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Part 1 - Project Details

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Plain English Summary

Recent research on the mechanisms by which plants defend themselves against insect attack has suggested a novel approach to crop protection that could be adopted for control of *Helicoverpa* on cotton. *Helicoverpa* feed on the growing tips and reproductive tissues of cotton throughout all growth phases of the plant. Late season infestations of *H. armigera* in particular cause significant economic losses. Currently *Helicoverpa* control relies on the use of a variety of chemicals, usually applied several times during the season. Most of these chemicals have been lost through the development of multi-resistant strains of *H. armigera* and although new generation chemicals are becoming available, they will be significantly more expensive than endosulfan and the synthetic pyrethroids. The advent of Ingard® cotton will provide some respite in the short to medium term, but Bt resistance will inevitably develop. To reduce reliance on chemical control, alternative *H. armigera* resistance genes are urgently required to support the development of sustainable IPM strategies for the cotton industry.

The project objective was to contribute to production of *Helicoverpa*-resistant cotton cultivars by identifying proteins capable of blocking insect digestion. Plants normally attempt to protect themselves from insect feeding by making protein inhibitors which block the action of essential digestive enzymes. Recent work has shown that insects are able to overcome the effects of these inhibitors by producing new enzymes that are less sensitive to the inhibitors. This project characterised the digestive proteinases of *H. armigera* and investigated the effects of different classes of proteinase inhibitors on the types proteinases produced by the insect larvae. The effects on insect growth and survival of combining multiple proteinase inhibitors were also determined.

It was shown that *H. armigera* produces a very large number of subtly different proteinases and is able to adjust the composition of the total complement of enzymes in response to inhibitors in its diet. Although the more effective inhibitors had some effects on insect growth, these were not sufficient to provide effective levels of plant protection. In light of the extreme genetic diversity present in these proteolytic enzymes it was concluded that a transgenic anti-nutritional strategy relying solely on this class of digestive enzymes was not likely to provide stable resistance against *H. armigera.*

Background to Project

The heliothine moths *Helicoverpa armigera* and *H. punctigera* are the most significant insect pests of cotton in all growing regions. Both species feed preferentially on growing tips and reproductive structures thus causing significant reductions in yield and consequent economic losses. Traditionally heliothis control has relied on the frequent application of chemical insecticides and this has resulted in *H. armigera* becoming resistant to most of the major insecticide classes. In addition to resistance problems and the undesirable environmental impact of intensive insecticide application, early season spraying with these chemicals is highly disruptive to the build-up of predatory species that will be an essential component of any IPM program for cotton. Recently the industry has seen commercial introduction of transgenic Bt cotton (INGARD) for heliothis control. Although INGARD cotton has performed well in field trials over several years, with large-scale commercial introduction will come strong selection pressure in *H. armigera* to develop resistance to the protein toxin encoded by the Cry1A(c) gene. Other genes for conferring resistance to *H. armigera* are urgently required either for use in combination with Bt cotton to help manage resistance development, or to replace it in the event of failure. One approach to heliothis control suggested by recent research is the disruption of feeding and nutrient absorption by inhibition of key digestive enzymes.

Plants arm themselves against attack by leaf-feeding insects by producing proteinaceous enzyme inhibitors. A wide variety of inhibitory peptides affecting different classes of enzymes have been identified from plants including inhibitors of serine and cysteine proteinases and α-amylases. These proteins are either expressed in seeds or induced in leaves in response to tissue damage caused by insect feeding. Attempts to generate insect-resistant plants by constitutive expression of proteinase inhibitor genes have not been generally successful. For example, recent efforts by CSIRO Plant Industry to engineer resistance to *Helicoverpa armigera* by over-expression of the Giant Taro proteinase inhibitor gene did not result in economically useful levels of plant protection (D. Llewellyn, personal communication), and this has been the experience of several groups working on lepidopteran pests. However, there have been a few cases where useful protection has been achieved with both proteinase inhibitor (eg Duan *et al.*, 1996) and α-amylase inhibitor transgenes (Morton *et al.*, 2000), suggesting that this approach to insect control will work provided the physiologically relevant set of enzymes has been targetted.

Recently, some reasons for the limited effectiveness of this technology have been identified for lepidopteran pests. Caterpillars feeding on plants containing high levels of proteinase inhibitors respond by producing novel enzyme activities that are insensitive to the inhibitory mechanism. The details of this adaptive response differ between species - some insects express a different sub-class of the same enzyme group, for example producing a chymotrypsin in place of a trypsin, whereas others produce an inhibitor-insensitive variant of the same enzyme sub-class (Jongsma *et al.*, 1995). It appears that the capacity for this type of "rescue" response may be limited. In the few cases investigated in any detail, the activity of insensitive proteinase was not sufficient to restore normal enzyme levels, and this probably accounts for the delayed development of insects reared on plants expressing inhibitor transgenes. This limited ability to compensate for inhibition of the normal complement of digestive enzymes suggests that expression of additional inhibitors directed against the insensitive enzymes could be sufficient to provide effective plant protection (Jongsma *et al.*, 1996b).

H. armigera is a highly polyphagous species with a proven capacity to feed on a variety of crop plants. A limited survey of *H. armigera* midgut proteolytic enzymes indicated that serine proteinases are the major class (Johnston *et al.*, 1991;1993) but the number and diversity of isozymes and sub-classes has not been investigated. Feeding trials with soybean trypsin inhibitor incorporated into the diet revealed that gut proteolytic activity was markedly reduced, and larval growth was severely retarded. Larvae survived for up to 14 days on inhibitor-containing diet, but then died suddenly (Johnston *et al.*, 1993). There have been no published studies of *H. armigera* involving transgenic plants expressing the soybean trypsin inhibitor, but one study deploying the cowpea trypsin inhibitor in transgenic tobacco failed to provide effective protection against *H. armigera* (Gatehouse *et al.*, 1992).

Previous research by Dr Danny Llewellyn from CSIRO Division of Plant Industry established that the Giant Taro proteinase inhibitor was unable to provide effective resistance to *H. armigera* in a

transgenic tobacco model, and the reason for this appeared to be the induction of inhibitor-insensitive chymotrypsin-like enzymes in place of the trypsin-like isozymes present in the absence of inhibitor (Wu *et al.*, 1997). This result suggests that transgenic cotton carrying a multi-domain inhibitor expressing optimised, high affinity trypsin and chymotrypsin/elastase inhibitors may provide effective resistance to *H. armigera*.

What were the project objectives and to what extent were these achieved?

The major objective was to contribute to the development of an alternative to Bt crystal toxins for protection of cotton plants against *H. armigera*. Specifically the project sought to evaluate the potential for dietary proteinase inhibitors to disrupt feeding and larval growth sufficiently to provide economic levels of plant protection. The project consisted of four components, identification of digestive proteinases, selection of novel inhibitors and characterisation of proteinase inhibitor effects *in vitro*, characterisation of inhibitor effects on *H. armigera* larval growth and development and characterisation of inhibitor-insensitive enzymes induced by caterpillar feeding on proteinase inhibitors.

H. armigera larvae were shown to produce a highly complex set of digestive proteinases of the trypsin- and chymotrypsin-like classes. Substantial progress was made in characterising the proteinase isozymes but ultimately this was incomplete. We suceeded in resolving 25 – 30 different proteinases on high resolution 2D gel systems and were able to tentatively group the majority of these into trypsin- or chymotrypsin-like families on the basis of peptide signatures. We established that *H. armigera* digestive proteinases are primarily of the serine proteinase class and that chymotrypsin-like enzymes predominate on both synthetic and natural plant diets. Approximately 90% of the proteolytic activity could be inhibited by the addition of two plant proteinase inhibitors to the enzyme assays but addition of these same inhibitors to artificial diet caused only a minor delay in larval development. Addition to the diet of inhibitors belonging to different plant inhibitor families did not give any further growth inhibition.

The very large number of proteins, combined with the existence of families of closely related enzymes prevented a complete characterisation of the *H. armigera* digestive proteinases. Although some progress was made in identifying inhibitor-insensitive enzymes the poor correlation between levels of enzyme inhibition observed *in vitro* and those achieved *in vivo* suggests that this approach does not have a strong predictive value for inhibitor selection. Thus, we were not able to achieve our overall objective of identifying a combination of inhibitors that could completely block larval growth. The adaptive flexibility conferred by the extreme genetic variation of the proteinase superfamily in *H. armigera* suggests that an approach to control based solely on this class of proteins is not likely to be effective.

What methodolgy was used, and a justification for the use of this methodology?

Insect Culture

A wild-type stock of *H. armigera* was maintained in laboratory culture at CSIRO Entomology. *H. armigera* adults, larvae and pupae were maintained at 25°C and 50% relative humidity with a 14hr light, 10 hr dark light cycle. Eggs were collected collected and stored at 10°C for up to 7 days before hatching at 25°C. Adult moths were maintained on a honey, sugar and water solution. Eggs and pupae were bleached in 0.2% sodium hypochlorite to prevent bacterial and viral infections.

Bioassays

Seed-based diet

Neonate larvae were raised in individual cells containing sufficient diet to allow development through to the final instar. Two different seed-based diets were tested extensively. The first used soybean meal with or without prior autoclaving to inactivate soy bean proteinase inhibitors (soy PIs, Birk, 1985). Heat-treated and non-heat-treated soy-meal was also mixed in various proportions to prepare diets to test for dosage effects of the soy PIs. We could not discount the possibility that the observed effects were due to some heat-labile, anti-nutritional factor other than the soy PIs in raw soy meal. We therefore tested diet based on chick-pea meal with or without

the addition of soy PIs or other proteinase inhibitors.

Two types of effect of diet, growth and proteinase expression, were measured routinely. To examine effects on growth, larvae were weighed after 1 week on the diet by which time they had generally reached 4th instar. Weights and mortality of larvae from proteinase inhibitor-containing diets were compared with larvae from control diets to test for any anti-nutritive effects of the proteinase inhibitors. Experiments comparing the effects of particular inhibitors on weights of larvae were repeated with at least three different generations of larvae. The mean weights of larvae from each inhibitor-containing diet were standardised to the corresponding control diet for each generation. Then the data were subjected to analysis of variance or t-tests to test the significance of any differences due to inhibitors.

Proteinase expression was compared among the different diets by analysing the soluble proteins recovered from the guts of animals on the various diets. Soluble gut proteins were collected from larvae raised on each of the diets, with and without various inhibitors. The cuticle of chilled larvae was slit longitudinally, then the gut tissue was slit longitudinally and the peritrophic membrane was lifted out together with most of the contents of the lumen of the midgut. This material was homogenised with cold 0.1 M Tris/HCl, pH 6.8 buffer and a few crystals of phenylthiourea (to inhibit phenoloxidase), centrifuged (16000*g* for 5 min at 4°C) and aliquots of the supernatants were stored at –80°C prior to analysis by various electrophoretic methods and enzyme assays outlined below.

