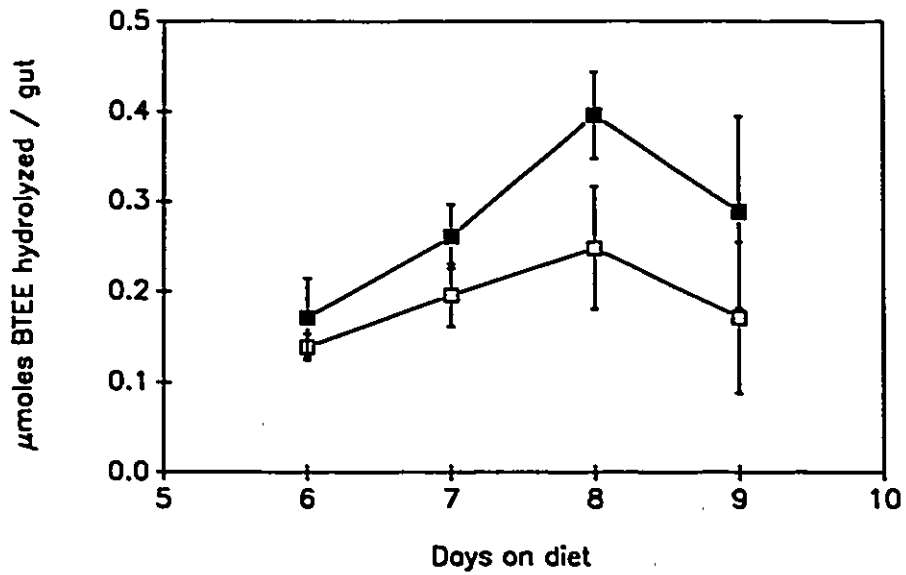


Figure 7d. Long term exposure study with 2% (W/V) casein: Effect on chymotryptic activity of *O. nubilalis* larvae.

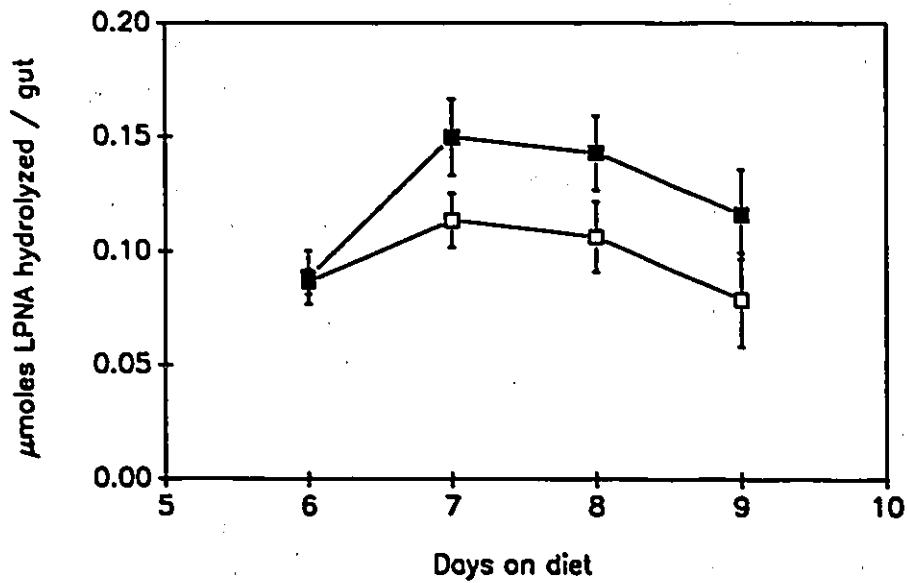


Figure 7e. Long term exposure study with 2% (W/V) casein: Effect on aminopeptidase activity of *O. nubilalis* larvae.

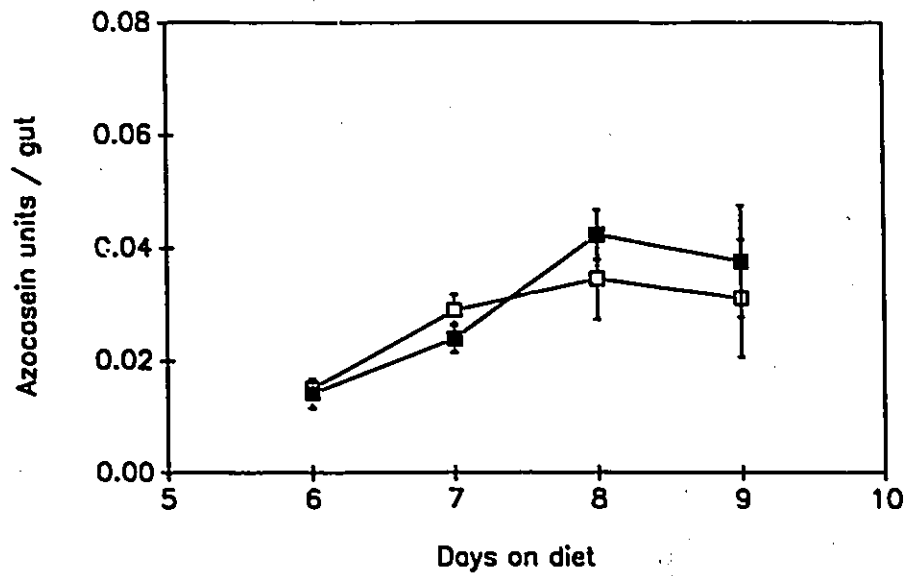


Figure 7f. Long term exposure study with 2% (W/V) casein: Effect on total proteolytic activity of *O. nubilalis* larvae.

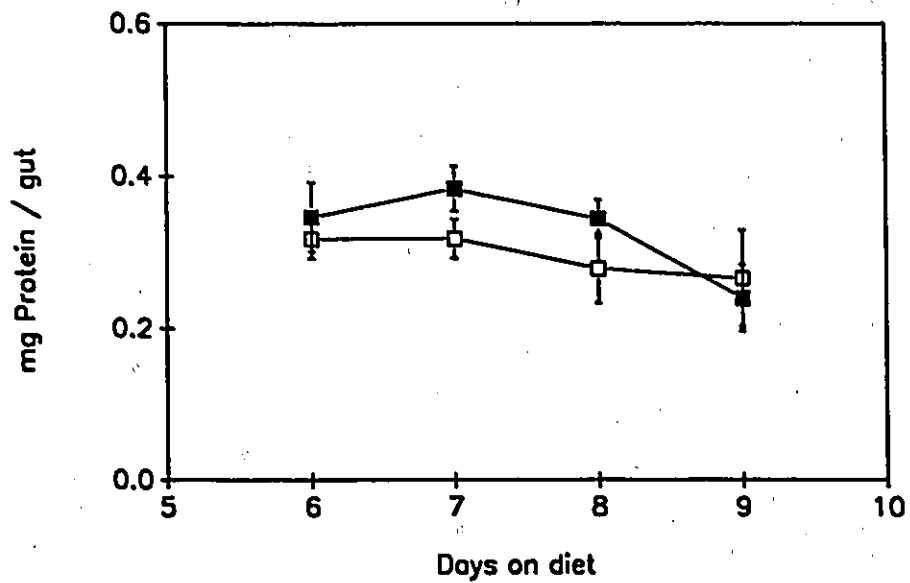


Figure 7g. Long term exposure study with 2% (W/V) casein: Effect on gut protein content of *O. nubilalis* larvae.

3.2 EFFECT OF TEST DIETS ON DEVELOPMENT

When insects ingested a diet containing either 1% (W/V) casein, soybean trypsin inhibitor or chicken ovomucoid trypsin inhibitor the average number of days to pupation was 13 for both control and treated insects. The average number of days to reach the adult stage was 21 for control, soybean and ovomucoid inhibitor treated insects. For casein treated insects the average number of days to reach the adult stage was 20.

Chapter IV

DISCUSSION

4.1 EFFECT OF TEST DIETS ON DIGESTION AND DEVELOPMENT

The fact that a crude gut homogenate was used to measure proteolytic activity was one factor that accounted for the variability seen in this study. Proteases were separated on the basis of the pH at which maximal substrate hydrolysis occurs and the effect of potential activators and inhibitors (Houseman *et al.* 1989). However, under such conditions there was the possibility for cross reaction. Both high and low alkaline trypsin prefer to cleave peptide bonds adjacent to an arginine and although assay conditions may have been set for either trypsin the other trypsin may have also contributed to hydrolysis of the substrate but under conditions that were not optimal. Cross reaction by other proteases for chymotryptic and aminopeptidase activity was unlikely since synthetic substrates that are unique for either protease were used. Houseman (unpublished) found that when larval midgut tissue was homogenized in detergent (Tubrol px) that high alkaline tryptic activity increased and the activities of low alkaline trypsin and chymotrypsin did not change. These results indicated limitations of the extraction procedure used in this study to measure high alkaline tryptic activity accurately. However, the work of Houseman (unpublished) also pointed out that the extraction procedure used did not affect the activity of chymotrypsin and this combined with the unlikely possibility of cross reaction with other proteases explained why this enzyme was the least variable in its activity. Chymotryptic activity was a reliable indicator for proteolytic activity. To prevent changes in proteolytic activity due to differences in weight an initial weight within a limited range was used for both the short term and long term

exposure studies. However, the raw data for the development study showed that not all insects pupated on the same day but rather over a range of days. In a small sample size any insect that did not develop at the same rate biased the results for proteolytic activity and was another source of variability. In summary, the potential sources of variability in this study were a crude gut homogenate to measure proteolytic activity, the extraction procedure, and the insects.

Also essential before the discussion is a clarification of the terms used in this study. Enzyme activity, as measured in this study, refers to the number of micromoles of substrate hydrolyzed/gut. Enzyme levels or the amount of an enzyme present is a balance between enzyme production and enzyme elimination. Although enzyme levels were not measured in this study, it was assumed that the activity of a specific enzyme was proportional to the level of this enzyme within the midgut. According to this assumption then, a decrease in enzyme activity would mean either a decrease in production and/or an increase in elimination. Alternatively, if the role of the inhibitor was considered, a reduction in enzyme activity could be due to direct binding of the inhibitor to the enzyme rendering it inactive.

4.1.1 SOYBEAN TRYPSIN INHIBITOR

Three consistent results were seen when an insect ingested a diet containing 1% (W/V) soybean trypsin inhibitor. The first was a significant reduction in chymotryptic activity, as seen in the preliminary study (Fig. 2a), short term exposure study (Table 1) and the long term exposure study (Fig. 3a). The second result was a significantly higher protein content of the gut in treated insects (Fig. 2b, Table 1, Fig. 3c). Finally, a reduction in larval weight was observed in the preliminary study (Fig. 2c), short term exposure study (Table 1) and the long term exposure study (Fig. 3d) with the last two being significant. Although the conclusions stated are based on the combined data, the results of the individual runs also support them (Appendix A, B, C).

