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Inhibition of *Helicoverpa armigera* gut pro-proteinase activation in response to synthetic protease inhibitors

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Abstract

Protease inhibitors play an important role in host plant defence against herbivores. However, insects have the ability to elevate the production of proteinases or resort to production of a diverse array of proteinases to offset the effect of proteinase inhibitors. Therefore, we studied the inhibition of pro-proteinase(s) activation in the midgut of the polyphagous pest, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) in response to protease inhibitors to develop appropriate strategies for the control of this pest. Gelatin coating present on X-ray film was used as a substrate to detect electrophoretically separated pro-proteinases and proteinases of *H. armigera* gut extract on native- and sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Six activated pro-proteinase bands were detected in *H. armigera* gut lumen, which were partially purified and characterized using substrate assays. Activated *H. armigera* midgut pro-proteinase(s) showed activity maxima at pH 8 and 10, and exhibited optimal activity at 40 °C. The activation of *H. armigera* gut pro-proteinase isoforms was observed in the fraction eluted on benzamidine-sepharose 4B column. Purification and substrate assay studies revealed that 23–70 kDa polypeptides were likely the trypsin/chymotrypsin-like pro-proteinases. Larvae of *H. armigera* fed on a cocktail of synthetic inhibitors (antipain, aprotinin, leupeptin, and pefabloc) showed maximum activation of pro-proteinases compared with the larvae fed on individual inhibitors. The implications of these results for developing plants expressing proteinase inhibitors for conferring resistance to *H. armigera* are discussed.

Introduction

Cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), is one of the major pests of cereal, legume, oilseed, fodder, and horticultural crops in Asia, Africa, Australia, and Mediterranean Europe. It causes an estimated annual loss of USD 2 billion in the semi-arid tropics, despite USD 500 million worth of pesticides applied for controlling this pest (Sharma, 2005). The high pest status of *H. armigera* is due to its potential for polyphaghy, as it is capable of feeding on a diverse array of plant species. Host plant resistance is one of the important components for the management of this pest, and protease inhibitors play an important role in host plant defence against the herbivores.

Studies on the digestion of proteins in insect gut have mainly focused on the initial phases of protein and peptide digestion through endopeptidase activities (Terra & Ferreira, 1994; Harsulkar et al., 1998; Estébanez-Perpiñá et al., 2001). However, the products of such endopeptidase cleavages are peptides, which must be further degraded to be absorbed by the insect gut cells (Billingsley, 1990; Estébanez-Perpiñá et al., 2001). Analysis of digestive proteinases of *H. armigera* has revealed the presence of serine proteinases, predominantly trypsin- and chymotrypsin-like enzymes (Johnston et al., 1991; Xu & Qin, 1994; Bown et al., 1997, 1998; Srinivasan et al., 2006). Screening of the cDNA library prepared from the midgut of *H. armigera* reared on a high-protein and inhibitor-free diet has revealed 18 genes encoding trypsin-like proteinases, 14 chymotrypsin-like proteinases, and two elastase-like

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proteinases (Gatehouse et al., 1997). A number of enzymes and physiologically active proteins are synthesized as inactive precursors that are subsequently converted to the active forms by the selective cleavage (limited proteolysis) of peptide bonds. The specificity of each activation reaction is determined by complementarities of the zymogen substrate and the active site of the attacking protease (Neurath & Walsh, 1976). A pro-carboxypeptidase (PCPAHa) from *H. armigera*, the first enzyme of this class reported in a lepidopteran insect, has been characterized by expressing its encoding cDNA in insect cells (Bown et al., 1998).

Columnar cells in the midgut epithelium are involved in absorption of digested food as well as secretion of enzymes (Terra & Ferreira, 1994). Proteins secreted into the lumen by the surrounding cells may be blocked or trapped within the peritrophic matrix (PM) if they are too large to pass through the meshwork of chitin fibrils or may pass through to contact the bolus of food if sufficiently small. Once inside the endoperitrophic space, proteins are carried posteriorly along with the mass movement of the food bolus and eventually excreted with the frass. However, it has been proposed that some proteins could be recycled by passing through the PM, and carried anteriorly by a countercurrent operating in the ectoperitrophic space (Terra, 2001). The main function of the lumen proteins is digestion, as a result, it is expected that there will be high abundance and activity of enzymes that cleave lipids, carbohydrates, and proteins into smaller molecules, which would be efficiently absorbed by the transport systems in the microvillar membranes (Pauchet et al., 2008).