A more limited experiment involved some other seed-based diets. Small batches of diets were prepared in which the meal components were prepared from dried haricot beans, dried mung bean, or soybeans. A diet consisting entirely of homogenised fresh maize was prepared and another diet comprising of a mixture of the haricot, mung, soy and maize diet. Sixteen neonate larvae were raised on each diet, weighed and dissected to yield gut proteins.

Leaf-based diet
Larvae were raised on fresh leaves from transgenic tobacco, with and without the expression of a giant taro proteinase inhibitor (GTPI). Plastic containers (9cm diameter) were used to raise groups of 10 larvae. The base of the container was covered with about 1cm of agar gel (20g agar and 3 g sorbic acid per litre of water) to provide constant humidity in the containers. Tobacco leaf pieces (3-4) about 5cm diameter were placed in the container and neonate larvae distributed among the leaf pieces. The perforated container lid was sealed with a sheet of tissue paper to prevent larvae escaping. Every few days old leaf pieces were removed and fresh leaf pieces added. After 11 days at room temperature (about 24°C) the larvae (generally 4th instars) were weighed and dissected to yield soluble gut protein preparations. Expression of the taro inhibitor in the appropriate leaves was confirmed by western blot using the GTPI anti-body raised by Wu *et al.* (1997).

Preparation of proteinase inhibitors

Soybean:
Commercial preparations of the separate soybean proteinase inhibitors (SBTI and SBBI) proved expensive for routine addition to diets. Instead we followed the method of Cole (1992) to extract a mixture of both soy proteinase inhibitors from soy meal. Organically grown soymeal (20g) was mixed with 200 ml water, the pH adjusted to 8 with NaOH, stirred for 1 hour, centrifuged (23000*g* for 20 min) and the supernatant filtered through glass wool. Polyethylene glycol (PEG1000) was added slowly with stirring to a final concentration of 40% (v/v). After 30 min stirring the mixture was centrifuged again (23000*g* for 20 min) and the supernatant filtered through glass wool. Acetone (-20°C) was added to the supernatant with stirring on ice to a final concentration of 80% (v/v). The mixture was left overnight at –20°C, centrifuged (23000*g* for 20 min), and the pellet was dried, crushed and stored at room temperature. Electrophoretic and mass spectrometric analysis of this preparation demonstrated that it contained a mixture of active SBBI and SBTI proteinase inhibitors and was substantially pure of other proteins.

Canola:
A crude preparation of Rapeseed Trypsin Inhibitor (RTI) was made following the initial steps of a method of Ceciliani *et al.* (1994). Canola seeds (40g) were ground, mixed with water (150 ml) and heated to 80°C for 3 min. After cooling and centrifuging (47000*g* for 30 min), the supernatant was filtered through glass wool and lyophilised.

Pumpkin Seed:
Seed of the Cucurbit (Squash) family generally contain proteinase inhibitors of a distinct structural class from other plant inhibitors (Bode and Huber, 1992). These are small, stable, water soluble peptides that are readily extracted. Seeds from Queensland Blue pumpkins (80g) were ground in a blender with 400 ml of 5mM ammonium bicarbonate, stirred for 1 hour at room temperature, centrifuged (16000*g* for 20 min) and the supernatant filtered through glass wool. The supernatant was boiled for 10 minutes, cooled to 4°C overnight and recentrifuged. The supernatant was aliquoted and stored at –20°C. Electrophoresis showed that the pumpkin seed preparation was enriched for proteins of about 17-30 kDa, and a contact print from a replicate electrophoresis gel showed proteinase inhibitor activity in the corresponding region.

Insect extracts:
At least six different structural classes of serine proteinase inhibitors have been documented from the haemolymph of various arthropods, including two lepidopterans (Kanost, 1999). Hamdaoui *et al.* (1998) also purified and identified a group of novel proteinase inhibitors from the haemolymph and ovaries of locust species. One proposed role for the insect inhibitors is regulation of endogenous proteinases so *H. armigera* had potential to be an ideal source of suitable inhibitors but we also tested material from other insects.

Peptides were extracted from dissected ovaries of *H. armigera* or *Lucilia cuprina* (Diptera) while precipitating proteins by homogenising the tissue in a methanol/acetic acid solution, sonicating (0°C, 30 minutes) and centrifugation (16000*g*, 4°C, 30 minutes). The supernatant was collected and the solvent was removed by evaporation. The dried material was redissolved in aqueous buffers for testing. Crude haemolymph was collected from two lepidopterans, *H. armigera* and *Galleria mellonella*, and a locust *Locusta migratoria*, into capillary tubes then transferred to microfuge tubes containing a few crystals of phenylthiourea to inhibit phenoloxidase activity. Haemocytes and any other particulate material were removed by centifugation and the clear serum was diluted in aqueous buffers for testing.

The various insect extracts were most extensively tested by incubation with gut proteins before or after native PAGE zymography. Some were tested in enzyme assays alone and in combination with other inhibitors. Although inhibition of various proteinase activities was observed none of the insect extracts appeared superior or complementary to the soy PIs.

Contact print detection of proteinase inhibitors
Solutions containing inhibitors from various sources (eg. insect haemolymph or seed extracts) were partially characterised by electrophoretic separation on SDS-PAGE (16%T/6%C resolving, 5%T/3%C stacking) using the tricine buffer system (Westermeier, 1997). Replicate gels were either stained with a general protein stain as above or zones containing protease inhibitors were detected by a contact print method (Pichare and Kachole, 1994). Gels were washed three times for 15 minutes with a 2.5% solution of Triton X-100 in 0.1M Tris/HCl, pH 7.8. Gels were briefly washed in the same buffer without Triton X-100 then soaked in a solution of bovine trypsin (0.1 mg/ml) for 5-10 minutes. Gels were again briefly washed with buffer then placed in contact with exposed X-ray film for 20 minutes at 37°C. The gelatin-based emulsion was digested by trypsin except in zones corresponding to proteinase inhibitors in gels. The digested emulsion was removed by rinsing the X-ray film under running water while undigested emulsion remained.

Total Proteinase Activity
Relative estimates of proteinase activity were obtained using a protein with attached dye molecules as a substrate (azocasein, Sigma) (Beynon and Bond, 1989). Soluble gut protein samples (3 µg) were preincubated at 37°C for 30 minutes in a microfuge tube with and without

proteinase inhibitor(s) in 75 µl of 0.15M Tris/HCl buffer, pH 8.8. Up to 100µg of inhibitors were added for maximum inhibition. Reactions were started by the addition of 150 µl of 1% w/v azocasein and reactions proceeded for a further 30 minutes at 37°C. The reaction was stopped by the addition of 75 µl of 40% trichloroacetic acid with vortex mixing. Undigested protein is coagulated and removed by centrifugation (16000*g*, 5 min) while the supernatant contains peptides with attached dye molecules. Aliquots (200µl) of the supernatant of each reaction were transferred to a 96 well titre tray and absorbances were measured at 340 nm.

Specific Proteinase Activities

Trypsin-like enzymes were detected with an artificial substrate, N-benzoyl-L-arginine p-nitroanilide (BApNA), while chymotrypsin-like enzymes were detected with N-succinyl-Ala-Ala-Pro-Phe p-nitroanilide (SA_2PFpNA) (Beynon and Bond, 1989). In both cases enzyme activity releases p-nitroaniline which is detected by its absorbance at 405 nm. Aliquots of gut protein (generally 3µg with BApNA or 2.5µg with SA_2PFpNA) were preincubated for 10 minutes with or without added inhibitor(s) in 100µl 0.15M Tris/HCl buffer, pH 8.8 in a 96 well microtitre tray at room temperature. Up to 100µg of inhibitors were added for maximum inhibition. Reactions were started by the addition of the appropriate substrate (2mM) dissolved in 100µl buffer. Stocks of the substrates were prepared as 40mM solutions in dimethylformamide. The reactions were followed for 10 minutes using BioRad 3550-UV microplate reader controlled by Microplate Manager/ Macintosh software (BioRad) to record reaction rates in each well. Elastase-like activities can be assayed with the artificial substrate, N-succinyl-Ala-Ala-Ala p-nitroanilide (SA_3pNA). Activity against this substrate was always less than 10% of the activity towards trypsin and chymotrypsin substrates for a variety of diets so this activity not investigated further.

Affinity purification of *H. armigera* Midgut Proteinases

Soluble gut proteins (2 mg) were applied to a column containing about 100µl of agarose beads with attached SBTI (Trypsin inhibitor agarose, T-0637, Sigma) equilibrated with 50 mM Tris/HCl, pH 8.0. Proteinases were allowed to bind for at least 10 minutes before the column was washed twice with 1ml of the same buffer, then eluted with 1 ml of 50 mM glycine/HCl, pH 2. Generally, at least 80% of the recovered proteinase activities were in the low pH eluate rather than the column washes. The low pH eluate was desalted and concentrated by diafiltration (Millipore Ultrafree Biomax 5K spin columns) and characterised by 2D-PAGE, mass spectral analysis of the whole sample, SDS-PAGE, and peptide mass fingerprinting of the material after SDS-PAGE.

Electrophoretic analysis:

Most published methods for proteinase zymography use the strongly denaturing detergent, sodium dodecyl sulphate (SDS) to inhibit proteinase activity during electrophoresis and incorporate a substrate such as gelatin into the acrylamide gel. It is intended that the substrate will only be hydrolysed after the electrophoretic separation when the SDS is washed out of the gel. We had two concerns with such methods. Firstly, we found that some *H. armigera* proteinases were not fully inactivated during electrophoresis and tended to clear the entire upper portion of the gels of substrate. Secondly, we were concerned that other proteinases might not reactivate when the SDS was removed. We therefore used two native PAGE systems that did not require denaturing and renaturing steps.

Standard Native PAGE

Native (non-denaturing) PAGE (7.5%T/3.3%C resolving gel, 4%T/3.3%C stacking gel) was used with the conventional Tris/glycine buffer system and the BioRad Mini Protean II electrophoresis apparatus to separate aliquots of soluble gut protein (5µg). This system only resolves proteins with neutral and acidic pIs. Following electrophoresis the gels were processed to visualise zones containing active proteinases. Gels were soaked for 20 min at 37°C in a solution of casein (2%) in 100 mM glycine, adjusted to pH10 with NaOH. The high pH was chosen to mimic the high pH of the caterpillar's midgut and the casein acts as a general substrate for proteinases. The gels were briefly rinsed with water then fixed and stained overnight with a solution of 0.01% w/v Coomassie G250 in 50% methanol, 10% acetic acid. Undigested casein causes the gel to stain uniformly blue except for clear zones where proteolytic activity had digested the casein.