If the role of the soybean trypsin inhibitor is considered, a reduction in chymotryptic activity could be due to direct binding of the inhibitor to the enzyme rendering it inactive. Knowledge of the binding interaction between bovine chymotrypsin and the soybean trypsin inhibitor and characteristics of the chymotrypsin-like proteinase from the corn borer (Houseman *et al.* 1989) can be used to support the concept of inhibition of corn borer chymotryptic activity by the soybean trypsin inhibitor. There are two binding sites on the soybean trypsin inhibitor (Quast and Steffen 1975) for bovine chymotrypsin. The first binding site is also the trypsin binding site, Arg⁶³-Ile⁶⁴ (De Vonis Bidlingmeyer *et al.* 1972) and the second site is thought to be around the Met⁸⁴-Leu bond (Bosterling and Quast 1981). Houseman *et al.* (1989) report that a chymotrypsin-like proteinase from the corn borer shares several features with bovine chymotrypsin including inhibition by the soybean trypsin inhibitor and optimal hydrolysis of the chymotrypsin substrate BTEE at pH 7.5-8.0. Houseman *et al.* (1989) show that when the soybean trypsin inhibitor is added to the assay for chymotrypsin it is capable of binding to the enzyme resulting in a reduction of corn borer chymotryptic activity. When the inhibitor is ingested, inhibition of chymotryptic activity was reflected in a significant reduction of chymotryptic activity.

Possible explanations for an increased gut protein content in treated insects include decreased assimilation of the diet or delayed movement of the diet. One could argue that addition of 1% (W/V) soybean trypsin inhibitor is equivalent to the addition of 1% (W/V) protein to the diet which should result in a significantly higher gut protein content when ingested. However, ingestion of 1% (W/V) casein by insects did not result in a significant increase in gut protein content of treated insects but rather a significant increase in aminopeptidase activity (Table 4). If an equivalent amount of casein ingested did not result in a significantly higher gut protein content of treated insects, ingestion of 1% (W/V) soybean trypsin inhibitor must have affected the effi-

ciency of the midgut proteases to break down protein. Consistent with undigested protein remaining in the midgut is a reduction in chymotryptic activity. To test this idea that there is undigested protein in the gut a study could be done similar to that of Broadway and Duffey (1986a) where casein in the diet is replaced by an equivalent amount of azocasein, the logic being that when the protein is digested in the gut a chromophore is released which can be detected in the insect frass.

A significant reduction in larval weight together with a significant reduction in chymotryptic activity and a significantly higher gut protein content indicate that the digestive system of the corn borer had been disrupted. The combined data did not indicate a significant reduction of the activities for high or low alkaline trypsin, aminopeptidase or total proteolysis. No reduction trends were seen in the results of the individual runs for any of these activities. Another indication of disruption is seen in the long term exposure study. Activity levels for high and low alkaline trypsin, chymotrypsin and total proteolysis drop between days 8 and 9 in control insects (Fig. 3e, 3f, 3a, 3g). This was not seen in inhibitor treated insects. This type of pattern, where proteolytic activity peaks at some point during the larval life cycle and subsequently declines prior to pupation is observed in larvae of other lepidoptera such as *B. mori* (Eguchi and Iwamoto 1976), *S. litura* (Ahmad *et al.* 1976), *S. exigua* (Broadway and Duffey 1986a) and *H. zea* (Broadway and Duffey 1986a). This disruption, however, is not detrimental to the insect since it was able to complete its development at a rate comparable to control insects.

Two unexpected results were seen with low alkaline tryptic activity and aminopeptidase activity. The combined results showed that low alkaline tryptic activity was significantly higher in treated insects in the short term exposure study (Table 1). Assuming that the activity of low alkaline trypsin is proportional to the level of this enzyme within the midgut of the corn borer, then higher activity levels for this enzyme could be

due to increased production or reduced elimination. Although production or elimination of this enzyme were not measured, one can speculate on the cause of this increase in activity based on other results within this study and on work done in the literature. It is possible that the corn borer compensated for the reduction in chymotryptic activity by increasing the production of low alkaline trypsin. This situation is similar to the study of Broadway and Duffey (1986b) where chronic ingestion of the soybean trypsin inhibitor results in hyperproduction of trypsin in *S. exigua* and *H. zea*. Assuming that the significantly higher gut protein content within the midguts of treated corn borer larvae represents undigested diet, our results differ from Broadway and Duffey (1986b) in that the level of digested protein decreased while in the cited study there was no change in the level of digested protein in treated insects. Aminopeptidase activity was significantly higher on days 5 and 8 in treated insects for the combined results (Fig. 3b). Since aminopeptidase is known to be an intracellular enzyme and would therefore not come into contact with the inhibitor which is in the lumen of the midgut, then these results seen are not related to ingestion of the inhibitor but may be due to some unrelated factor.

Houseman *et al.* (1989) found that high and low alkaline tryptic activities are inhibited when the soybean trypsin inhibitor is added to the respective assays for the corn borer. The question arises as to why inhibition of both tryptic activities was seen when the inhibitor was added to the respective enzyme assay but not when the inhibitor was ingested. Inhibition of the tryptic activities when the inhibitor was added to the enzyme assays shows that the inhibitor is physically capable of binding to either enzyme. Assuming that the chymotrypsin-like proteinase from the corn borer binds to the same two sites on the soybean trypsin inhibitor as bovine chymotrypsin, one of which is the trypsin binding site, perhaps either trypsin is prevented from binding to this site due to blockage by chymotrypsin.

4.1.2 CHICKEN OVOMUCOID TRYPSIN INHIBITOR

The results indicated that proteolytic activity was significantly higher in insects that consumed a diet containing either 1.5% (W/V) or 2% (W/V) chicken ovomucoid trypsin inhibitor. This was seen in the preliminary study with 1.5% (W/V) inhibitor where high and low alkaline tryptic activities and total proteolysis were significantly higher in treated insects (Fig. 4a, 4b, 4c). The results of the individual replicates support the conclusions based on the combined data for ingestion of 1.5% (W/V) ovomucoid trypsin inhibitor (Appendix D). Higher proteolytic activity was also seen with 2% (W/V) inhibitor where treated insects had significantly higher chymotryptic activity in both the short term (Table 2) and long term exposure study (Fig. 5c) in addition to significantly higher values for both tryptic activities in the long term exposure study (Fig. 5a, 5b).

Two interesting questions can be posed from these results. The first addresses the question of why chicken ovomucoid is not an effective inhibitor of corn borer proteases and the second asks why there is higher proteolytic activity in ovomucoid inhibitor treated insects.

The chicken ovomucoid molecule is composed of three domains where each domain contains a potential reactive site for a serine proteinase (Kato *et al.* 1987). The reactive site in the first domain, Lys²⁴-Asp, inhibits a trypsin-like enzyme from *Streptomyces erythraeus* (Nagata and Yoshida 1984) while the reactive site in the second domain, Arg⁸⁹-Ala, inhibits bovine trypsin. Kato *et al.* (1987) refer to a study where the reactive site in the third domain, Ala¹⁴⁸-Glu is a weak inhibitor of bovine chymotrypsin. Kato *et al.* (1987) state that each of these domains is active as an inhibitor. Houseman *et al.* (1989) found that when 5 or 10 μ g of this inhibitor are added to the assays for high or low alkaline trypsin or chymotrypsin that activities of the respective enzymes are not reduced. Based on the substrates used to measure both tryptic activities of the corn borer, BAPNA and BAEE, it can be assumed that these two pro-

teinasases prefer to cleave internal peptide bonds adjacent to arginine. The second domain of the inhibitor has the reactive site Arg⁸⁹-Ala. Based on the preferences of the two trypsins, one would expect association between inhibitor and either enzyme at this reactive site. A possible explanation of why this does not occur is that the site is recognized by either trypsin but for conformational reasons neither enzyme can bind. Based on the preference of the chymotrypsin-like proteinase from the corn borer for peptide bonds adjacent to tyrosine, one would not expect association with any of the three possible reactive sites on the inhibitor.

Insects that had ingested a diet containing either 2% (W/V) inhibitor (Table 2) or 2% (W/V) casein (Table 5) had significantly higher chymotryptic activity in the short term exposure study but casein treated insects also had significantly higher gut protein content and larval weight (Table 5). The results for the long term exposure study were not similar. In inhibitor treated insects activity of high and low alkaline trypsin were significantly higher on days 7 and 9 while chymotryptic activity was significantly higher on days 6 and 7 (Fig. 5a, 5b, 5c). If the confidence level is reduced to 90%, significantly higher chymotryptic activity and total proteolytic activity are seen on day 9 (Fig. 5c, 5f). A significant increase in proteolytic activity was not observed in casein treated insects (Fig. 7b, 7c, 7d, 7e, 7f). Although activity was higher in casein treated insects for both trypsins, chymotrypsin and aminopeptidase, the overall pattern of activity for each enzyme was similar to control insects over the period day 6 to day 9 (Fig. 7b, 7c, 7d, 7e). Since the results for the long term exposure study were not similar for both casein and ovomucoid treated insects it seems unlikely that an increase in protein content alone can explain the results seen for ovomucoid treated insects. An alternative explanation is protein quality. It is known that ovomucoid inhibitors contain large amounts of cysteine (Osuga and Feeney 1968). Broadway and Duffey (1986b) state that sulphur-containing amino acids are required for production of proteolytic enzymes.

The sulphur-rich diet of the ovomucoid inhibitor may result in an increase in the production of proteolytic enzymes. This increase in production was perhaps reflected in the significantly higher activities for either trypsins and chymotrypsin seen in the long term exposure study. Although larval weight in ovomucoid treated insects was significantly higher at the 90% confidence level at day 9 (Fig. 5g), the time to pupation was identical for both control and treated insects.

An indication that the digestive system of the corn borer had been disrupted is seen on day 9 of the long term exposure study where activities for both trypsins are significantly higher at the 95% confidence level (Fig. 5a, 5b) while chymotryptic activity and total proteolysis are significantly higher at the 90% confidence level (Fig. 5c, 5f). Looking at the control values for each enzyme over the 6-9 day period one can see that enzyme activity peaks and then declines between days 8 and 9. This decrease in activity must be due to decreased production and/or increased elimination. Since proteolytic activity and presumably enzyme levels are higher in inhibitor treated insects perhaps the time for elimination was prolonged in treated insects as a result of increased enzyme levels compared to control insects.

4.1.3 CORN TRYPSIN INHIBITOR

Both affinity chromatography (Hochstrasser *et al.* 1967, 1970, Swartz *et al.* 1977, Johnson *et al.* 1980 and Mahoney *et al.* 1984) and the immunoabsorbent technique (Corfman and Reeck 1982) have been used in the purification procedure for the corn trypsin inhibitor. Hochstrasser *et al.* (1967) report a molecular weight of approximately 19,000 daltons for the intact inhibitor. The intact inhibitor consists of three subunits where each subunit has a molecular weight of 6,500 daltons (Hochstrasser *et al.* 1970). Based on amino acid sequence Mahoney *et al.* (1984) report a molecular weight of 12,028 daltons and Swartz *et al.* (1977) report a molecular weight of 12,500 daltons. Using an extraction procedure similar to that of Mahoney *et al.* (1984) and Swartz *et*

al. (1977) , the molecular weight determined in this study using gel filtration chromatography was found to be 11,500 daltons (Figure 6). This value is in agreement with that obtained by Mahoney *et al.* (1984) and Swartz *et al.* (1977).

When insects ingested a diet containing 1% (W/V) corn trypsin inhibitor for a period of two days, proteolytic activity was disrupted (Table 3). High alkaline tryptic activity was significantly lower in treated insects while chymotryptic activity and total proteolytic activity were significantly higher in treated insects. Gut protein content of treated insects was significantly higher compared to control insects. There was not a significant difference in larval weight between control and treated insects.