Earlier studies on the occurrence of inactive precursors (zymogens) of insect digestive proteinases have largely been unsuccessful (Applebaum, 1985). Graf et al. (1986) suggested the occurrence of an inactive form of trypsin (trypsinogen) in midgut cells of *Aedes aegypti* (L.), based on trypsin immunoreactivity in midgut cells, and their failure to assay trypsin activity in the homogenate of washed midgut cells. The failure to assay trypsin in midgut homogenates indicated low sensitivity of the procedure employed, rather than the existence of trypsinogen. A precursor of midgut trypsin has been sequenced, although it showed significant differences from the vertebrate trypsin precursors in the region of the activation peptide (Barillas-Mury et al., 1991). Similar results have also been observed with a putative trypsinogen from *Drosophila melanogaster* Meigen (Davis et al., 1985) and *Simulium vittatum* Zetterstedt (Ramos et al., 1993). Processing of precursors of insect trypsins may be different from that of vertebrates. Recently, Liu et al. (2009) observed that trypsins and other serine endoproteases are the most important proteases in *H. armigera*, because of their key roles in food digestion and zymogen activation. A serine protease is present in the gut lumen, which activates the zymogen phenol oxidase of *Spodoptera litura* (Fabricius) (Arora et al., 2009). There is a need to study the activation processes, which might provide the basis for a detailed understanding of the physiology of digestion in insects. Therefore, the present studies were undertaken on inhibition of pro-proteinase(s) activation in *H. armigera* midgut in response to protease inhibitors, which will be useful to develop strategies for deployment of protease inhibitors in transgenic plant for the control of *H. armigera*.

Materials and methods

Chemicals

Bovine trypsin, chymotrypsin, benzoyl-DL-arginyl-*p*-nitroanilide (BA*p*NA), *N*-α-benzoyl-L-arginine ethyl ether (BAEE), *N*-α-benzoyl-L-tyrosine ethyl ether, azocasein, bovine serum albumin, sepharose 4B, the proenzymes trypsinogen and α-chymotrypsinogen A, and benzamidine hydrochloride were procured from Sigma Chemicals (St. Louis, MO, USA). Protease inhibitors containing synthetic inhibitors (SIs), such as antipain-dihydrochloride, pepstatin, chymostatin, E-64, leupeptin, pefabloc, EDTA-Na_2, and aprotinin, were obtained from Roche Diagnostics (Mannheim, Germany). High molecular weight markers were procured from Bangalore Genei (Bangalore, India). Acrylamide, *N*, *N*′-methylene bisacrylamide, Tris-Cl, and glycine were of analytical grade, and obtained from Sisco Research Laboratory (SRL, Mumbai, India). All other chemicals used were of analytical grade, and purchased from SRL and Qualigens (Mumbai, India). Agfa X-ray films were obtained from Selvas Photographic (Silvassa, India).

Rearing of *Helicoverpa armigera* larvae and bioassays

Helicoverpa armigera larvae were collected from the pigeonpea and chickpea fields at the International Crops Research Institute for the Semi-Arid Tropics (ICISAT), Patancheru, Andhra Pradesh, India (17°31′48″N, 78°16′12″E), and reared on chickpea flour-based artificial diet (Armes et al., 1992) at ICRISAT, Patancheru, India. To ensure greater genetic homogeneity, the insects were reared on a chickpea-based artificial diet for a minimum of three generations. Bioassays were conducted by feeding the *H. armigera* larvae on a control artificial diet (chickpea protease inhibitors removed) and a diet with synthetic protease inhibitors added.