Alkaline Native PAGE
An alternative native PAGE separation (10%T/3.3%C resolving gel, 4%T/3.3%C stacking gel) used a nonconventional histidine/TAPS buffer system to separate soluble gut protein (1µg). Cathodal migration rather than conventional anodal migration resolves proteins with basic pIs (Säftel *et al.*, BioRad Technical Note). Once the pyronine Y tracking dye reached the end of the resolving gel, the gels were soaked with casein, fixed and stained as above to reveal zones of protease activity.

Two dimensional (2D) -PAGE
2D-PAGE was used to resolve a greater number of proteinases than could be visualised by either 1D-PAGE system. Soluble midgut protein (5µg) was dissolved with rehydration solution (8 M urea, 2% ampholytes (IPG buffer 3-10, Pharmacia), 2% w/v CHAPS detergent, a trace of bromophenol blue dye, and water to final volume of 120 µl) which was used to rehydrate 7cm polyacrylamide immobilised pH gradient strips (IPG 3-10, Pharmacia) overnight at 4°C. The strips were focussed at 10°C using a Pharmacia Multiphor apparatus with an initial voltage of 150V ramped up to 3000V over about 2 hours. The voltage was then maintained at 3000V for 2.5-3hr for a total of about 9000VHrs. Focussed strips were equilibrated for 20-30 minutes with two changes of equilibration solution (6M urea, 2% w/v SDS, 50 mM Tris/HCl pH 6.8 and 20% v/v glycerol) and placed in "preparative" wells of SDS-PAGE gels (1mm thick, 12%T/3.3%C resolving gel, 4%T/3.3%C stacking gel) using the conventional Tris/Glycine buffer system (Laemmli, 1970) and the BioRad Mini-Protean II apparatus. Prestained molecular weight marker proteins (5µl, MultiMark, Invitrogen) were placed in reference wells. When the lowest molecular weight marker proteins were near the bottom of the resolving gels, SDS was removed from gels by gentle agitation for 30 minutes with two changes of a 2.5% (v/v) Triton X-100 solution. Gels were then soaked with casein, fixed and stained as above to reveal zones of proteinase activity.

The 2D-PAGE system was also used with a general protein stain (Zoion Fast Blue) to visualise all separated proteins. Compared with 2D-PAGE zymograms the amount of protein was greatly increased. In some experiments 800 µg of soluble gut protein was separated and in other experiments proteinases were first extracted from 2 mg of soluble gut protein by SBTI affinity chromatography (above). Both types of sample were desalted and concentrated by diafiltration (Millipore Ultrafree Biomax 5K spin columns).

Mass Spectrometry
Matrix assisted laser desorption/ionisation time of flight mass spectroscopy (MALDI-TOF-MS) was used in delayed extraction, linear mode to characterise several protein mixtures. Samples were mixed with 10 mg/ml sinapinic acid dissolved in 50% acetonitrile/0.1% trifluoroacetic acid and 2µl of each mixture was allowed dried on the sample plate of a Voyager Elite MALDI-TOF mass spectrometer (Perseptive Biosystems). Adjacent to each sample spot was placed a similar spot containing horse myoglobin (Sigma) for close external calibration (MH^+ = 16952.56 with M-$2H^+$ = 8476.78 or 2M-H^+ = 33904.1 peaks).

Peptide mass fingerprinting
In-gel digestion of proteins was performed with reduction and carbamidomethylation of any cysteine residues. 2D-PAGE gel spots (or bands from 1D gels) were cut from gels with a clean scalpel and incubated with 2.8 mM dithiothrietol in ammonium bicarbonate solution (160 µl, 100 mM) for 30 minutes at 60°C. Iodoacetamide (10 µl, 5.9 mM final conc.) was added and the reaction was kept for a further 30 minutes at room temperature in the dark. The gel pieces were washed twice with 50% acetonitrile/0.1% trifluoroacetic acid, dried under vacuum, rehydrated with 150 ng of trypsin (Promega sequencing grade) in 10 µl ammonium bicarbonate (100 mM), incubated overnight at 37°C, then extracted twice with 50% acetonitrile/0.1% trifluoroacetic acid in a sonicating water bath. For each gel piece, the extracts were pooled and dried in a vacuum centrifuge, redissolved in 2µl of matrix solution (10 mg/ml of α-cyano, 4-hydroxy, cinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid), and dried on the sample plate of a Voyager Elite MALDI-TOF mass spectrometer (Perseptive Biosystems). The instrument was used in delayed extraction, reflector mode. Peptides in a trypsin digest of bovine serum albumin (927.4940 and 2045.0285) were used for a close external calibration of digests. Mass list from digests were

used to search the NCBInr database using ProFound software (Version 4.8.5, Rockefeller University). Searches were restricted to the "Other Metazoa" taxonomic category to eliminate spurious matches. Where possible the largest and smallest masses from the best matching sequences were used for internal calibration of the mass spectrum and the search was repeated with a mass tolerance of 30 ppm.

Peptide sequence tagging
Trypsin digests from several of the most intensely stained spots on 2D gels were processed according to Keough *et al.* (2000) to add a sulphonic acid group at the N-terminus of each of the digest peptides. With suitable peptides this modification enhances post-source fragmentation in MALDI-TOF-MS while simplifying the pattern of fragmentation. Individual derivatised peptides were isolated from the other peptides using the Timed Ion Selector feature of the Voyager Elite instrument (Perseptive Biosystems). Fragments from selected peptides were separated in the ion mirror. The voltage on the ion mirror was altered incrementally to resolve fragments of different masses then the individual fragment spectra were stitched together using the Voyager Elite software. Composite spectra were smoothed and the mass differences between adjacent peaks were labelled using PerSeptive GRAMS software. These mass differences usually correspond to the masses of individual amino acid residues, often allowing sequences of about 6-12 amino acid residues to be deduced. Peptides chosen for sequencing by this method corresponded to masses that were found in fingerprint spectra of more than one 2D-PAGE spot but did not correspond to the masses of any predicted tryptic peptides from any known *H. armigera* serine proteinase gene.

Detailed results including statistical analysis of results

(1) Identification of midgut proteinases

Two complementary approaches were developed for proteinase characterisation; first, enzyme assays using a general non-specific substrate for total activity measurement, plus a panel of p-nitroaniline substrates specific for different serine proteinase subclasses and second, a gel electrophoretic zymogram analysis that allowed us to visualise the effects of different experimental treatments on individual isozymes. Most of the electrophoretic studies employed a novel native zymogram method developed specifically for this project.

The enzyme assays indicated that both trypsin-like and chymotrypsin-like activities are present in *H. armigera* larvae and are secreted into the midgut lumen as determined by hydrolysis of specific p-nitroaniline substrates. Essentially all of this activity could be inhibited by addition of different proteinase inhibitors to the assay (Fig. 1). When the non-specific substrate azocasein was used, approximately 70-80% of the proteolytic activity was inhibited by addition of the most effective proteinase inhibitors. The same proportion of activity was inhibited by the general serine hydrolase inhibitors DFP and PMSF, indicating that the majority of secreted midgut proteinases belong to the serine hydrolase class. The effects of other inhibitors on the different serine proteinase subclasses are presented in more detail in the results Section 2 below.

Electrophoretic analyses of *H. armigera* midgut proteinases were performed using non-denaturing polyacrylamide gel electrophoresis (PAGE) followed by incubation of the gel slab with casein as a general proteinase substrate. This system routinely allowed us to detect 12-16 electrophoretically distinct proteinase isozymes. Prior to making a more detailed analysis of the effects of dietary inhibitors, we used the zymogram system to show that the complement of proteinases expressed in the midgut changes little between different phases of larval development (Fig.2).

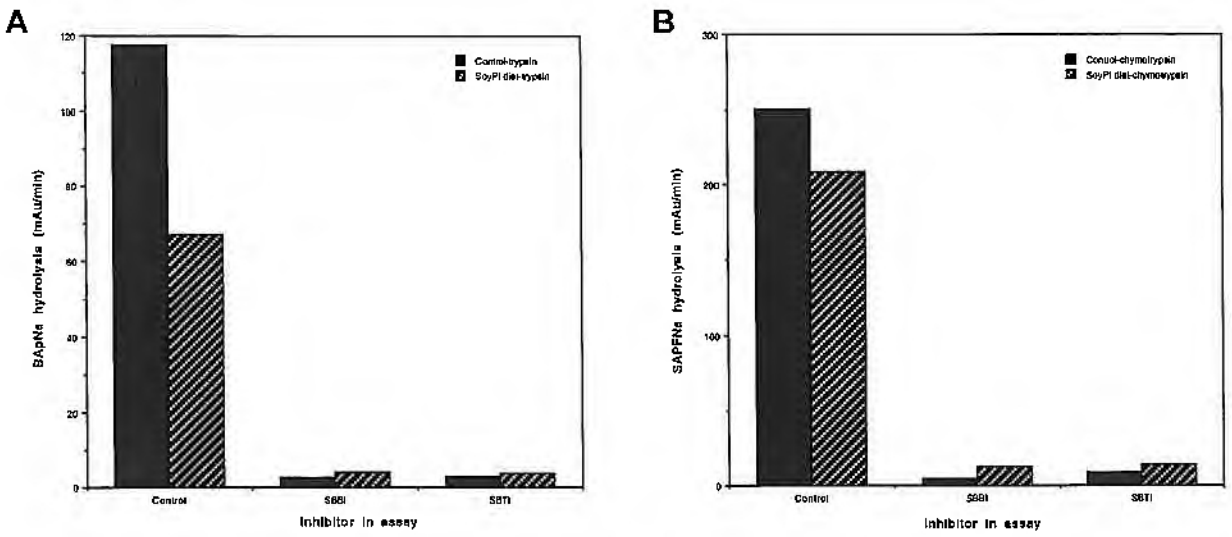

Fig. 1 Presence of trypsin- and chymotrypsin-like activities in secreted midgut enzymes of fourth instar *H. armigera* larvae determined by hydrolysis of the class-specific substrates BapNa and SAPFNa respectively. Panel A is the trypsin-like activity for larvae reared on control diet and diet containing soybean proteinase inhibitors and shows the effect of adding soybean Bowman-Birk inhibitor (SBBI) and soybean Kunitz trypsin inhibitor (SBTI) to the enzyme assay. Panel B shows chymotrypsin-like activity for the same sample treatments as in A.