This disruption of proteolytic activity in inhibitor treated insects appeared not to be detrimental since there was not a significant difference in weight between the two groups. Of course this cannot be confirmed until the effects on development are measured by addition of the inhibitor at the 1% (W/V) level to diet. Houseman and Larocque (1987) found that when 5 or 10 μg of crude corn trypsin inhibitor extract was added to the assays for both trypsins and chymotrypsin that no reduction in proteolytic activity was observed. When the same amounts were added to the assays for bovine trypsin and chymotrypsin reduction in the activity of either proteinase was seen. The reactive site for bovine trypsin is at Arg³⁶-Ile³⁷ on the corn trypsin inhibitor (Mahoney *et al.* 1984). The results of Houseman *et al.* (1989) with corn trypsin inhibitor and corn borer proteases cannot be explained by amino acid preference at the reactive site alone. Both trypsins prefer to cleave peptide bonds adjacent to an arginine and one would expect association between either enzyme and the reactive site on the corn trypsin inhibitor. Possible explanations as to why inhibition of the trypsins was not seen is that the inhibitor is not in a conformation that allows combination with either trypsin even though an arginine is at the reactive site. Alternatively impurities in the crude extract may have prevented combination. The chymotrypsin-like proteinase, based on

the use of BTEE to measure activity, prefers to cleave a peptide bond adjacent to tyrosine. Based on amino acid preference one would not expect association between the inhibitor at the Arg³⁶-Ile³⁷ reactive site and the chymotrypsin-like proteinase from the corn borer.

If combination of inhibitor and corn borer proteinases did not occur when the inhibitor was added to the assays for either trypsins or chymotrypsin, then why was disruption of proteolytic activity seen when the inhibitor was ingested? The following is proposed to explain the results observed. Within the gut of the corn borer the inhibitor undergoes a conformational change that allows combination with high alkaline trypsin. This combination between inhibitor and enzyme is reflected in a significant reduction in high alkaline tryptic activity. Significantly higher gut protein content in treated insects represents undigested protein. Houseman *et al.* (1989) report that high alkaline trypsin appears to be the major gut proteinase since its pH activity curve resembles that of total proteolysis. The corn borer compensates for the reduction in the activity of this proteinase by increasing the levels of chymotrypsin. This increase is reflected in the increase in activity of chymotrypsin. The increase in activity of chymotrypsin would also explain why total proteolytic activity was significantly higher in treated insects. This increase in proteolytic activity does not appear to completely compensate for the reduction in high alkaline tryptic activity since protein content of the gut is significantly higher in treated insects. Whether this significantly higher gut protein content actually represents undigested diet cannot be determined for certain until the level of digested protein is measured. Assuming there is undigested protein left within the gut, the amount digested by treated insects appeared to be sufficient for growth since there was not a significant difference between the two groups for larval weight. A better understanding could be ascertained if the inhibitor was also studied in a long term exposure study and in a developmental study. This was not possible in the current study due to low yields obtained in the extraction procedure for the corn trypsin inhibitor.

4.1.4 PROTEASE INHIBITORS AND THE CORN BORER: A SUMMARY

The hypothesis of this work was that inhibitors within the host plant range of corn borer larvae would not affect proteolytic activity or development while inhibitors outside of its host plant range would have a negative effect on these two factors, and that there would be an inverse relationship between host plant preference and the ability of ingested protease inhibitors to disrupt digestive processes. To test this hypothesis inhibitors were chosen from the insect's preferred host corn, from soybeans and from a source not normally encountered in nature, chicken egg white. All three inhibitors, regardless of source, are bovine trypsin inhibitors where the reactive site on each respective inhibitor is known as well as the kinetics of complex formation with bovine trypsin. Although corn is the major host for the corn borer, Hudon and LeRoux (1986) state that infestations of other large stem plants are not uncommon, with soybeans being reported as one of the several examples. It was surprising to see disruption of proteolytic activity in corn inhibitor treated insects. The insect appears to compensate for the reduction in activity of high alkaline trypsin by increased activities of chymotrypsin and total proteolysis. Although a developmental study was not done, this disruption of proteolytic activity does not appear to be detrimental since there was not a significant difference in larval weight between treatments. These results differ from the hypothesis because the corn trypsin inhibitor which is present within the corn borer's primary host, corn, did disrupt proteolytic activity but apparently not growth. It appears that the corn borer has adapted to feed on its host by compensating for a reduction in the activity of one of its proteinases and is able to break down enough protein to complete development. A similar pattern was seen with the soybean trypsin inhibitor although larval weight was significantly lower in treated insects. With the soybean trypsin inhibitor chymotryptic activity was significantly reduced in treated insects while significantly higher low alkaline tryptic activity was also seen in the short term

exposure study. As with the corn trypsin inhibitor despite a reduction in proteolytic activity as indicated by significantly higher gut protein content, the insect is able to hydrolyze enough protein to complete its development. Based on these results the hypothesis of this study is rejected and it is concluded that inhibitors within the host plant range of corn borer larvae may disrupt proteolytic activity but the insect is able to compensate for this and complete its development. An inverse relationship between host plant preference and the ability of ingested protease inhibitors to disrupt digestive processes was not seen in this study. Perhaps this is due to the fact that the corn borer is a polyphagous insect and has adapted to feed on a broad host plant range that may contain numerous inhibitors. This inverse relationship would perhaps be more evident in an insect with a restricted host plant range. For example, Shukle and Murdock (1983) found that when *M. sexta* larvae, whose preferred host is tobacco, consumes a diet containing 5% soybean trypsin inhibitor that there is a significant decrease in body weight compared to control insects. Similarly, Hilder *et al.* (1987) found that when *H. virescens* larvae, whose preferred host is tobacco, consumes tobacco plants transformed with the cowpea trypsin inhibitor that insect survival is reduced compared to control insects. The lack of a reduction in proteolytic activity when the chicken ovomucoid trypsin inhibitor was ingested was not surprising since this inhibitor would not be normally encountered in nature by the insect. The increase in proteolytic activity in treated insects however was a surprise.

Steffens *et al.* (1978) found that when corn borer larvae consume a diet containing 3% soybean trypsin inhibitor that weight is reduced and pupation is delayed compared to control insects. However, it was not stated if these results were statistically significant. Comparison of these results to the current work is difficult since 1% (W/V) soybean trypsin inhibitor was used. Concentrations above 1.5% (W/V) soybean trypsin inhibitor were not attempted due to the difficulty of getting 1.5% (W/V) inhibitor into

solution within the diet mixture. A significant reduction in larval weight was seen in the short term exposure study and on day 7 of the long term exposure study. However, a delay in pupation was not seen in soybean inhibitor treated insects. Whether this difference between the current study and the work of Steffens *et al.* (1978) is due to the inhibitor or some unrelated factor is unknown. The current work showed that in a short term exposure study with 1% (W/V) corn trypsin inhibitor that weight in treated insects was not statistically different from control insects. This result is similar to the work of Steffens *et al.* (1978) where there was not a difference in weight between control insects and insects treated with 3% (W/V) corn trypsin inhibitor throughout the larval life cycle.

Based on the current work with the corn borer, the role of ingested protease inhibitors appears to be different in phytophagous insects compared to haematophagous insects. In haematophagous insects it is speculated that ingested inhibitors are used as the stimulants for protease production (Gooding 1977). Houseman *et al.* (1988) show that when stable flies are fed whole blood, serum, or plasma, all of which contain protease inhibitors, tryptic activity in the posterior midgut increases more than 10-fold and protein content of the posterior midgut correlates significantly with tryptic activity. The mechanism of protease production is not well understood in corn borer larvae. Ingestion of 1% (W/V) soybean trypsin inhibitor resulted in a significant reduction in chymotryptic activity but also significantly higher low alkaline tryptic activity in the short term exposure study while ingestion of 1% (W/V) corn trypsin inhibitor resulted in a significant reduction of high alkaline tryptic activity but also significantly higher chymotryptic and total proteolytic activity. This pattern of both an increase and a decrease in proteolytic activity when either the soybean trypsin inhibitor or corn trypsin inhibitor were ingested indicate that it is unlikely that the ingested inhibitors are acting as the stimulants for protease production. Alternately, increased proteolytic activity could be in response to enzyme inhibition.

A discussion of the practical implications of these results is important. Based on the current study neither the soybean trypsin inhibitor nor the corn trypsin inhibitor are ideal candidates for plant breeding programs. Despite inhibition of chymotryptic activity with the soybean trypsin inhibitor and high alkaline tryptic activity with the corn trypsin inhibitor the corn borer is able to complete its development at a rate comparable to control insects. Although a developmental study was not done with the corn trypsin inhibitor, it is assumed that lack of a significant difference in larval weight for treated insects for the short term exposure study indicates that treated insects are developing at a rate comparable to control insects. The question can be raised as to the relevance of these findings to the natural situation in the corn plant where protein quality and quantity are lower. Perhaps the toxicity of the soybean trypsin inhibitor would be manifested if the insect was reared on a protein poor source. Broadway and Duffey (1988) found that protein quality, in particular the arginine and lysine content, changes the toxicity of the soybean trypsin inhibitor to larvae of *S. exigua* where the inhibitor is less toxic when the insect is fed a nutritiously high protein source. The work with the chicken ovomucoid trypsin inhibitor and corn borer proteases has an important theoretical implication. The majority of plant protease inhibitors have been screened using bovine trypsin and chymotrypsin. The current work showed that chicken ovomucoid, an inhibitor of bovine trypsin, did not inhibit either trypsins of the corn borer when ingested. It is important that defense theories for plant protease inhibitors be based on studies with insect proteases.

4.1.5 SUGGESTED FUTURE WORK

The effect of protein quality and/or quantity on the results observed with the soybean trypsin inhibitor would be interesting to pursue. A combination of protein quality and quantity similar to that in the corn plant could be incorporated into the artificial diet for the corn borer in order to mimic conditions in the corn plant.

Despite differences in proteolytic activity between the different test diets the average number of days to reach the pupal and adult stages were similar. It would also be interesting to enlarge this developmental study to include pupal weights and fecundity. The implication of higher larval weight in ovomucoid inhibitor treated insects at day 9 of the long term exposure study with respect to pupal weight and fecundity is also interesting.

Regulation of protease production in corn borer larvae and the effect of ingested protease inhibitors on this would be of interest. When either the soybean or chicken ovomucoid trypsin inhibitors were ingested in the long term exposure study proteolytic activity did not decline between days 8 and 9 as seen in control insects suggesting that either inhibitor may affect protease regulation.

4.2 GENERAL DISCUSSION

Plant protease inhibitors appear to be an ideal control agent for insect pests. Is this actually the case?

Green and Ryan (1972) present an attractive model where wounding of leaf tissue causes accumulation of digestive enzyme inhibitors throughout the whole plant. This model was reinforced when the gene encoding for the cowpea trypsin inhibitor, when transferred to tobacco, enhanced resistance to the plants own pest, *H. virescens* (Hilder *et al.* 1987).

The majority of plant protease inhibitors have been screened for using bovine trypsin and chymotrypsin. Work on ingested inhibitors for insects has been focussed on growth studies (Broadway *et al.* 1986, Shukle and Murdock 1983, Steffens *et al.* 1978, Hilder *et al.* 1987, Gatehouse and Boulter 1983, Benz *et al.* 1985), with less work done on proteolytic studies (Broadway and Duffey 1986b, 1988). The current work is the first that has used optimized enzyme assays for the study of ingested enzyme inhibitors.

Despite disruption of proteolytic activity this study has shown that the corn borer is able to compensate for this and complete its development at a rate comparable to control insects.

Appendix A

Preliminary study with 0.5, 1 and 1.5% (W/V) soybean trypsin inhibitor: Results of individual runs and values for standard error of the mean.

Chymotrypsin (μ Moles BTEE hydrolyzed/gut)

	0	0.5%	1%	1.5%
Combined	0.186 \pm 0.027	0.076 \pm 0.008*	0.080 \pm 0.007*	0.077 \pm 0.010*
Run 1	0.146 \pm 0.039	0.058 \pm 0.012	0.079 \pm 0.008	0.060 \pm 0.010
Run 2	0.237 \pm 0.028	0.095 \pm 0.007*	0.081 \pm 0.012*	0.095 \pm 0.015*

mg Protein/gut

	0	0.5%	1%	1.5%
Combined	0.292 \pm 0.015	0.345 \pm 0.025	0.394 \pm 0.020*	0.404 \pm 0.020*
Run 1	0.308 \pm 0.022	0.363 \pm 0.033	0.379 \pm 0.024*	0.375 \pm 0.020*
Run 2	0.271 \pm 0.019	0.327 \pm 0.039	0.410 \pm 0.033*	0.434 \pm 0.031*

Values are mean \pm S.E.M. * Indicates significant differences between treated and control insects, $p \leq 0.05$. For Run 1 N=9 and N=7 for control and treated insects respectively and for Run 2 N=7 for control and treated insects.

Preliminary study with 0.5, 1 and 1.5% (W/V) soybean trypsin inhibitor: Results of individual runs and values for standard error of the mean.

		Larval Weight (g)			
		0	0.5%	1%	1.5%
Combined		0.0819 ± 0.0030	0.0769 ± 0.0032	0.0757 ± 0.0025	0.0706 ± 0.0024*
Run 1		0.0816 ± 0.0051	0.0748 ± 0.0052	0.0738 ± 0.0034	0.0685 ± 0.0039
Run 2		0.0822 ± 0.0025	0.0790 ± 0.0039	0.0776 ± 0.0039	0.0727 ± 0.0031*

High Alkaline Trypsin (μ Moles BAPNA hydrolyzed/gut)

		0	0.5%	1%	1.5%
Combined		0.255 ± 0.024	0.247 ± 0.028	0.244 ± 0.039	0.230 ± 0.023
Run 1		0.201 ± 0.028	0.260 ± 0.051	0.283 ± 0.074	0.221 ± 0.034
Run 2		0.325 ± 0.024	0.233 ± 0.028*	0.205 ± 0.020*	0.240 ± 0.034

Values are mean \pm S.E.M. * Indicates significant differences between treated and control insects, $p \leq 0.05$. For Run 1 N=9 and N=7 for control and treated insects respectively and for Run 2 N=7 for control and treated insects.

Preliminary study with 0.5, 1 and 1.5% (W/V) soybean trypsin inhibitor: Results of individual runs and values for standard error of the mean.

Low Alkaline Trypsin (μ Moles BAAE hydrolyzed/gut)

	0	0.5%	1%	1.5%
Combined	0.572 \pm 0.052	0.624 \pm 0.067	0.753 \pm 0.095	0.698 \pm 0.074
Run 1	0.442 \pm 0.058	0.569 \pm 0.098	0.795 \pm 0.151	0.664 \pm 0.104
Run 2	0.738 \pm 0.039	0.679 \pm 0.096	0.711 \pm 0.124	0.731 \pm 0.113

Amino-peptidase (μ Moles LPNA hydrolyzed/gut)

	0	0.5%	1%	1.5%
Combined	0.111 \pm 0.007	0.120 \pm 0.009	0.121 \pm 0.011	0.112 \pm 0.010
Run 1	0.115 \pm 0.008	0.099 \pm 0.009	0.095 \pm 0.010	0.083 \pm 0.007*
Run 2	0.106 \pm 0.012	0.141 \pm 0.013	0.147 \pm 0.013*	0.140 \pm 0.010*

Values are mean \pm S.E.M. * Indicates significant differences between treated and control insects, $p \leq 0.05$. For Run 1 N=9 and N=7 for control and treated insects respectively and for Run 2 N=7 for control and treated insects.

Preliminary study with 0.5, 1 and 1.5% (W/V) soybean trypsin inhibitor: Results of individual runs and values for standard error of the mean.

	Total Proteolysis (Azocasein units/gut)			
	0	0.5%	1%	1.5%
Combined	0.054 ± 0.005	0.043 ± 0.004	0.050 ± 0.005	0.047 ± 0.005
Run 1	0.042 ± 0.006	0.039 ± 0.006	0.048 ± 0.009	0.037 ± 0.005
Run 2	0.069 ± 0.005	0.047 ± 0.005*	0.052 ± 0.005*	0.056 ± 0.005

Values are mean ± S.E.M. * Indicates significant differences between treated and control insects, $p \leq 0.05$. For Run 1 N=9 and N=7 for control and treated insects respectively and for Run 2 N=7 for control and treated insects.

Appendix B

Short term exposure study with 1% (W/V) soybean trypsin inhibitor.

		Total Proteolysis (Azocasein)	mg Protein/gut	Larval Weight (g)
Combined	C	0.025 ± 0.003	0.310 ± 0.012	0.0739 ± 0.0015
	T	0.020 ± 0.002	0.372 ± 0.015*	0.0663 ± 0.0017*
Run 1	C	0.053 ± 0.007	0.245 ± 0.020	0.0748 ± 0.0056
	T	0.028 ± 0.003*	0.340 ± 0.022*	0.0653 ± 0.0030
Run 2	C	0.022 ± 0.003	0.316 ± 0.024	0.0741 ± 0.0022
	T	0.026 ± 0.004	0.292 ± 0.017	0.0573 ± 0.0027*
Run 3	C	0.016 ± 0.001	0.330 ± 0.014	0.0735 ± 0.0017
	T	0.016 ± 0.001	0.419 ± 0.018*	0.0710 ± 0.0017

Values are mean ± S.E.M. Enzyme activity as micromoles substrate hydrolyzed/gut. * Indicates significant differences between treated and control insects, $p \leq 0.05$. For Run 1 N=7 and N=4 for control and treated insects respectively, for Run 2 N=9 and N=10 for control and treated insects respectively and for Run 3 N=20 for control and treated insects.

Short term exposure study with 1% (W/V) soybean trypsin inhibitor.

		High Alkaline Trypsin (BAPNA)	Low Alkaline Trypsin (BAEE)	Chymotrypsin (BTEE)	Aminopeptidase (LPNA)
Combined	C	0.180 ± 0.014	0.407 ± 0.031	0.159 ± 0.013	0.086 ± 0.004
	T	0.177 ± 0.016	0.551 ± 0.038*	0.058 ± 0.006*	0.078 ± 0.004
Run 1	C	0.273 ± 0.045	0.602 ± 0.106	0.166 ± 0.044	0.098 ± 0.008
	T	0.117 ± 0.014*	0.383 ± 0.054	0.034 ± 0.011*	0.091 ± 0.005
Run 2	C	0.145 ± 0.022	0.364 ± 0.054	0.119 ± 0.020	0.074 ± 0.007
	T	0.198 ± 0.038	0.503 ± 0.074	0.052 ± 0.015*	0.073 ± 0.005
Run 3	C	0.164 ± 0.010	0.357 ± 0.023	0.175 ± 0.014	0.086 ± 0.005
	T	0.179 ± 0.018	0.608 ± 0.049*	0.066 ± 0.007*	0.078 ± 0.005

Values are mean ± S.E.M. Enzyme activity as micromoles substrate hydrolyzed/gut. * Indicates significant differences between treated and control insects, $p \leq 0.05$. For Run 1 N=7 and N=4 for control and treated insects respectively, for Run 2 N=9 and N=10 for control and treated insects respectively and for Run 3 N=20 for control and treated insects.

Appendix C

Long term exposure study with 1% (W/V) soybean trypsin inhibitor: Results of individual runs and values for standard error of the mean.

Chymotrypsin (μ Moles BTEE hydrolyzed / gut)

Day		5	6	7	8	9
Combined	C	0.020 \pm 0.008	0.189 \pm 0.026	0.237 \pm 0.028	0.296 \pm 0.039	0.122 \pm 0.082
	T	0.017 \pm 0.008	0.082 \pm 0.010*	0.115 \pm 0.013*	0.143 \pm 0.019*	0.113 \pm 0.038
Run 1	C	-	0.246 \pm 0.060	0.273 \pm 0.063	0.354 \pm 0.095	0.040 \pm 0.019
	T	-	0.082 \pm 0.020*	0.146 \pm 0.035	0.103 \pm 0.015*	0.127 \pm 0.036*
Run 2	C	-	0.115 \pm 0.028	0.238 \pm 0.070	0.270 \pm 0.092	0.265 \pm 0.222
	T	-	0.073 \pm 0.012	0.165 \pm 0.027	0.244 \pm 0.033	0.105 \pm 0.058
Run 3	C	-	0.230 \pm 0.036	0.246 \pm 0.045	0.306 \pm 0.062	-
	T	-	0.109 \pm 0.017*	0.094 \pm 0.017*	0.132 \pm 0.025*	-
Run 4	C	0.020 \pm 0.008	0.051 \pm 0.007	0.151 \pm 0.024	0.245 \pm 0.084	-
	T	0.017 \pm 0.008	0.033 \pm 0.005	0.064 \pm 0.007*	0.069 \pm 0.011	-

Values are mean \pm S.E.M. * Indicates significant differences between treated and control insects, $p \leq 0.05$. For Run 1 and Run 2 N=4-7 for control and treated insects, for Run 3 N=7-10 for control and treated insects and for Run 4 N=2-7 for control and treated insects.

Long term exposure study with 1% (W/V) soybean trypsin inhibitor: Results of individual runs and values for standard error of the mean.

Aminopeptidase (μ Moles LPNA hydrolyzed / gut)

Day		5	6	7	8	9
Combined	C	0.015 \pm 0.003	0.096 \pm 0.008	0.132 \pm 0.011	0.096 \pm 0.007	0.103 \pm 0.014
	T	0.029 \pm 0.005*	0.094 \pm 0.007	0.109 \pm 0.009	0.145 \pm 0.013*	0.113 \pm 0.018
Run 1	C	-	0.104 \pm 0.013	0.141 \pm 0.014	0.104 \pm 0.009	0.100 \pm 0.014
	T	-	0.128 \pm 0.010	0.152 \pm 0.010	0.162 \pm 0.020*	0.126 \pm 0.024
Run 2	C	-	0.100 \pm 0.014	0.160 \pm 0.027	0.141 \pm 0.008	0.108 \pm 0.034
	T	-	0.101 \pm 0.009	0.147 \pm 0.015	0.195 \pm 0.025	0.105 \pm 0.025
Run 3	C	-	0.107 \pm 0.007	0.139 \pm 0.014	0.089 \pm 0.006	-
	T	-	0.091 \pm 0.011	0.083 \pm 0.009*	0.135 \pm 0.019	-
Run 4	C	0.015 \pm 0.003	0.018 \pm 0.001	0.050 \pm 0.008	0.064 \pm 0.014	-
	T	0.029 \pm 0.005*	0.048 \pm 0.009	0.068 \pm 0.006	0.086 \pm 0.013	-

Values are mean \pm S.E.M. * Indicates significant differences between treated and control insects, $p \leq 0.05$. For Run 1 and Run 2 N=4-7 for control and treated insects, for Run 3 N=7-10 for control and treated insects and for Run 4 N=2-7 for control and treated insects.

Long term exposure study with 1% (W/V) soybean trypsin inhibitor: Results of individual runs and values for standard error of the mean.