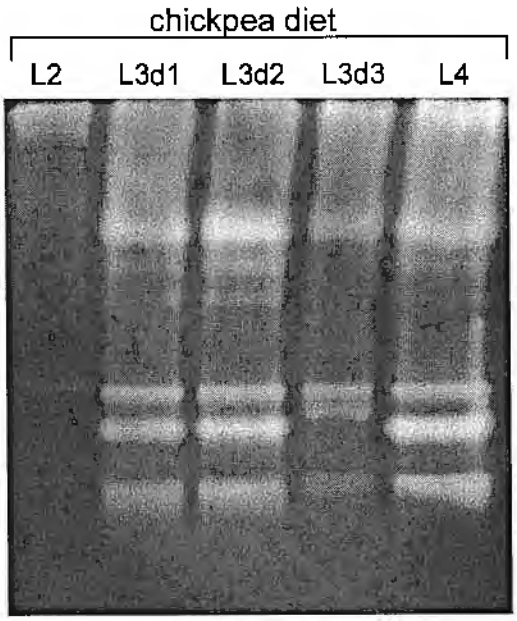

Fig. 2 Developmental profile of secreted midgut proteinases determined by zymogram method. Proteinases appear as white bands on a dark background. Equivalent amounts of total protein were loaded in each lane.

There was a major difference in the amount of proteinase present early in development (second instar) compared to the later stages (third and fourth instar). However, although the level of proteinase activity in second instar larvae was very low, and it is not obvious on Fig. 2, analysis of larger quantities of material indicated that the zymogram pattern was essentially the same as that observed for the later instars.

Several replicated bioassay experiments using different diet formulations established that the complement of enzymes produced in larval *H. armigera* is quite stable and relatively independent of diet (Fig. 3).

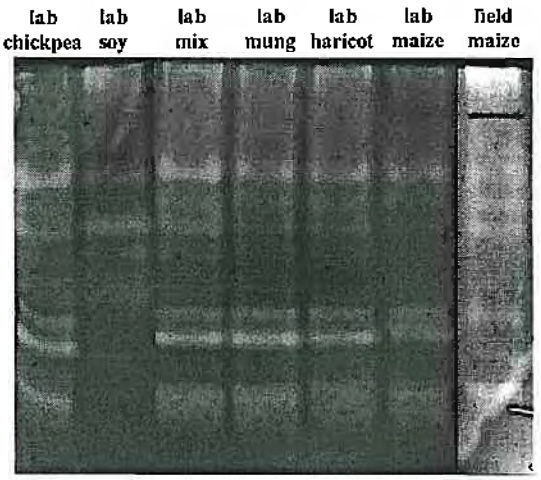

Fig.3 The *H. armigera* lab strain was raised on a standard diet differing only by the use of chickpea, soy, mung or haricot meals. The lab strain was also raised on a puree of fresh maize. For comparison larvae were collected directly from ears of maize collected in the field. "Lab mix" indicates the lab strain raised on a mixture of 25% each of soy-, mung- and haricot-based diet and maize puree.

We compared zymogram patterns for larvae of our laboratory strain raised on various diets and some field collected larvae (from maize). The diets were based on different legumes (mung bean, haricot beans, soy beans, chickpeas) or maize, or combinations of these ingredients, that might contain unidentified proteinase inhibitors. Essentially the same zymogram pattern was observed in all cases except for the soy-based diet. The field-collected larvae had bands that corresponded with the lab strain. Inclusion of proteinase inhibitors in the diet above a threshold level changed the pattern of enzyme expression, and we believe this is the reason for the different pattern of enzyme expression in the soymeal diet. In the absence of inhibitors the pattern of proteinases observed on the zymograms was essentially the same for caterpillars fed either on different synthetic diets or on live plant material (eg "field maize" in Fig. 3 above).

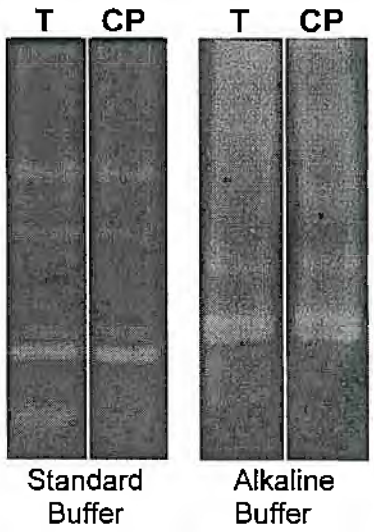

Fig. 4 Detection of *H. armigera* midgut proteinases for larvae reared on tobacco (T) and laboratory chickpea (CP) diets, using standard and alkaline buffer systems.

The electrophoresis buffers used for the native PAGE system would only resolve proteinases with neutral and acidic isoelectric points (pI), so similar experiments were performed using a different

(alkaline) native PAGE system to separate basic (i.e. high pI) proteins (Fig. 4). A larger proportion of the proteinase activity appeared to have a high pI, as strong clearing of zones of the basic, native PAGE gels were seen with five-fold less midgut extract than the standard native PAGE system. Although the resolution was not as good as the standard native PAGE system, at least 6 bands were reproducibly detected. In common with the standard native PAGE system, there were no dramatic differences between the zymogram patterns for tobacco leaf vs. chickpea diets.

The zymogram method was subsequently adapted to a high resolution two-dimensional electrophoretic system (Fig. 5). This highly sensitive method enabled us to visualise at least 25 active proteinases in the *H. armigera* larval gut covering a range of isoelectric points from pH 3 to 10 and apparent molecular weights from about 30 to 60 kDa. Many of the proteinase activities corresponded with major proteins of the gut.

This type of analysis would normally cover the majority of proteins expressed in a tissue. However, the lepidopteran larval midgut has an unusually high luminal pH of 10-11, and it is probable that at least some enzymes adapted to this environment will have very high isoelectric points. Although immobilised pH gradients have recently become available that extend the 2D PAGE analysis up to pH 11 we were unable to reproducibly resolve additional proteinases with this system.

Fig. 5 Two dimensional PAGE zymogram showing resolution of at least 25 distinct proteinase enzymes in secreted midgut fluid of fourth instar *H. armigera* larvae.

From the 2D gels it was clear that there were several proteinases with high isoelectric points that were not observed in our one dimensional native PAGE zymogram system. In addition to the native single dimension and two-dimensional electrophoretic systems we also used denaturing single dimension PAGE and showed that there was likely to be at least one major isozyme not detected at the pH used for the native gels. Combining the results of the zymogram assays we have now conservatively identifed a minimum of 30 different proteinases.

Prior to making a more detailed study of these enzymes we conducted a bioinformatic analysis of the 22 published *H. armigera* serine proteinase gene sequences (Bown *et al.*, 1997) that became available in the GenBank database in the course of this project. These 22 proteinases can be grouped into trypsin-like and chymotrypsin-like enzymes on the basis of structural features. Approximately half were trypsin-like and had relatively high calculated pI values (>8.3). We expect most of these enzymes would be detected on the alkaline gel assay system. The chymotrypsin-like enzymes were much more variable in structure with pI values ranging from 6.9 to 11.7. We would expect some of these to be resolved on our standard native gel system and others to be detectable only on the alkaline buffer system.

Once it became clear that the *H. armigera* caterpillar expresses a very large number of proteinases we focussed the majority of our effort on development of techniques that would allow us to identify individual proteins. The availability of a sensistive 2D electrophoretic system provided a potential solution through the use of peptide mass fingerprinting and database searching. Although this technology is exceptionally sensitive, the particular problem posed by the *H. armigera* proteinases is a challenging one, as it is now clear that we are dealing with several different multi-member gene families, where members within a family are structurally quite similar. To provide sufficient material for biochemical analysis we partially purified the proteinases on a Kunitz soybean trypsin inhibitor affinity column. Figure 6 shows the electrophoretic analysis of the affinity purified material.

Although many of the proteinases were present in only small amounts we were able to derive mass fingerprints for the majority of protein spots. This allowed us to group enzymes into "families" based on the presence of diagnostic peptides that were either conserved, or present as single amino acid substitutions in all members of a "family".

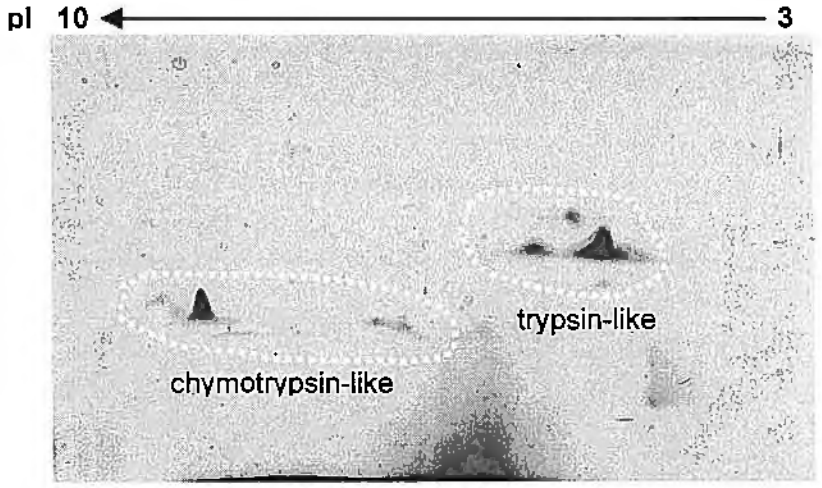

Fig. 6 Two dimensional electrophoresis of midgut proteinases partially purified by SBTI affinity chromatography resolved approximately 18 proteins.

Curiously, we were not able to identify a single enzyme from the existing *H. armigera* proteinase database on the basis of peptide mass fingerprinting. However, combination of diagnostic peptide masses and partial amino acid sequencing by derivatisation and peptide fragmentation by post source decay enabled us to identify groups of proteins as belonging to either the trypsin- or chymotrypsin superfamilies.

(2) Identification of enzyme inhibitors effective against H. armigera proteinases in vitro

Initially we evaluated the use of heat-treated and non-heat-treated soy meal as a basis for diets with and without active soybean proteinase inhibitors and the effect of these diets on *H. armigera* midgut proteinase expression are discussed in results Section 3 below. As this appeared to be a promising system we developed a rapid method for preparing a substantially pure extract containing both the soybean trypsin inhibitor and the soybean Bowman-Birk inhibitor. This provided a simple and cost-effective means for preparing diets containing two of the major plant proteinase inhibitor classes.

As illustrated in Figure 7, inclusion of the two classes of soybean inhibitors in the diet induced a substantial alteration in the pattern of proteinase expression in *H. armigera* larvae. Some enzymes disappeared or had greatly reduced activity on diet with inhibitors (eg those indicated by small arrows) whereas others appeared to be expressed at higher levels (eg the band marked with a large arrow). There did not appear to be any marked differential effect associated with

developmental stage of the insect, and all subsequent work was done using fourth instar caterpillars.