Day		5	6	7	8	9
Combined	C	0.169 ± 0.086	0.338 ± 0.034	0.283 ± 0.019	0.295 ± 0.018	0.218 ± 0.024
	T	0.159 ± 0.043	0.339 ± 0.025	0.399 ± 0.027*	0.483 ± 0.039*	0.249 ± 0.034
Run 1	C	-	0.292 ± 0.035	0.200 ± 0.010	0.293 ± 0.026	0.235 ± 0.033
	T	-	0.402 ± 0.040	0.366 ± 0.041*	0.526 ± 0.075*	0.325 ± 0.067
Run 2	C	-	0.218 ± 0.024	0.246 ± 0.019	0.224 ± 0.041	0.190 ± 0.032
	T	-	0.287 ± 0.013*	0.386 ± 0.038*	0.366 ± 0.034*	0.205 ± 0.028
Run 3	C	-	0.524 ± 0.017	0.395 ± 0.029	0.342 ± 0.034	-
	T	-	0.373 ± 0.050*	0.484 ± 0.061	0.627 ± 0.082*	-
Run 4	C	0.169 ± 0.086	0.111 ± 0.007	0.243 ± 0.026	0.288 ± 0.034	-
	T	0.159 ± 0.043	0.234 ± 0.037	0.316 ± 0.027	0.387 ± 0.033	-

Values are mean ± S.E.M. * Indicates significant differences between treated and control insects, $p \leq 0.05$. For Run 1 and Run 2 N=4-7 for control and treated insects, for Run 3 N=7-10 for control and treated insects and for Run 4 N=2-7 for control and treated insects.

Long term exposure study with 1% (W/V) soybean trypsin inhibitor: Results of individual runs and values for standard error of the mean.

Day	Larval Weight (g)					
	5	6	7	8	9	
Combined	C	0.0387 ± 0.0060	0.0718 ± 0.0032	0.0891 ± 0.0031	0.0934 ± 0.0035	0.0865 ± 0.0047
	T	0.0477 ± 0.0072	0.0642 ± 0.0031	0.0732 ± 0.0036*	0.0859 ± 0.0036	0.0749 ± 0.0059
Run 1	C	-	0.0727 ± 0.0074	0.1008 ± 0.0040	0.0965 ± 0.0075	0.0910 ± 0.0069
	T	-	0.0705 ± 0.0600	0.0858 ± 0.0077	0.0867 ± 0.0044	0.0882 ± 0.0121
Run 2	C	-	0.0780 ± 0.0046	0.0904 ± 0.0059	0.1018 ± 0.0053	0.0785 ± 0.0013
	T	-	0.0732 ± 0.0031	0.0824 ± 0.0046	0.0946 ± 0.0032	0.0672 ± 0.0047
Run 3	C	-	0.0731 ± 0.0029	0.0807 ± 0.0053	0.0833 ± 0.0030	-
	T	-	0.0580 ± 0.0060*	0.0587 ± 0.0062*	0.0830 ± 0.0094	-
Run 4	C	0.0387 ± 0.0060	0.0446 ± 0.0041	0.0855 ± 0.0085	0.0974 ± 0.0103	-
	T	0.0477 ± 0.0072	0.0590 ± 0.0060	0.0732 ± 0.0036	0.0790 ± 0.0065	-

Values are mean ± S.E.M. * Indicates significant differences between treated and control insects, $p \leq 0.05$. For Run 1 and Run 2 N=4-7 for control and treated insects, for Run 3 N=7-10 for control and treated insects and for Run 4 N=2-7 for control and treated insects.

Long term exposure study with 1% (W/V) soybean trypsin inhibitor: Results of individual runs and values for standard error of the mean.

Day	High Alkaline Trypsin (μ Moles BAPNA hydrolyzed / gut)								
		5	6	7	8	9			
Combined	C	0.048 \pm 0.016	0.224 \pm 0.028	0.319 \pm 0.046	0.394 \pm 0.058	0.247 \pm 0.073			
	T	0.044 \pm 0.012	0.223 \pm 0.032	0.256 \pm 0.027	0.382 \pm 0.039	0.462 \pm 0.141			
Run 1	C	-	0.337 \pm 0.059	0.541 \pm 0.117	0.671 \pm 0.158	0.223 \pm 0.066			
	T	-	0.359 \pm 0.096	0.369 \pm 0.057	0.380 \pm 0.109	0.683 \pm 0.151*			
Run 2	C	-	0.182 \pm 0.031	0.261 \pm 0.063	0.289 \pm 0.058	0.289 \pm 0.180			
	T	-	0.163 \pm 0.045	0.289 \pm 0.073	0.450 \pm 0.073	0.336 \pm 0.196			
Run 3	C	-	0.180 \pm 0.036	0.263 \pm 0.057	0.360 \pm 0.074	-			
	T	-	0.202 \pm 0.030	0.198 \pm 0.039	0.373 \pm 0.084	-			
Run 4	C	0.048 \pm 0.016	0.126 \pm 0.022	0.156 \pm 0.025	0.234 \pm 0.076	-			
	T	0.044 \pm 0.012	0.137 \pm 0.014	0.197 \pm 0.019	0.315 \pm 0.057	-			

Values are mean \pm S.E.M. * Indicates significant differences between treated and control insects, $p \leq 0.05$. For Run 1 and Run 2 N=4-7 for control and treated insects, for Run 3 N=7-10 for control and treated insects and for Run 4 N=2-7 for control and treated insects.

Long term exposure study with 1% (W/V) soybean trypsin inhibitor: Results of individual runs and values for standard error of the mean.

Day	Low Alkaline Trypsin (μ Moles BAEE hydrolyzed / gut)					
	5	6	7	8	9	
Combined	C	0.082 \pm 0.026	0.529 \pm 0.066	0.769 \pm 0.105	0.916 \pm 0.150	0.525 \pm 0.150
	T	0.114 \pm 0.034	0.619 \pm 0.083	0.733 \pm 0.070	1.05 \pm 0.112	1.27 \pm 0.453
Run 1	C	-	0.762 \pm 0.143	1.24 \pm 0.275	1.70 \pm 0.441	0.469 \pm 0.142
	T	-	0.889 \pm 0.267	1.17 \pm 0.181	0.975 \pm 0.257	1.64 \pm 0.518
Run 2	C	-	0.413 \pm 0.074	0.667 \pm 0.159	0.677 \pm 0.125	0.624 \pm 0.362
	T	-	0.511 \pm 0.114	0.720 \pm 0.093	1.27 \pm 0.158*	1.05 \pm 0.663
Run 3	C	-	0.492 \pm 0.097	0.661 \pm 0.126	0.798 \pm 0.156	-
	T	-	0.639 \pm 0.072	0.556 \pm 0.084	1.20 \pm 0.257	-
Run 4	C	0.082 \pm 0.026	0.210 \pm 0.034	0.372 \pm 0.056	0.455 \pm 0.125	-
	T	0.114 \pm 0.034	0.307 \pm 0.045	0.571 \pm 0.055*	0.633 \pm 0.098	-

Values are mean \pm S.E.M. * Indicates significant differences between treated and control insects, $p \leq 0.05$. For Run 1 and Run 2 N=4-7 for control and treated insects, for Run 3 N=7-10 for control and treated insects and for Run 4 N=2-7 for control and treated insects.

Long term exposure study with 1% (W/V) soybean trypsin inhibitor: Results of individual runs and values for standard error of the mean.

Day	Total Proteolysis (Azocasein units / gut)					
	5	6	7	8	9	
Combined	C	0.007 ± 0.002	0.038 ± 0.005	0.064 ± 0.009	0.068 ± 0.010	0.045 ± 0.014
	T	0.008 ± 0.003	0.034 ± 0.004	0.055 ± 0.008	0.058 ± 0.007	0.055 ± 0.015
Run 1	C	-	0.056 ± 0.012	0.106 ± 0.018	0.124 ± 0.023	0.039 ± 0.012
	T	-	0.048 ± 0.013	0.066 ± 0.007	0.071 ± 0.015	0.089 ± 0.018*
Run 2	C	-	0.034 ± 0.004	0.056 ± 0.014	0.074 ± 0.014	0.054 ± 0.033
	T	-	0.040 ± 0.007	0.066 ± 0.014	0.094 ± 0.008	0.036 ± 0.018
Run 3	C	-	0.028 ± 0.004	0.038 ± 0.007	0.042 ± 0.007	-
	T	-	0.031 ± 0.004	0.059 ± 0.022	0.033 ± 0.004	-
Run 4	C	0.007 ± 0.002	0.024 ± 0.009	0.066 ± 0.035	0.040 ± 0.010	-
	T	0.008 ± 0.003	0.016 ± 0.002	0.026 ± 0.004	0.040 ± 0.006	-

Values are mean ± S.E.M. * Indicates significant differences between treated and control insects, $p \leq 0.05$. For Run 1 and Run 2 N=4-7 for control and treated insects, for Run 3 N=7-10 for control and treated insects and for Run 4 N=2-7 for control and treated insects.

Appendix D

Preliminary study with 0.5, 1 and 1.5% (W/V) chicken ovomucoid trypsin inhibitor: Results of individual runs and values for standard error of the mean.

High Alkaline Trypsin (μ Moles BAPNA hydrolyzed/gut)

	0	0.5%	1%	1.5%
Combined	0.219 \pm 0.020	0.255 \pm 0.039	0.256 \pm 0.038	0.287 \pm 0.020*
Run 1	0.214 \pm 0.029	0.234 \pm 0.057	0.240 \pm 0.064	0.287 \pm 0.021
Run 2	0.223 \pm 0.029	0.276 \pm 0.056	0.272 \pm 0.045	0.288 \pm 0.037

Low Alkaline Trypsin (μ Moles BAEE hydrolyzed / gut)

	0	0.5%	1%	1.5%
Combined	0.462 \pm 0.044	0.616 \pm 0.090	0.654 \pm 0.092	0.632 \pm 0.047*
Run 1	0.430 \pm 0.054	0.563 \pm 0.134	0.540 \pm 0.137	0.608 \pm 0.053*
Run 2	0.494 \pm 0.073	0.668 \pm 0.127	0.768 \pm 0.115	0.655 \pm 0.082

Values are mean \pm S.E.M. * Indicates significant differences between treated and control insects, $p \leq 0.05$. For Run 1 and for Run 2 N=7 for control and treated insects.

Preliminary study with 0.5, 1 and 1.5% (W/V) chicken ovomucoid trypsin inhibitor: Results of individual runs and values for standard error of the mean.

Total Proteolysis (Azocasein units/gut)

	0	0.5%	1%	1.5%
Combined	0.043 ± 0.005	0.050 ± 0.008	0.056 ± 0.007	0.058 ± 0.005*
Run 1	0.042 ± 0.004	0.046 ± 0.011	0.048 ± 0.012	0.063 ± 0.007*
Run 2	0.044 ± 0.010	0.055 ± 0.012	0.063 ± 0.007	0.053 ± 0.006

Larval Weight (g)

	0	0.5%	1%	1.5%
Combined	0.0815 ± 0.0024	0.0794 ± 0.0034	0.0781 ± 0.0037	0.0766 ± 0.0029
Run 1	0.0793 ± 0.0028	0.0777 ± 0.0064	0.0786 ± 0.0056	0.0768 ± 0.0031
Run 2	0.0836 ± 0.0038	0.0811 ± 0.0028	0.0777 ± 0.0054	0.0763 ± 0.0052

Values are mean ± S.E.M. * Indicates significant differences between treated and control insects, $p \leq 0.05$. For Run 1 and for Run 2 N=7 for control and treated insects.