Most effort was concentrated on peptide inhibitors of serine proteinases from plant sources. In addition to soybean Kunitz trypsin inhibitor (SBTI) and soybean Bowman-Birk trypsin/ chymotrypsin inhibitor (SBBI), we investigated inhibitors from two other plant sources. One of these was derived from seeds of the curcurbit (squash) family of plants. These inhibitors are a distinct structural family from other inhibitors and are unusually small (only about 30 amino acids) and stable. The other class of inhibitors tested were a newly described family from rape seed. We prepared a crude inhibitor extract from canola seed to represent this class.

Fig. 7 Effect of dietary proteinase inhibitors on expression of secreted midgut proteinases for different developmental stages as determined by zymogram method.

The pumpkin seed inhibitors were very effective against trypsin activity from the gut of larvae raised without inhibitors in their diet (up to 90% inhibition). However, they were less effective against the trypsin activity of larvae raised with soybean inhibitors in their diet (23% inhibition). This observation is consistent with the effect of combining the soy and pumpkin seed inhibitors in diet. Alone each inhibitor preparation caused about a 25% reduction in growth but no further effect was obtained when both inhibitors were included in diet. The canola seed inhibitor did not seem to be effective but further testing is required.

Proteinase inhibitors from several different animal sources were tested since these are not encountered naturally in the diet of *H. armigera* and therefore have not been subjected to adaptive development of inhibitor-insensitive isozymes. An interesting source of inhibitors are insects themselves. A recent paper reported inhibition of the gut proteinases of a locust by extracts of its thoracic glands or ovaries (Hamdaoui *et al.*, 1998). Similar ovary extracts have been prepared from *H. armigera* and *L. cuprina.* There have also been reports of proteinase inhibitors in the haemolymph of Lepidoptera and other insects, with some examples of activity against gut proteinases. A possible function of these inhibitors is to control the insect's own enzymes and *H. armigera* haemolymph therefore might be an ideal source of proteinase inhibitors that have been honed by evolution to fit the enzymes we are targetting. When testing for *H. armigera* haemolymph inhibitors we also tested haemolymph from several lepidopteran and other insect species available in culture at the Division of Entomology.

Insect ovary extracts were tested for their ability to inhibit proteinases by incubation with larval gut extracts before or after separation by native PAGE. No inhibitory effects were observed and this potential inhibitor source was not pursued further. Each of five haemolymph types was tested for

its ability to inhibit *H. armigera* proteinases by incubation with zymogram strips prior to the addition of casein as the proteinase substrate. The two most promising were haemolymph from *H. armigera* and *G. mellonella*. Both of these inhibited multiple proteinase bands in zymograms of gut extracts from larvae raised with or without soybean inhibitors in their diet. Although these haemolymph inhibitors were highly effective against both trypsin- and chymotrypsin-like activities, they appeared to act on the same enzymes as the plant proteinase inhibitors (Fig. 8).

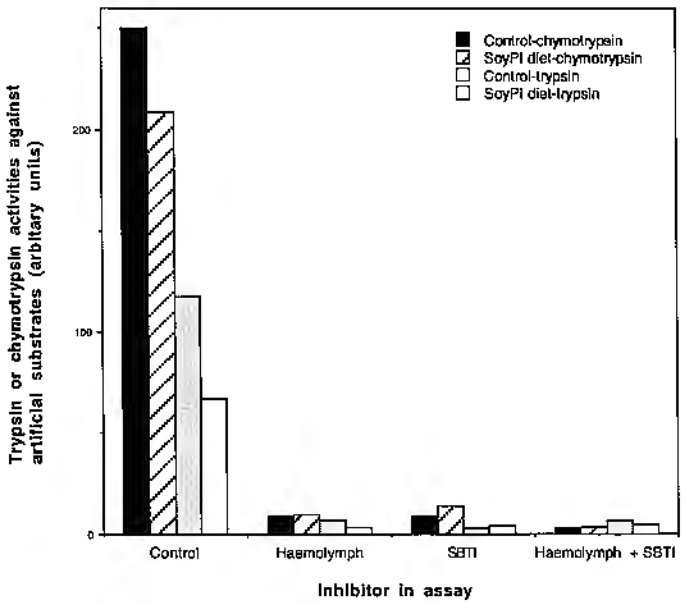

Fig. 8 Effects of plant and insect-derived proteinase inhibitors on tryptic and chymotryptic activity in midgut extracts of larvae reared on control and soy-inhibitor containing diets.

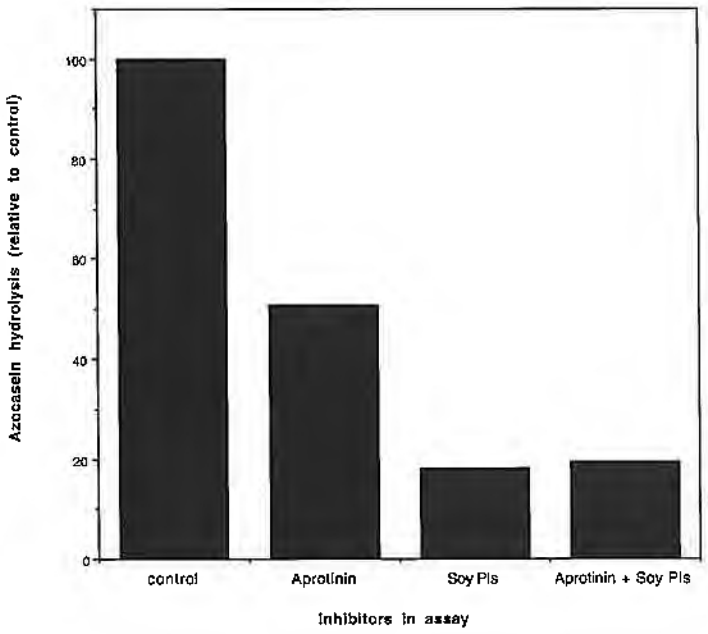

Fig. 9 Effect of aprotinin on *H. armigera* proteinase activity, alone and in combination with soybean inhibitors.

We investigated two proteinase inhibitors from vertebrates, aprotinin from bovine lung and a semipurified inhibitor from human serum (donated by Dr. D. Ollis, Australian National University). These two vertebrate inhibitors, which belong to a different structural class to the plant and insect inhibitors, blocked part of the larval gut proteinase activity, but no more than was inhibited by the soybean inhibitors. This was the case whether larvae were raised with or without dietary soybean inhibitors, i.e. the enzymes that were not inhibited by soybean inhibitors were also not inhibited by the vertebrate inhibitors. Results for one of these experiments, with aprotinin, are shown in Figure 9.

Although there were obvious differences in the efficacy of different inhibitor preparations, we did not attempt any further work on identification of new inhibitors. We concluded that there was nothing to be gained from random screening of additional inhibitors until there is a more complete understanding of the functional significance of the extreme diversity of proteinases produced by larval *H. armigera* and described in the Results Section 1 above.

(3) Effects of candidate inhibitors on growth and enzyme activity

The effects of proteinase inhibitors on larval growth were tested in four different diets. The first was based on soybean meal which was used with or without prior autoclaving to inactivate soybean proteinases. We found dose-dependant variation in the growth of larvae on diet prepared with soy meal that was heat-treated, non-heat-treated or a mixture of both. Larvae were 32±6% less heavy ($F_{5,372}$=11, $p < 0.01$) after 1 week on diet made with 25% or more non-heat-treated soy meal (Fig. 10). We attributed this to the effects of active proteinase inhibitors but we could not discount the possibility of some other heat-labile, anti-nutritional factor in raw soy meal. We therefore tested a second diet based on chick-pea meal with or without the addition of soy bean proteinase inhibitors (SBPIs). The addition of SBPIs to this diet reduced larval weight after one week by 24±9% (t_5 = 2.6 , $p < 0.05$), an effect similar to that seen with the heat-treated/non-heat-treated soy diets.

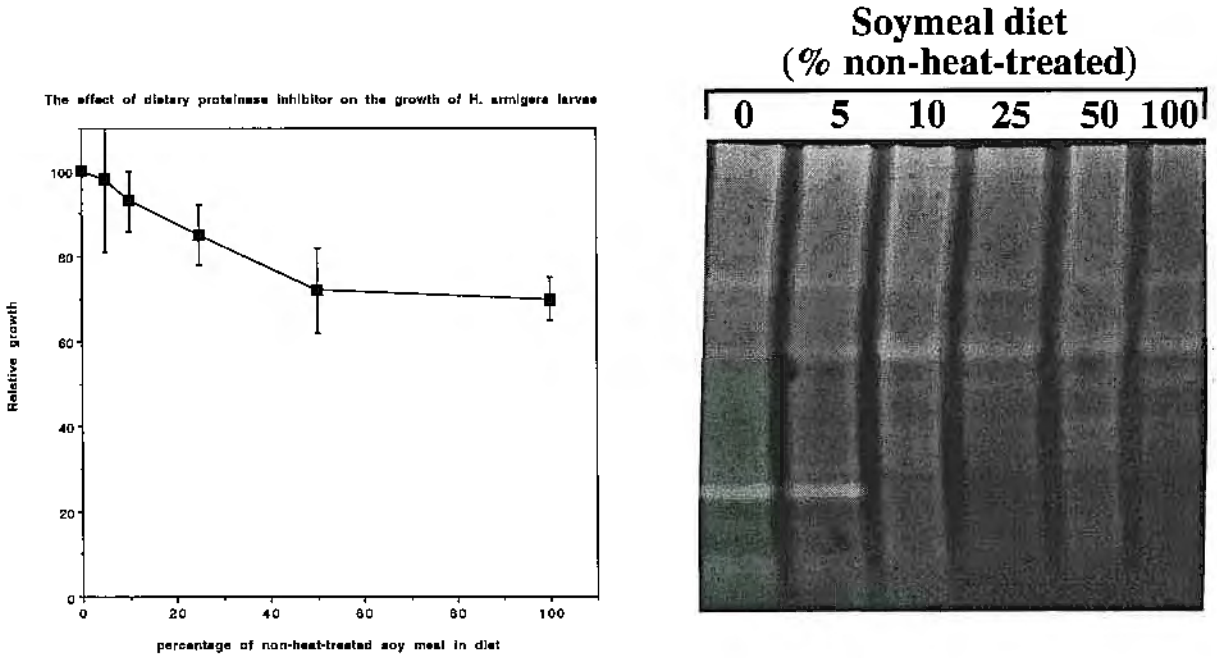

Fig. 10 Effect of dietary proteinase inhibitors on larval growth and proteinase expression patterns. Panel A shows the effect of increasing concentrations of proteinase inhibitor in the larval diet on larval weight at 12 days after emergence. Panel B shows the effect of increasing concentration of proteinase inhibitor on the expression of individual proteinase isozymes.