Preliminary study with 0.5, 1 and 1.5% (W/V) chicken ovomucoid trypsin inhibitor: Results of individual runs and values for standard error of the mean.

Amino peptidase (μ Moles LPNA hydrolyzed / gut)

	0	0.5%	1%	1.5%
Combined	0.101 \pm 0.005	0.108 \pm 0.006	0.104 \pm 0.009	0.111 \pm 0.007
Run 1	0.101 \pm 0.006	0.096 \pm 0.006	0.083 \pm 0.010	0.090 \pm 0.005
Run 2	0.101 \pm 0.008	0.121 \pm 0.008	0.125 \pm 0.008	0.132 \pm 0.006*

Chymotrypsin (μ Moles BTEE hydrolyzed/gut)

	0	0.5%	1%	1.5%
Combined	0.225 \pm 0.031	0.217 \pm 0.044	0.246 \pm 0.034	0.272 \pm 0.023
Run 1	0.284 \pm 0.042	0.216 \pm 0.082	0.191 \pm 0.050	0.288 \pm 0.038
Run 2	0.165 \pm 0.033	0.218 \pm 0.040	0.300 \pm 0.039*	0.257 \pm 0.029

Values are mean \pm S.E.M. * Indicates significant differences between treated and control insects, $p \leq 0.05$. For Run 1 and for Run 2 N=7 for control and treated insects.

Preliminary study with 0.5, 1 and 1.5% (W/V) chicken ovomucoid trypsin inhibitor: Results of individual runs and values for standard error of the mean.

	mg Protein/gut			
	0	0.5%	1%	1.5%
Combined	0.307 ± 0.016	0.259 ± 0.011*	0.277 ± 0.016	0.267 ± 0.016
Run 1	0.342 ± 0.021	0.251 ± 0.013*	0.291 ± 0.023	0.279 ± 0.028
Run 2	0.273 ± 0.017	0.267 ± 0.019	0.262 ± 0.022	0.255 ± 0.016

Values are mean ± S.E.M. * Indicates significant differences between treated and control insects, $p \leq 0.05$. For Run 1 and for Run 2 N=7 for control and treated insects.

Appendix E

Short term exposure study with 1% (W/V) casein.

		High Alkaline Trypsin (BAPNA)	Low Alkaline Trypsin (BAEE)	Chymotrypsin (BTee)	Amino-peptidase (LPNA)
Combined	C	0.198 ± 0.014	0.436 ± 0.028	0.169 ± 0.011	0.082 ± 0.003
	T	0.184 ± 0.012	0.426 ± 0.026	0.167 ± 0.009	0.094 ± 0.004*
Run 1	C	0.145 ± 0.022	0.364 ± 0.054	0.119 ± 0.020	0.074 ± 0.007
	T	0.184 ± 0.020	0.365 ± 0.037	0.137 ± 0.017	0.090 ± 0.009
Run 2	C	0.164 ± 0.010	0.357 ± 0.023	0.175 ± 0.014	0.086 ± 0.005
	T	0.162 ± 0.014	0.418 ± 0.035	0.197 ± 0.013	0.077 ± 0.005
Run 3	C	0.246 ± 0.024	0.529 ± 0.050	0.182 ± 0.019	0.082 ± 0.005
	T	0.201 ± 0.021	0.458 ± 0.047	0.156 ± 0.015	0.109 ± 0.006*

Values are mean ± S.E.M. Enzyme activity as micromoles substrate hydrolyzed/gut. For Run 1 N=9 and N=10 for control and treated insects respectively, for Run 2 N=20 for control and treated insects, and for Run 3 N=24 and N=25 for control and treated insects respectively. * Indicates significant differences between treated and control insects, $p \leq 0.05$.

Short term exposure study with 1% (W/V) casein

		Total Proteolysis (Azocasein)	mg Protein/gut	Larval Weight (g)
Combined	C	0.019 ± 0.001	0.296 ± 0.010	0.0750 ± 0.0012
	T	0.023 ± 0.002	0.309 ± 0.009	0.0765 ± 0.0014
Run 1	C	0.022 ± 0.003	0.316 ± 0.024	0.0741 ± 0.0022
	T	0.035 ± 0.005*	0.268 ± 0.015	0.0715 ± 0.0020
Run 2	C	0.016 ± 0.001	0.330 ± 0.014	0.0735 ± 0.0017
	T	0.021 ± 0.003	0.340 ± 0.013	0.0771 ± 0.0023
Run 3	C	0.021 ± 0.001	0.260 ± 0.013	0.0766 ± 0.0022
	T	0.020 ± 0.001	0.301 ± 0.013*	0.0781 ± 0.0023

Values are mean ± S.E.M. Enzyme activity as micromoles substrate hydrolyzed/gut. For Run 1 N=9 and N=10 for control and treated insects respectively, for Run 2 N=20 for control and treated insects, and for Run 3 N=24 and N=25 for control and treated insects respectively. * Indicates significant differences between treated and control insects, $p \leq 0.05$.

BIBLIOGRAPHY

- Abe, M., Arai, S., Kato, H. and Fujimaki, M. 1980. Thiol-protease inhibitors occurring in endosperm of corn. *Agric. Biol. Chem.* 44: 685-686.
- Ahmad, Z., Saleemuddin, M., and Siddiqi, M. 1976. Alkaline protease in the larvae of the army worm, *Spodoptera litura*. *Insect Biochem.* 6: 501-505.
- Ahmad, Z., Saleemuddin, M. and Siddi, M. 1980. Purification and characterization of three alkaline proteases from the gut of the larva of army worm, *Spodoptera litura*. *Insect Biochem.* 10: 667-673.
- Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J.D. 1983. The fractionation of cells and their contents. In *Molecular Biology of the Cell*. Garland publishing, New York. pp. 170-173.
- Applebaum, S.W. 1985. Biochemistry of digestion. In *Comparative Physiology, Biochemistry, and Pharmacology of Insects*. Vol IV. Edited by G.A. Kerkut and L.I. Gilbert. Pergamon Press, Toronto. pp. 279-311.
- Baker, J.E., Woo, S.M. and Mullen M.A. 1984. Distribution of proteinases and carbohydrases in the midgut of larvae of the sweet potato weevil *Cylas formicarius elegantulus* and response of proteinases to inhibitors from sweet potatoes. *Entomol. Exp. Appl.* 36: 97-105.
- Barrett, A.J. 1981. Cystatin, the egg white inhibitor of cysteine inhibitors. In *Methods in Enzymology*. Edited by L. Lorand. Academic Press, New York. 80: 771-778.
- Bates, R.P., Knapp, F.W. and Araujo, P.E. 1977. Protein quality of green mature, dry mature and sprouted soybeans. *J. Food Sci.* 42: 271-272.

- Baumgartner, B. and Chrispeels, M.J. 1976. Partial characterization of a protease inhibitor which inhibits the major endopeptidase present in the cotyledons of mung beans. *Plant Physiol.* 58: 1-6.
- Baydoun, E.A.H. and Fry, S.C. 1985. The immobility of pectic substances in injured tomato leaves and its bearing on the identity of the wound hormone. *Planta* 165: 269-276.
- Benz, G., Jeker, T. and Mueller, C. 1985. The influence of artificial wounds on the leaves of *Solanum tuberosum* on the larval development of *Leptinotarsa decemlineata* and *Leptinotarsa haldemani* (Coleoptera, Chrysomelide). *Mitt. Schweiz. Entomol. Ges.* 58: 39-46.
- Birk, Y. and Applebaum, S.W. 1960. Effect of soybean trypsin inhibitors on the development and midgut proteolytic activity of *Tribolium castaneum* larvae. *Enzymologia* 22: 318-326.
- Birk, Y., Harpaz, I., Ishaaya, I. and Bondi, A. 1962. Studies on the proteolytic activity of the beetles *Tenebrio* and *Tribolium*. *J. Insect Physiol.* 8: 414-429.
- Birk, Y. 1968. Chemistry and nutritional significance of proteinase inhibitors from plant sources. *Ann. N.Y. Acad. Sci.* 146: 388-399.
- Birk, Y. 1976. Proteinase inhibitors from legume seeds. In *Methods in Enzymology*. Edited by L. Lorand. 45: 697-700. Academic Press, New York.
- Bishop, P.D., Pearce, G., Bryant, J.E. and Ryan, C.A. 1984. Isolation and characterization of the proteinase-inhibitor inducing factor from tomato leaves: Identity and activity of poly- and oligogalacturonide fragments. *J. Biol. Chem.* 259: 13172-13176.
- Blow, D.M., Janin, J. and Sweet, R.M. 1974. Trypsin-inhibitor (Kunitz) as a model for specific protein-protein interactions. *Nature* 249: 54-57.

- Bosterling, B. and Quast, U. 1981. Soybean trypsin inhibitor (Kunitz) is doubleheaded. Kinetics of the interaction of α -chymotrypsin with each side. *Biochim. Biophys. Acta.* 657: 58-72.
- Bramhall, S.N., Noack, N., Wu, M. and Loewenberg, J.R. 1969. A simple colorimetric method for determining protein. *Anal. Biochem.* 31: 146-148.
- Broadway, R.M., Duffey, S.S., Pearce, G. and Ryan, C.A. 1986. Plant proteinase inhibitors: A defense against herbivorous insects? *Entomol. Exp. Appl.* 41: 33-38.
- Broadway, R.M. and Duffey, S.S. 1986a. The effect of dietary protein on the growth and digestive physiology of larval *Heliothis zea* and *Spodoptera exigua*. *J. Insect Physiol.* 32: 673-680.
- Broadway, R.M. and Duffey, S.S. 1986b. Plant proteinase inhibitors : Mechanism of action and effect on the growth and digestive physiology of larval *Heliothis zea* and *Spodoptera exigua*. *J. Insect Physiol.* 32: 827-833.
- Broadway, R.M. and Duffey, S.S. 1988. The effect of plant protein quality on insect digestive physiology and the toxicity of plant proteinase inhibitors. *J. Insect Physiol.* 12: 1111- 1117.
- Brown, W.E., Takio, K., Titani, K. and Ryan, C.A. 1985. Wound-induced trypsin inhibitor in alfalfa leaves: Identity as a member of the Bowman-Birk inhibitor family. *Biochem.* 24: 2105-2108.
- Chen, I. and Mitchell, H.L. 1973. Trypsin inhibitors in plants. *Phytochem.* 12: 327-330.
- Collins, J.L. and Sanders, G.G. 1976. Changes in trypsin inhibitory activity in some soybean varieties during maturation and germination. *J. Food Sci.* 41: 168-172.
- Corfman, R.S. and Reeck, G.R. 1982. Immunoabsorbent isolation of trypsin inhibitors from corn and teosinte seeds. *Biochim. Biophys. Acta.* 715: 170-174.

- De Vonis Bidlingmeyer, V., Leary, T.R. and Laskowski, M. Jr. 1972. Identity of the tryptic and α -chymotryptic reactive sites on soybean trypsin inhibitor (Kunitz). *Biochem. 11: 3303-3310.*
- Edelhoch, H. and Steiner, R.F. 1963. Structural transitions of soybean trypsin inhibitor II. The denatured state in urea. *J. Biol. Chem. 238: 931-938.*
- Eguchi, M. and Iwamoto, A. 1976. Alkaline proteases in the midgut tissue and digestive fluid of the silkworm, *Bombyx mori*. *Insect Biochem. 6: 491-496.*
- Eguchi, M. and Iwamoto, A. 1982. Comparison of three alkaline proteases from digestive fluid of the silkworm *Bombyx mori* L. *Comp. Biochem. Physiol. 71B: 663-668.*
- Erlanger, B.F., Kokowsky, N. and Cohen, W. 1961. The preparation and properties of two new chromogenic substrates of trypsin. *Arch. Biochem. Biophys. 95: 271-278.*
- Feeney, R.E., Means, G.E. and Bigler, J.C. 1969. Inhibition of human trypsin, plasmin and thrombin by naturally occurring inhibitors of proteolytic enzymes. *J. Biol. Chem. 244: 1957-1960.*
- Fossum, K. and Whitaker, J.R. 1968. Ficin and papain inhibitor from chicken egg white. *Arch. Biochem. Biophys. 125: 367- 375.*
- Freed, R.C. and Ryan, D.S. 1978. Changes in Kunitz trypsin inhibitor during germination of soybeans: An immunoelectrophoresis assay system. *J. Food Sci. 43: 1316-1319.*
- Freed, R.C. and Ryan, D.S. 1980. Isolation and characterization of genetic variants of the Kunitz soybean trypsin inhibitor. *Biochim. Biophys. Acta. 624: 562-572.*
- Gatehouse, A.M.R. and Boulter, D. 1983. Assessment of the antimetabolic effects of trypsin inhibitors from cowpea (*Vigna unguiculata*), and other legumes on development of the Bruchid beetle *Callosobruchus maculatus*. *J. Sci. Food Agric. 34: 345-350.*

- Gatehouse A.M.R., Butler, K.J., Fenton, K.A., and Gatehouse, J.A., 1985. Presence and partial characterization of a major proteolytic enzyme in the larval gut of *Callosobruchus maculatus*. *Entomol. Exp. Appl.* 39: 279-286.
- Goldstein, Z., Trop, M. and Birk, Y. 1973. Multifunctional proteinase inhibitor from *Bauhinia* seeds. *Nature New Biology* 246: 29-31.
- Gooding, R.H. 1975. Digestive enzymes and their control in haematophagous arthropods. *Acta Tropica* 32: 96-111.
- Gooding, R.H. 1977. Digestive processes in haematophagous insects : Secretion of trypsin and carboxypeptidase B by *Glossina morsitans morsitans* Westwood *Can. J. Zool.* 55: 215-222.
- Green, T.R. and Ryan, C.A. 1972. Wound induced proteinase inhibitor in plant leaves: A possible defense mechanism against insects. *Science* 175: 776-777.
- Guthrie, W.D., Robbins, J.C. and Jarvis, J.L. 1985. *Ostrinia nubilalis*. In *Handbook of Insect Rearing*. Vol II. Edited by P. Singh and R.F. Moore. Elsevier, New York. pp. 407-413.
- Halim, A.H., Wassom, C.E. and Mitchell, H.L. 1973. Trypsin inhibitor in corn (*Zea mays* L.) as influenced by genotype and moisture stress. *Crop Sci.* 13: 405-407.
- Hamed M.B.B. and Attias J. 1987. Isolation and partial characterization of two alkaline proteases of the greater wax moth *Galleria mellonella* (L.). *Insect Biochem.* 17: 653-658.
- Hass, G.M. and Ryan, C.A. 1980. Carboxypeptidase inhibitor from ripened tomatoes; purification and properties. *Phytochem.* 19: 1329-1333.
- Hass, G.M., Ager, S.P., Le Tourneau, D. and Derr-Makus, J.E. 1981. Specificity of the carboxypeptidase inhibitor from potatoes. *Plant Physiol.* 67: 754-758.
- Heimberger, N. 1975. Protease inhibitors of human plasma. Their properties and control functions. In *Proteases and Biological Control*. Edited by E. Reich, D.B. Rifkin and E. Shaw. Cold Spring Harbor Laboratory, USA. pp 367-386.

- Hilder, V.A., Gatehouse, A.M.R., Sheerman, S.E., Barker, R.F. and Boulter, D. 1987. A novel mechanism of insect resistance engineered into tobacco. *Nature* 330: 160-163.
- Hobday, S.M., Thurman, D.A. and Barber, D.J. 1973. Proteolytic and trypsin inhibitory activities in extracts of germinating *Pisum sativum* seeds. *Phytochem.* 12: 1041-1046.
- Hochstrasser, V.K., Muss, M. and Werle, E. 1967. Reindarstellung und charakterisierung des trypsin-inhibitors aus mais. *Hoppe-Seyler's Z. Physiol. Chem.* 348: 1337-1340.
- Hochstrasser, K., Ilchmann, K. and Werle, E. 1970. Die aminosäuresequenz des spezifischen trypsininhibitors aus maissamen, charakterisierung als polymers. *Hoppe-Seyler's Z. Physiol. Chem.* 351: 721-728.
- Hoff, J., Jones, C.M., Sosa, M.P. and Rodis, P. 1972. Naturally occurring crystals in the potato: Isolation and identification as a protein. *Biochem. Biophys. Res. Commun.* 49: 152-1529.
- Hollander-Czytko, H., Anderson J.K., and Ryan, C.A. 1985. Vacuolar localization of wound-induced carboxypeptidase inhibitor in potato leaves. *Plant Physiol.* 78: 76-79.
- Horiguchi, T. and Kitagishi, K. 1971. Studies on rice seed protease V. Protease inhibitor in rice seed. *Plant Cell Physiol.* 12: 907-915.
- Houseman J.G. and Larocque A.M. 1987. Phytochemical control of the European corn borer : Strategy II Efficacy of plant protease inhibitors as control agents. In *Interm Report Ontario Ministry of Agriculture and Food.*
- Houseman, J.G., Aresta, F., Mark, W.A. and Morrison, P.E. 1988. Effect of fractionated blood components on trypsin activity in the stable fly, *Stomoxys calcitrans* (L.) (Diptera: Muscidae). *Can. J. Zool.* 66: 1188-1190.

- Houseman, J.G., Downe, A.E.R. and Philogene, B.J.R. 1989. Partial characterization of proteinase activity in the larval midgut of the European corn borer *Ostrinia nubilalis* Hubner (Lepidoptera: Pyralidae). *Can. J. Zool.* 67: 864-868.
- Hudon, M. and Le Roux, E.J. 1986. Biology and population dynamics of the European corn borer (*Ostrinia nubilalis*) with special reference to sweet corn in Quebec. I. Systematics, morphology, geographical distribution, host range, economic importance. *Phytoprot.* 67: 39-54.
- Hummel, C.W. 1959. A modified spectrophotometric determination of chymotrypsin, trypsin and thrombin. *Can. J. Biochem. Physiol.* 37: 1393-1399.
- Hymowitz, T. and Hadley, H.H. 1972. Inheritance of a trypsin inhibitor variant in seed protein of soybeans. *Crop Sci.* 12: 197-198.
- Hymowitz, T., Orf, J.H., Kaizuma, N. and Skorupska, H. 1978. *Soybean Genet. Newsl.* 5: 19-22.
- Johnson, R., Mitchell, H.L., Wassom, C.E. and Reeck, G.R. 1980. Comparison of trypsin inhibitors from several types of corn *Phytochem.* 19: 2749-2750.
- Kassel, B. 1970. Inhibitors of proteolytic enzymes. In *Methods in Enzymology*. Edited by G.E. Perlmann and L. Lorand. Academic Press, New York. 19: 839-906.
- Kato, I., Schrode, J., Kohr, W.J., and Laskowski, M. Jr. 1987. Chicken ovomucoid: Determination of its amino acid sequence, determination of the trypsin reactive site, and preparation of all three of its domains. *Biochem.* 26: 193-201.
- Keilova, H. and Tomasek, V. 1974. Effect of papain inhibitor from chicken egg white on cathepsin B. *Biochem. Biophys. Acta.* 334: 179-186.
- Keilova, H. and Tomasek, V. 1976. Isolation and properties of cathepsin D inhibitor from potatoes. *Collection Czechoslov. Chem. Commun.* 41: 489-497.
- Kezdy, F.J., Lorand, L. and Miller, K.D. 1965. Titration of active centres in thrombin solutions. Standardization of the enzyme. *Biochem.* 4: 2302-2308.

- Kim, S.H., Hara, S., Hase, S., Ikenaka, T., Toda, H., Kitamura, K. and Kaizuma, N. 1985. Comparative study on amino acid sequences of Kunitz-type soybean trypsin inhibitors Ti^a, Ti^b, and Ti^c. *J. Biochem.* 98: 435-448.
- Kowalski, D. and Laskowski, M. Jr. 1976a. Chemical-enzymatic replacement of Ile⁶⁴ in the reactive site of soybean trypsin inhibitor (Kunitz). *Biochem.* 15: 1300-1308.
- Kowalski, D. and Laskowski, M. 1976b. Chemical-enzymatic insertion of an amino acid residue in the reactive site of soybean trypsin inhibitor (Kunitz). *Biochem.* 15: 1309-1315.
- Klocke, J.A. and Chan, B.G. 1982. Effects of cotton condensed tannin on feeding and digestion in the cotton pest, *Heliothis zea*. *J. Insect Physiol.* 28: 911-915.
- Koide, T. and Ikenaka, T. 1973a. Studies on soybean trypsin inhibitors. 1. Fragmentation of soybean trypsin inhibitor (Kunitz) by limited proteolysis and by chemical cleavage. *Eur. J. Biochem.* 32: 401-407.
- Koide, T., Tsunasawa, S. and Ikenaka, T. 1973. Studies on soybean trypsin inhibitors. 2. Amino-acid sequence around the reactive site of soybean trypsin inhibitor (Kunitz). *Eur. J. Biochem.* 32: 408-416.
- Koide, T. and Ikenaka, T. 1973b. Studies on soybean trypsin inhibitors. 3. Amino-acid sequence of the carboxyl-terminal region and the complete amino acid sequence of soybean trypsin inhibitor (Kunitz). *Eur. J. Biochem.* 32: 417-431.
- Koshiyama, I., Kikuchi, M., Harada, K. and Fukushima, D. 1981. 2S globulins of soybean seeds. 1. Isolation and characterization of protein components. *J. Agric. Food Chem.* 29: 336-340.
- Kraemer, M.E., Rangappa, M., Gade, W. and Benepal, P.S. 1987. Induction of trypsin inhibitors in soybean leaves by Mexican bean beetle (Coleoptera: Coccinellidae) defoliation. *J. Econ. Entomol.* 80: 237-241.