Because the effect of increasing proteinase inhibitor reached a plateau at around 50% of non-heat treated soy meal in the diet we were concerned to demonstrate that under these conditions there was excess inhibitor present in the insect gut. Figure 11 shows the gut contents of fourth instar larvae fed on inhibitor containing diet. The most intensely stained region from the centre of the abundant protein present in the lower right hand corner of the gel was cut out, digested and analysed by mass spectrometry. The resultant peptide mass fingerprint identified it unambiguously as the soybean Kunitz trypsin inhibitor (SBTI), clearly indicating a massive excess of undigested inhibitor in the gut.

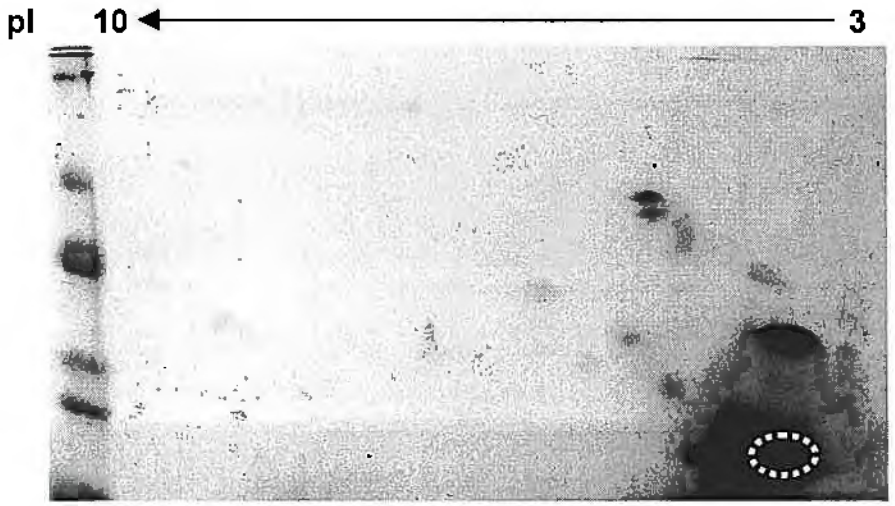

Fig. 11 The 2D gel is of whole gut lumen contents from larvae fed on diet prepared from untreated soy meal containing active proteinase inhibitors. Circled area indicates region of gel excised for mass spectrometric analysis.

The third diet involved tests of the cucurbit (pumpkin) seed inhibitor alone and in combination with the two soybean inhibitors. Alone each inhibitor preparation caused about a 25% reduction in growth but no further effect was obtained when both inhibitors were included in diet (Fig. 12). The canola seed inhibitor did not affect larval growth or survival.

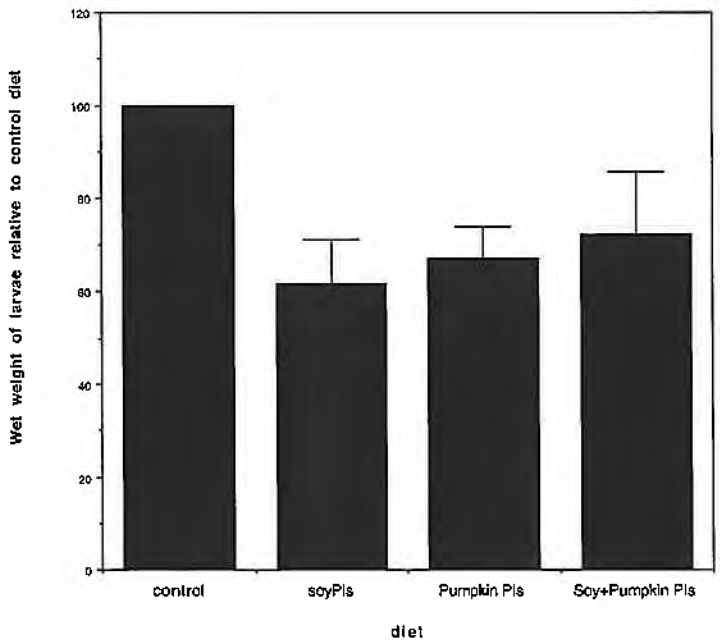

Fig. 12 Effect on *H. armigera* growth of diet containing the two soybean inhibitors, the cucurbit inhibitor and a mixture of all three classes of plant proteinase inhibitor.

The fourth diet we tested was fresh leaves from transgenic tobacco, with and without the expression of a giant taro proteinase inhibitor. In contrast to an earlier study (Wu *et al.* 1997) we did not see any consistent effects on *H. armigera* larvae grown for 11 days on transgenic tobacco with or without the giant taro proteinase inhibitor.

A major focus of this work was to identify changes in proteinase expression resulting from feeding *H. armigera* larvae on diets supplemented with selected plant proteinase inhibitors, and to compare that with the profile of enzyme expression in insects fed on a susceptible host plant constitutively expressing proteinase inhibitor.

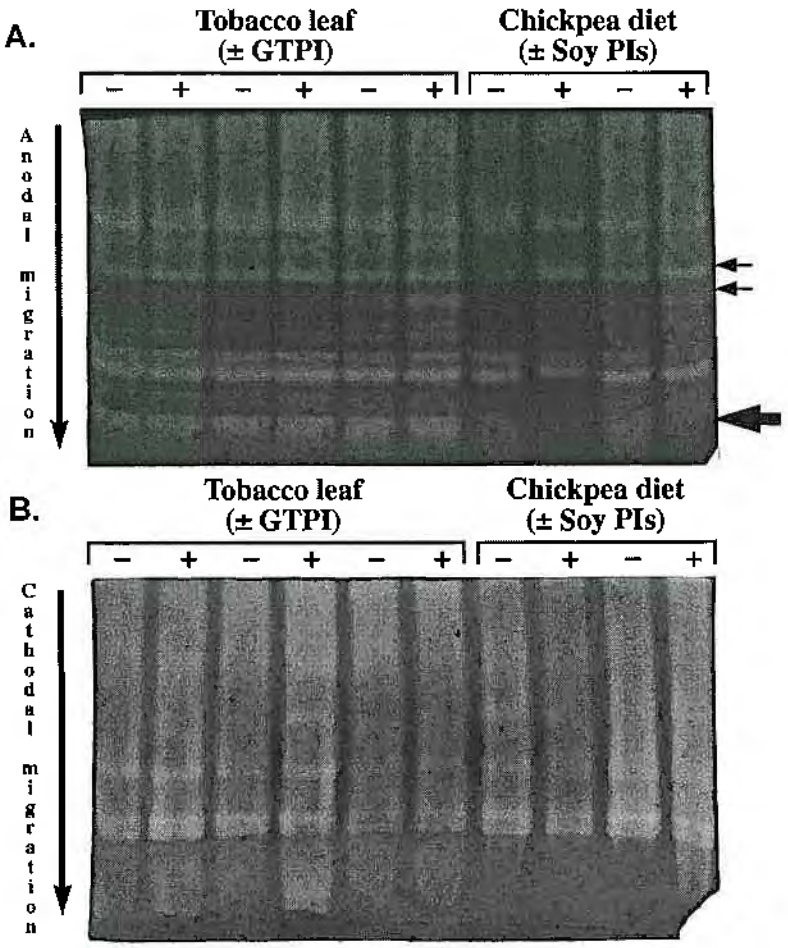

Fig. 13. Native PAGE zymograms for *H. armigera* larvae reared on synthetic laboratory (chickpea) diet either lacking, or supplemented with, purified soybean proteinase inhibitors (±Soy PI), or fresh plant material (tobacco) either untransformed or transformed to express giant taro proteinase inhbitor (± GTPI). Panel A. shows the standard native PAGE system. Panel B. shows the use of a native alkaline buffer system to identify enzymes with high pI.

With the exception of a group of rapidly-migrating isozymes (indicated by large arrow, Fig. 13), the pattern of proteinase expression in larvae grown on synthetic diets and live plant material was essentially the same suggesting a stable, constitutive synthesis of one group of enzymes. The pattern of proteinase expression was also very similar in insects reared on transgenic plants expressing a proteinase inhibitor compared with those raised on synthetic diet containing proteinase inhibitor. However, when proteinase inhibitor was included in the diet, the expression of at least two isozymes was increased substantially compared to inhibitor-free diet (indicated by small arrows, Fig. 13).

(4) Characterisation of inhibitor-insensitive proteinases

One of the characteristic changes in proteinase patterns of larvae from diet containing soy inhibitors was the partial or complete "loss" of proteinase activity bands from the high mobility

zone of native PAGE zymograms. This might have been due to the presence of dietary inhibitor remaining bound to these proteinases throughout the process of gut extraction, dilution, electrophoresis and zymogram staining. Alternatively, the dietary inhibitors might have repressed the expression of those particular enzymes.

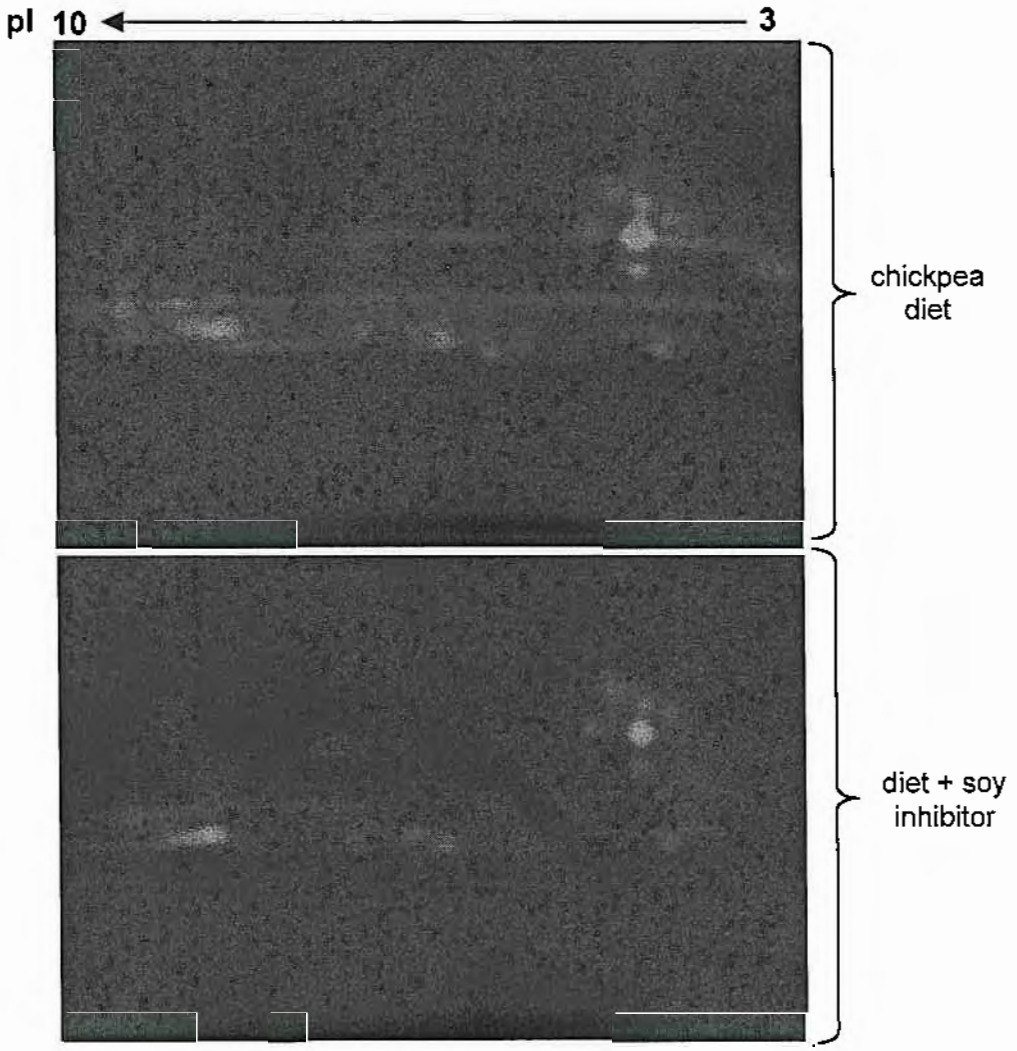

Fig. 14 Effect of dietary inhibitors on proteinase expression profile as determined by high resolution 2D zymograms.

A feature of the 2D zymogram system is that proteins are denatured before electrophoretic separation and refolded after separation. Thus proteinases are uncovered that are present but inactive because they are complexed with inhibitor from the diet. The 2D zymogram patterns of larvae fed with soy inhibitors and larvae not fed with inhibitors are very similar (Fig.14) , unlike the native zymograms, suggesting that the "loss" of proteinase bands was due to inhibition of enzymes which continue to be expressed in the presence of soy inhibitors. Overall there was an apparent reduction in proteolytic activity in extracts from animals reared on diet containing proteinase inhibitors, consistent with enzyme assay data presented above (Fig. 1) showing reduced trypsin- and chymotrypsin-like activity under these conditions.

In order to characterise the changes in expression of individual isozymes in more detail, proteinases were partially purified by affinity chromatography on a Kunitz SBTI-agarose column. Midgut luminal contents from fourth instar *H. armigera* larvae were collected from animals reared on either standard chickpea diet, or diet suplemented with purified soybean proteinase inhibitors. Approximately equal amounts of protein were loaded onto the affinity column and the eluted

proteinases were concentrated and analysed by 2D PAGE (Fig. 15). There appeared to be both qualitative and quantitative differences between the two samples. Approximately 20 proteins were visible on the stained second dimension gels and most of these corresponded with proteinases visualised on zymograms of the starting material before it had been processed on the affinity column (*cf* Fig. 14). Less protein was recovered from the material from insects raised on inhibitor-containing diet and the reason for this is not clear. Overall, the pattern of staining was quite similar for the two samples with the exception of a major trypsin-like enzyme (indicated by the arrow on Fig. 15) that was present in animals fed on on control diet and absent or greatly reduced in animals fed on diet containing proteinase inhibitor.

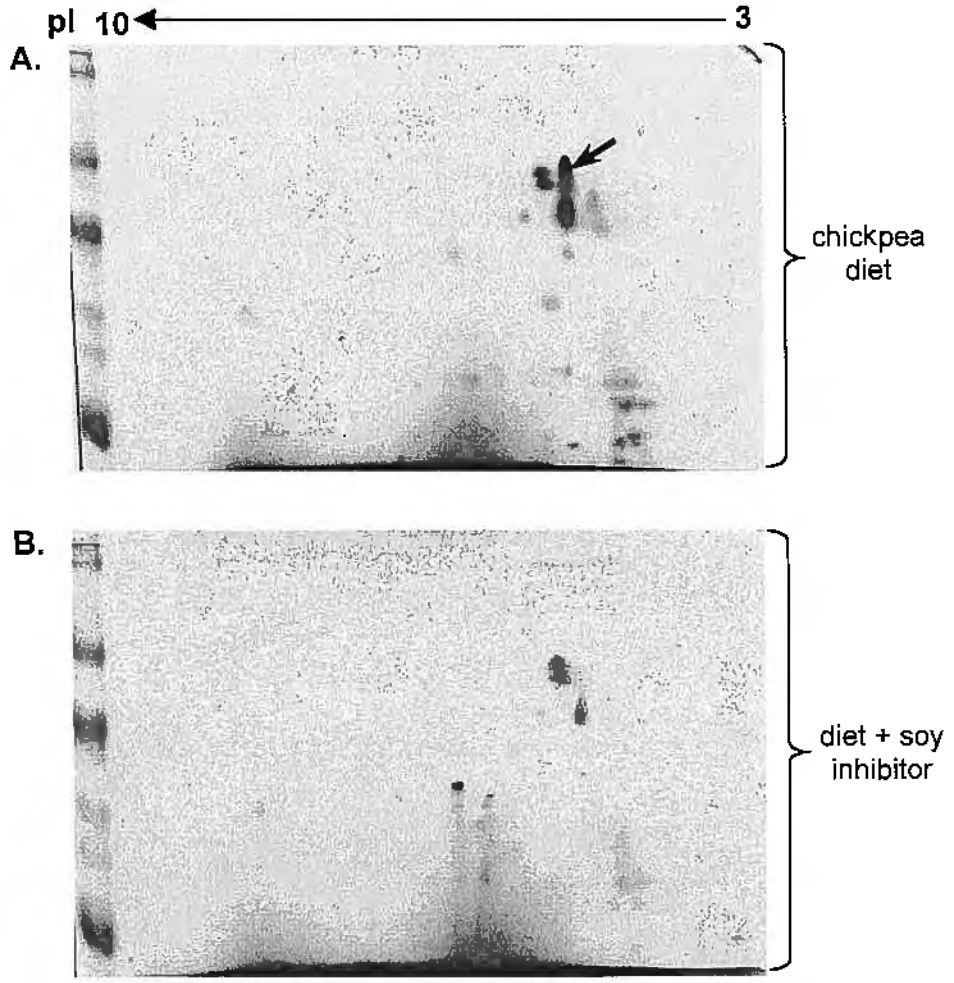

Fig. 15 2D PAGE analysis of affinity purified midgut proteinases from *H. armigera* larvae. Panel A is material from insects raised on standard chickpea diet. Panel B shows proteinases from larvae grown on chickpea diet containing Soybean proteinase inhibitors. A major protein that is reduced in animals reared in the presence of soy inhibitors is indicated by an arrow.

As noted in Results section 1 above, soy inhibitor-fed larvae had reduced trypsin and chymotrypsin-like activities (20-40% reductions), and the residual activity was almost completely inhibited by addition of excess soybean inhibitors to assays employing p-nitrophenyl esters (Fig. 1). However, when the non-specific substrate azocasein was used, a reduction in total proteolytic activity was observed for larvae fed on inhibitor-containing diet, but approximately 15-20% of this activity remained after excess inhibitor was added to the assay (Fig. 16).

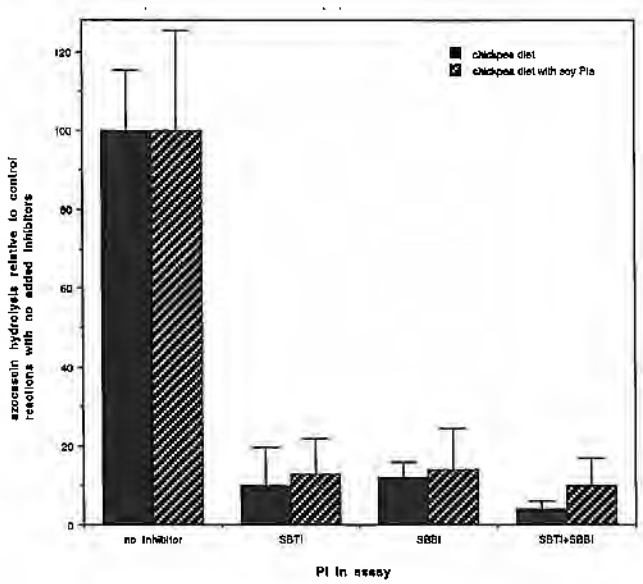

Fig. 16 Effects of proteinase inhibitors on residual proteinase activity in midgut extracts of larvae raised on standard chickpea diet and diet supplemented with soybean inhibitors.

This effect was even more pronounced when larvae were raised on fresh plant material, irrespective of whether or not the plants were expressing a transgenic proteinase inhibitor. Under these growth conditions 20-30% of the activity against azocasein could not be inhibited by addition of excess soybean inhibitors to the enzyme assays (Fig. 17).

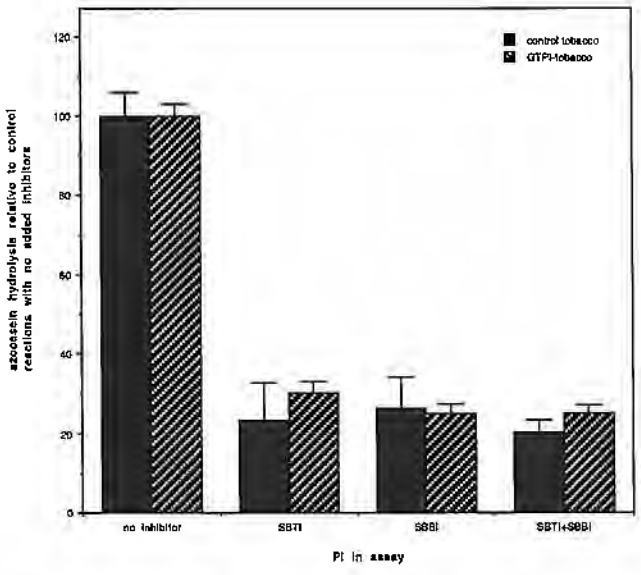

Fig. 17 Effects of proteinase inhibitors on residual proteinase activity in midgut extracts of larvae raised on control or transgenic tobacco expressing giant taro proteinase inhibitor.

Figure 18 shows that a similar effect was also observed when the enzyme class-specific substrates were used to measure trypsin- and chymotrypsin-like activities.