- Kunitz, M. 1947. Crystalline soybean tyrpsin inhibitor. 2. General properties. *J. Gen. Physiol.* 30: 291-307.
- Laskowski, M. Jr. and Sealock, R.W. 1971. Protein proteinase inhibitors-Molecular aspects. In *The Enzymes. Vol III.* Edited by P.D. Boyer. Academic Press, New York. p.p. 375-473.
- Laskowski, M. Jr., Kato, J. and Kohr, W.J. 1978. Protein inhibitors of serine proteinases; Convergent evolution , multiple domains and hypervariability of reactive sites. In *Versatility of Proteins.* Edited by C.H. Li. Academic Press, New York. pp. 307-318.
- Laskowski, M. Jr. and Kato, I. 1980. Protein inhibitors of proteinases. *Ann. Rev. Biochem.* 49: 593-626.
- Leary, J.R. and Laskowski, M. Jr. 1973. *Fed. Proc. Am. Soc. Exp. Biol.* 32: 1363.
- Lehninger, A.L. 1982. Enzymes. In *Principles of Biochemistry.* Worth Publishers, New York. pp. 223-224.
- Liener, I.E. and Kakade, M.L. 1980. Protease inhibitors. In *Toxic Constituents of Plant Foodstuffs.* Edited by I.E. Leiner. Academic Press, New York. pp. 7-71.
- Lineweaver, H. and Murray, C.W. 1947. Identification of the trypsin inhibitor of egg white with ovomucoid. *J. Biol. Chem.* 171: 565- 581.
- Lipke, H. and Fraenkel, G.S. 1954. Growth inhibitors: Effect of soybean inhibitors on growth of *Tribolium confusum.* *J. Agric. Food Chem.* 2: 410-414.
- Mahoney, W.C., Hermodson, M.A., Jones, B., Powers, D.D., Corfman , R.S. and Reeck, G.R. 1984. Amino acid sequence and secondary structural analysis of the corn inhibitor of trypsin and activated hageman factor. *J. Biol. Chem.* 259: 8412-8416.
- McFarland, D. and Ryan, C.A. 1974. Proteinase inhibitor-inducing factor in plant leaves. *Plant Physiol.* 54: 706-708.

- Melville, J.C. and Scandalios, J.G. 1972. Maize endopeptidase: Genetic control, chemical characterization, and relationship to an endogenous trypsin inhibitor. *Biochem. Genet.* 7: 15-31.
- Mikola, J. and Kirsi, M. 1972. Differences between endospermal and embryonal trypsin inhibitors in barley, wheat and rye. *Acta Chem. Scand.* 26: 787-795.
- Miller, J.W., Kraemer, K.J. and Law, J.H. 1974. Isolation and partial characterization of the larval midgut trypsin from the tobacco hornworm, *Manduca sexta*, Johannson (Lepidoptera: Sphindidae). *Comp. Biochem. Physiol.* 48B: 117-129.
- Mullen, M.A., Jones, A., Arbogast R.T., Schalk, J.M., Paterson, D.R., Boswell, T.E. and Earhart, D.R. 1980. Field selection of sweetpotato lines and cultivars for resistance to the sweetpotato weevil. *J. Econ. Entomol.* 73: 288-290.
- Nagase, H., Harris, E.D. Jr., Woessner, J.F. and Brew, K. 1983. Ovostatin: A novel proteinase inhibitor from chicken eggwhite I. Purification, physiochemical properties and tissue distribution of ovostatin. *J. Biol. Chem.* 258: 7481-7489.
- Nagata, K. and Yoshida, N. 1984. Interaction between trypsin-like enzyme from *Streptomyces erythraeus* and chicken ovomucoid. *J. Biochem.* 96: 1041-1049.
- Orf, J.H. and Hymowitz, T. 1979. Genetics of the Kunitz trypsin inhibitor: An antinutritional factor in soybeans. *J. Am. Chem. Soc.* 56: 722-726.
- Osuga, D.T. and Feeney, R.E. 1968. Biochemistry of the egg-white proteins of the ratite group. *Archiv. Biochem. Biophys.* 124: 560-574.
- Pritchett, D.W., Young, S.Y. and Geren, C.R. 1981. Proteolytic activity in the digestive fluid of larvae of *Trichoplusia ni*. *Insect Biochem.* 11: 523-526.
- Quast, U. and Steffen, E. 1975. The soybean trypsin inhibitor (Kunitz) is a double-headed inhibitor. *Hoppe-Seyler's Z. Physiol. Chem.* 356: 617-620.
- Rackis, J.J. and Anderson, R.L. 1964. Isolation of four soybean trypsin inhibitors by DEAE-cellulose chromatography. *Biochem. Biophys. Res. Commun.* 15: 230-235.

- Reddy, M.N., Keim, P.S., Heinrickson, R.L. and Kezdy, F.J. 1975. Primary structural analysis of sulfhydryl protease inhibitors from pineapple stems. *J. Biol. Chem.* 250: 1741-1750.
- Rhodes, M.B., Bennett, N. and Feeney, R.E. 1960. The trypsin and chymotrypsin inhibitors from avian egg whites *J. Biol. Chem.* 235: 1686-1692.
- Richardson, M. 1977. Review. The proteinase inhibitors of plants and micro-organisms. *Phytochem.* 16: 159-169.
- Richardson, M. 1980-1981. Protein inhibitors of enzymes. *Food Chem.* 6: 235-253.
- Richardson, M., Valdes-Rodriguez, S. and Blanco-Labra, A. 1987. A possible function for thaumatin and a TMV-induced protein suggested by homology to a maize inhibitor. *Nature* 327: 432-434.
- Royer, A., Miede, M.N., Grange, A., Miede, J. and Mascherpa, J.M. 1974. Inhibiteurs anti-trypsine et activites proteolytiques des albumins de graine de *Vigna unguiculata*. *Planta* 119: 1-16.
- Ryan, C.A. 1973. Proteolytic enzymes and their inhibitors in plants. *Ann. Rev. Plant Physiol.* 24: 173-196.
- Ryan, C.A. 1974. Assay and biochemical properties of the proteinase inhibitor-inducing factor, a wound hormone. *Plant Physiol.* 54: 328-332.
- Ryan, C.A. 1978. Proteinase inhibitors in plant leaves: A biochemical model for pest-induced natural plant protection. *Trends Biochem. Sci.* 5: 148-150.
- Ryan, C.A. 1981. Proteinase inhibitors. In *The Biochemistry of plants. Vol. 6*. Edited by A. Marcus. Academic Press, New York. pp. 351-370.
- Ryan, C.A., Bishop, P., Pearce, G., Darvill, A.G., McNeil, M. and Albersheim, P. 1981. A sycamore cell wall polysaccharide and a chemically related tomato leaf polysaccharide possess similar proteinase inhibitor-inducing activities. *Plant Physiol.* 68: 616-618.

- Ryan, C.A. and Hass, G.M. 1981. Structural, evolutionary and nutritional properties of proteinase inhibitors from potatoes. In *Antinutrients and Natural Toxicants in Foods*. Edited by R.L. Ory. Food and Nutrition Press, Connecticut. pp. 169- 185.
- Ryan, C.A. and Walker-Simmons, M. 1981. Plant proteinases. In *The Biochemistry of Plants. Vol. 6*. Edited by A. Marcus. Academic Press, New York. pp. 321-322.
- Ryan, C.A., Bishop, P.D., Graham, J.S., Broadway, R.M. and Duffey, S.S. 1986. Plant and fungal cell wall fragments activate expression of proteinase inhibitor genes for plant defense. *J. Chem. Ecol.* 12: 1025-1036.
- Sanderson, J.E., Freed, R.C. and Ryan, D.S. 1982. Thermal denaturation of genetic variants of the Kunitz soybean trypsin inhibitor. *Biochem. Biophys. Acta.* 701: 237-241.
- Santos, C.D. and Terra, W.R. 1986. Distribution and characterization of oligomeric digestive enzymes from *Erinnyis ello* larvae and inferences concerning secretory mechanisms and the permeability of the peritrophic membrane. *Insect Biochem.* 16: 691-700.
- Schneider, F., Houseman, J.G. and Morrison, P.E. 1987. Activity cycles and the regulation of digestive proteases in the posterior midgut of *Stomoxys calcitrans* (L.) (Diptera: Muscidae). *Insect Biochem.* 17: 859-862.
- Sealock, R.W. and Laskowski, M. Jr. 1969. Enzymatic replacement of the arginyl by a lysyl residue in the reactive site of the soybean trypsin inhibitor. *Biochem.* 8: 3703-3710.
- Shain, Y. and Mayer, A.M. 1968. Activation of enzymes during germination -trypsin-like enzymes in lettuce. *Phytochem.* 7: 1491-1498.
- Shukle, R.H. and Murdock, L.L. 1983. Lipoxygenase, trypsin inhibitor and lectin from soybeans : Effect on larval growth of *Manduca sexta* (Lepidoptera: Sphingidae) *Environ. Ent.* 12: 787-791.

- Smirnoff, P., Khalet, S., Birk, Y. and Applebaum, S.W. 1979. Trypsin and chymotrypsin inhibitor from chickpeas. Selective chemical modification of the inhibitor and isolation of two isoinhibitors. *Int. J. Pept. Prot. Res.* 14: 186-192.
- Spates, G. 1979. Fecundity of the stable fly: Effect of soybean trypsin inhibitor and Phospholipase A inhibitor on the fecundity. *Ann. Entomol. Soc. Am.* 72: 845-849.
- Steffens, R., Fox, F.R. and Kassell, B. 1978. Effect of trypsin inhibitors on growth and metamorphosis of corn borer larvae *Ostrinia nubilalis* (Hubner). *J. Agric. Food Chem.* 26: 170-174.
- Stevens, F.C. and Feeney, R. 1963. Chemical modification of avian ovomucoids. *Biochem.* 2: 1346-1352.
- Swartz, M.J., Mitchell, H.L., Cox, D.J. and Reeck, G.R. 1977. Isolation and characterization of trypsin inhibitor from opaque-2 corn seeds. *J. Biol. Chem.* 252: 8105-8107.
- Sweet, R.M., Wright, H.T., Chothia, C.H. and Blow, D.M. 1974. Crystal structure of the complex of porcine trypsin with soybean trypsin inhibitor (Kunitz) at 2.6-A resolution. *Biochem.* 13: 4212-4228.
- Tomimatsu, Y., Clary, J.J. and Bartulovich, J.J. 1966. Physical characterization of ovinhibitor, a trypsin and chymotrypsin inhibitor from chicken egg white. *Arch. Biochem. Biophys.* 115: 536-544.
- Tranter, H.S. and Board, R.G. 1982. Review. The antimicrobial defense of avian eggs: Biological perspective and chemical basis. *J. Appl. Biochem.* 4: 295-338.
- Vithayathil, A.J., Buck, F., Bier, M. and Nord, F.F. 1961. On the mechanism of enzyme action. LXXII. Comparative studies on trypsins of various origins. *Arch. Biochem. Biophys.* 92: 532-540.
- Vogel, R., Trautschold, I. and Werle, E. 1968. Inhibitors from blood serum. In *Natural Proteinase Inhibitors*. Academic Press, New York. pp. 57-72.

- Walker-Simmons, M. and Ryan, C.A. 1977. Immunological identification of proteinase inhibitors I and II in isolated tomato leaf vacuoles. *Plant Physiol.* 60: 61-63.
- Warsy, A.S., Norton, G. and Stein, M. 1974. Protease inhibitors from broad bean; isolation and purification. *Phytochem.* 13: 2481-2486.
- Whitaker, J.R. 1981. Naturally occurring peptide and protein inhibitors of enzymes. In *Impact of toxicology on food processing*. Edited by Institute of Food Technologists, Chicago, Illinois. Avi Publishing Company, Inc. Westport, Connecticut. pp. 57-103.
- Wu, Y.V. and Scheraga, H.A. 1962. Studies of soybean trypsin inhibitor. II. Conformational properties. *Biochem.* 1: 905-911.
- Yamamoto, M. and Ikenaka, T. 1967. Studies on soybean trypsin inhibitors. I. Purification and characterization of two soybean trypsin inhibitors. *J. Biochem.* 62: 141-149.
- Zuroske, G., Makus, D.J. and Ryan, C.A. 1980. The direction and rate of transport of the proteinase inhibitor inducing factor out of wounded tomato leaves (Abstr.). *Plant Physiol.* 65, suppl., 150.