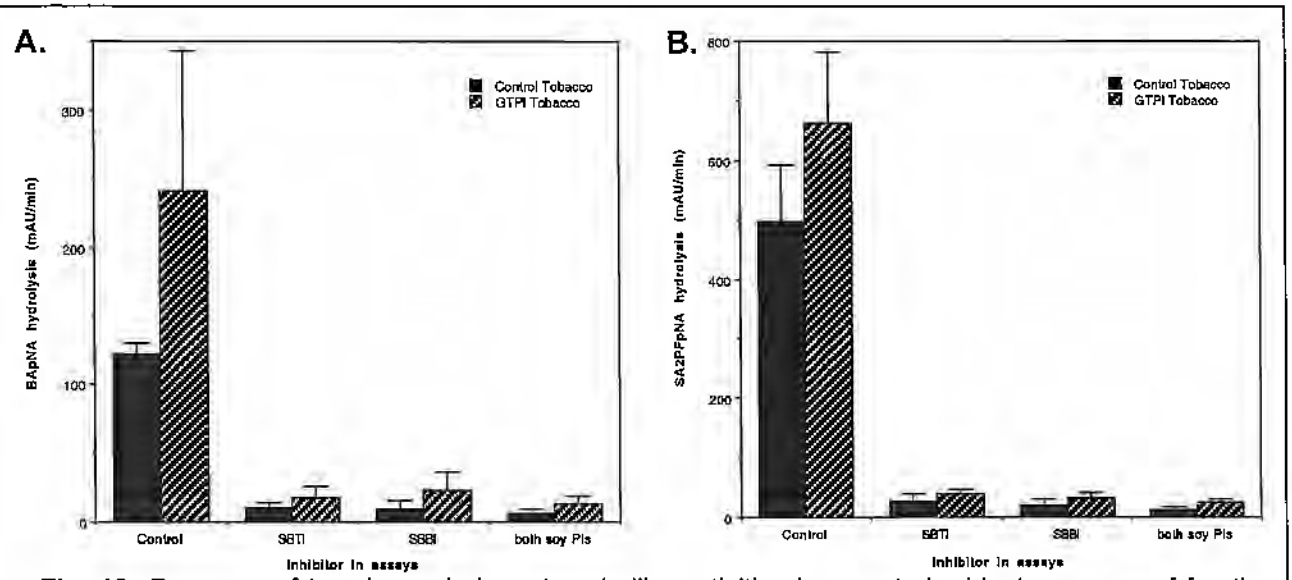

Fig. 18 Presence of trypsin- and chymotrypsin-like activities in secreted midgut enzymes of fourth instar *H. armigera* larvae raised on tobacco leaves, determined by hydrolysis of the class-specific substrates BapNa and SA_2PFpNa respectively. Panel A is trypsin-like activity for larvae reared on control tobacco and tobacco expressing GTPI and shows the effect of adding soybean Bowman-Birk inhibitor (SBBI) and soybean Kunitz trypsin inhibitor (SBTI), and a combination of both inhibitors to the enzyme assay. Panel B shows chymotrypsin-like activity for the same sample treatments as in A.

Taken together with the results from insects raised on inhibitor-free diet, these data suggest that about one fifth of the total proteolytic activity in larvae reared on diet containing soybean inhibitors is insensitive to a range of serine proteinase inhibitors. It has not been possible to determine if this is due to one or a small number of relatively abundant enzymes or to a larger number of moderately expressed enzymes.

A discussion of the results, including an anlysis of research outcomes compared with the objectives

It is clear from the results obtained in this project that *H. armigera* has a highly developed repertoire of digestive proteinases that enables it to feed successfully on a wide range of diets containing a variety of different proteinase inhibitors. A novel 2D-gel electrophoretic proteinase zymogram developed for this project has allowed us to visualise at least 25 distinct proteinases in the midgut contents of *H.armigera* caterpillars. We believe there are likely to be a significant number of additional proteinases not detected in this assay because their atypically high isoelectric points lie outside the pH range covered by this gel system.

The patterns of proteinase expression in larvae grown on synthetic diets and live plant material were quite similar suggesting a stable, constitutive synthesis of a large group of enzymes. However, the zymograms from larvae grown on fresh plant material were more complex, suggesting that a greater number of proteinases were being expressed under these conditions compared to the simpler and more stable synthetic laboratory diets.

When proteinase inhibitor was included in the diet the expression of at least two isozymes is increased substantially. The same isozymes appeared to be activated in response to a variety of different proteinase inhibitors. The native PAGE zymogram technique enabled us to resolve three distinct populations of proteinases from *H. armigera*; one group of high relative mobility that were consistently absent in animals fed on inhibitor-containing laboratory diet, a second group of low/intermediate mobility that were relatively unaffected by diet composition and a third group, represented by two isozymes, that were present in much higher levels in inhibitor-fed larvae. This analysis was based largely on the one-dimensional native zymogram gel system, and it should be stressed that this is likely to be an oversimplification of the true situation.

The high resolution 2D gel electrophoretic system established that each detectable protein spot in affinity-purified preparations corresponded to a proteinase activity on 2D zymograms of *H. armigera* midgut extracts. The results suggested that totally novel proteins were not expressed in response to soy inhibitors, but rather, the quantity of particular enzymes seemed to change. This is consistent with recent data showing several fold changes (up and down) in the level of serine proteinase cDNAs in response to dietary inhibitors (Gatehouse *et al.* 1997).

We have established that *H. armigera* larvae adapt to the presence of high levels of proteinase inhibitors in their diet by adjusting the balance of digestive proteinases secreted into the gut lumen. However, due to the very large number of enzymes involved, it has not been possible to determine the precise details of this adaptive response.

The substrate specificity of the inhibitor-insensitive proteinases is not yet clear, although limited data suggest they are trypsin-like. *H. armigera* larvae normally produce a mixture of trypsin- and chymotrypsin-like activities when assayed with p-nitroaniline substrates specific for the different subclasses of serine proteinases. Larvae raised on diet containing soybean inhibitors had significantly reduced trypsin- and chymotrypsin-like activities. However, essentially all of this activity could be abolished by the addition of soybean inhibitors to the *in vitro* enzyme assay. This contrasts with the results obtained with the general proteinase substrate azocasein. With this substrate, approximately the same reduction in total proteolytic activity was observed for insects raised on inhibitor-containing diet, but a significant proportion (up to 30%) of this activity was unaffected by inclusion of soybean inhibitors in the *in vitro* enzyme assay. Since more than 90% of the activity against azocasein was blocked *in vitro* by inclusion of the generic non-peptide serine proteinase inhibitors DFP and PMSF in the assay, we conclude that the soybean inhibitor-insensitive enzymes are probably serine proteinases. The difference in results obtained with the specific substrates compared to the general substrate has not been reported previously, possibly because most studies have relied on monitoring enzyme activities with the p-nitroaniline substrates. The reasons for the discrepancy are not clear but it is an important finding. Any future work to purify the inhibitor-insensitive enzyme(s) and identify effective new inhibitors ought to be done using the general proteinase substrate, though these assays are more cumbersome and slower to perform.

In the course of this project we established that the spectrum of serine proteinases expressed in the *H. armigera* larval gut is far more extensive and complex than previously suspected. Until recently it would not have been technically feasible to individually identify such a large group of closely related proteins. However, the combination of multiple high-resolution electrophoretic techniques with protein mass spectrometry provides a solution to the problem. Once an appropriate database of *H. armigera* midgut proteinase sequences becomes available it will be possible to obtain a quantitative measure of the changes in expression of individual proteinases in response to enzyme inhibitors in the insect's diet.

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An assessment of the likely impact of the results and conclusions of the Research project for the cotton industry and, where possible, a statement of the costs and potential benefits to the Australian cotton industry and future research needs

Although substantial progress was made against all the major experimental aims of the project, ultimately we were not able to identify a combination of proteinase inhibitors that would provide economically useful levels of protection against *H. armigera*.

In view of the great genetic diversity of midgut proteinases produced by *H. armigera* larvae and the flexibility this provides for adaptation to structurally diverse peptide inhibitors, a transgenic control strategy that targetted this one class of enzymes seems unlikely to succeed. However, the concept of anti-nutritional transgenes ought not be dismissed prematurely. Once there is a more detailed understanding of the rate-limiting processes in caterpillar digestion it would be appropriate to re-visit this type of control strategy, recognising the need to target synergising combinations of enzyme inhibitors.

Understanding the proteolytic capability of larval *Helicoverpa* has implications that extend well beyond the development of proteinase inhibitor transgenes. The success of *H. armigera* as a highly polyphagous species has been accompanied by the development of a formidable armoury of proteolytic enzymes. Any transgenic approach to *H. armigera* control must overcome the insect's capacity to rapidly digest protein toxins delivered through the host plant. For example, a recent paper showed that *H. armigera* was far more effective at digesting Bt δ-endotoxin than another lepidopteran species (Shao *et al.*, 1998. J. Invert. Path. **72**, 73-81). This may provide an explanation for the relatively poor performance of the Cry1Ac toxin used in Ingard cotton against *H. armigera* compared to other heliothis species. Knowledge of the inhibitor sensitivity and substrate specificity of *H. armigera* proteinases should aid in the development of methods to overcome toxin resistance resulting from proteolytic degradation.

A description of the project technology (e.g. commercially significant developments, patents applied for or granted, licences etc)

The most significant finding from the project was the very large number of proteolytic enzymes produced by *H. armigera* larvae and the evident flexibility that this provides the insect when confronted by plant-derived inhibitors of these enzymes. The high resolution 2D-gel zymogram technology developed for the project provides a sensitive diagnostic tool for the analysis of

alterations to proteinase expression in *H. armigera*.

A technical summary of any other information developed as part of the Research project including discoveries in methodology, equipment design, etc

Two items of technology developed specifically for this project were the native PAGE zymogram and the 2D-PAGE zymogram. Both represent a significant technical improvement over pre-existing methods for resolving complex mixtures of proteinases. Detailed desciptions of these techniques are provided in the Methods section of the Report.

Recommendations on the activities or the steps that may be taken to further develop, disseminate or exploit the project technology

The results of the project indicate that targetting of proteolytic digestion alone is unlikely to be a successful strategy for control of *H. armigera* on cotton. While dietary proteinase inhibitors have some detrimental effect on larval growth and survival, the process of identifying and optimising a population of inhibitors will be time-consuming and expensive. It is also likely, given the extreme diversity of proteinases in this species, that any given combination of inhibitors would not provide stable resistance, as there is considerable scope for rapid accumulation of new enzyme variants that are less sensitive to a particular inhibitor set.

A list of publications arising from the research project

Nothing has been published to date.

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