Extraction of inactive and active proteinases from *Helicoverpa armigera* midgut

Five-hour starved *H. armigera* larvae fed on artificial diet and on diets containing synthetic protease inhibitors

{aprotinin, E-64 or leupeptin, pefabloc SC PLUS [4-(2-aminoethyl)benzenesulphonyl fluoride hydrochloride], and pepstatin} were immobilized by keeping them at −20 °C for 30 min. The midguts of the larvae were dissected out and stored at −20 °C until needed. Tissues of larvae fed on artificial diet with and without protease inhibitors were weighed and homogenized separately in three volumes of 0.2 M glycine-NaOH buffer (pH 10.0). The homogenates were centrifuged at 12 000 ***g*** at 4 °C for 20 min, and the supernatants were used as crude preparations of *H. armigera* gut proteinases (HaGPs) and pro-proteinases (HaGPPs). Proteins in crude gut extracts comprising of HaGPs and HaGPPs were estimated by Lowry's method (Lowry et al., 1951), using bovine serum albumin as a standard.

Trypsinogen and proteinase assay

Trypsinogen and/or *H. armigera* gut trypsinogen, total gut pro-proteinases and proteinase activity, and trypsin were measured using trypsinogen assay (Perlmann & Lorand, 1970; Bergmeyer et al., 1974), azo-caseinolytic assay (Brock et al., 1982), and BA*p*NA assay (Erlanger et al., 1964), respectively. The activating reaction mixtures were prepared in proportions of standard enzyme solution. The total trypsin activity of trypsinogen and HaGPP were measured at the beginning (t = 0), and after addition of activating mixture (Parde et al., 2010). The assays were carried out spectrophotometrically (Hitachi U-2900; Hitachi, Tokyo, Japan), and the observations were recorded at $A_{253\ nm/minute}$ using maximum linear rate for both the test and the blank. One trypsinogen unit was defined as one BAEE unit that produced 0.001 increase in optical density (OD) per minute at $A_{253\ nm}$ with BAEE as a substrate at 37 °C (pH 8) in a reaction volume of 3.2 ml (1 cm light path).

The total gut proteinase activity and pro-proteinase activation were determined by azocaseinolytic assay (Parde et al., 2010). For the azocaseinolytic assay, diluted *H. armigera* gut enzyme was added to azocasein, and standard azocaseinolytic assay was performed. One proteinase unit was defined as the amount of enzyme that increased the absorbance by 1 OD under the given assay conditions. Activities of trypsin isoforms of HaGP were estimated using the chromogenic substrate *N* α-benzoyl-L-arginyl-*p*-nitroanilide (BA*p*NA). For the trypsin assay, diluted *H. armigera* gut extract enzyme was added to BA*p*NA and incubated at 37 °C, the reaction was terminated by the addition of acetic acid, and OD was measured at 410 nm. One unit of proteinase activity was defined as the amount of enzyme that caused an increase of 1 unit of OD at 410 nm due to the release of *p*-nitroaniline. The *H. armigera* gut extracts were treated with trypsin (0.0001%), and azocaseinolytic and BA*p*NA assays were carried out for determining the activation of pro-proteinase and trypsinogen isoforms.

Visualization of *Helicoverpa armigera* gut proteinase isoforms and activation of pro-proteinases on gel X-ray contact print

Visualization of activation of HaGPP isoforms on native (Davis, 1964) and denatured (sodium dodecyl sulphate, SDS) (Laemmli, 1970) polyacrylamide gel electrophoresis (PAGE) was carried out using the gel X-ray contact print (GXCP) technique (Pichare & Kachole, 1994). Samples for native- and SDS-PAGE were used without heating and without the addition of reducing agents, such as β-mercaptoethanol or dithiothreitol. Ten and 12% resolving gels were used for polymerization. The stacking gel (6% for native-PAGE and 3% for SDS-PAGE) was poured on top and electrophoresis was carried out at 150 V at a constant current of 30 mA. When the tracking dye front reached the bottom of the running gel, the gel was removed and shaken gently for 15 min in 0.2 M glycine-NaOH buffer at pH 10.0. Proteinase activity bands were visualized in native gel. In case of denatured gel, the SDS-PAGE was washed thrice for 5 min with 2.5% (wt/vol) Triton X-100 dissolved in the 0.2 M glycine-NaOH buffer, pH 10.0, prior to incubation in assay buffer. For activation of HaGPPs, trypsin (0.0001%) solution was used as an activator, which was prepared in 0.2 M glycine-NaOH buffer, pH 10.0. After activation, the gel was washed with the same buffer twice for removal of excess trypsin, and GXCP carried out. After equilibration, the gel was overlaid on unprocessed X-ray film for 30–40 min at 37 °C. For the second exposure, the gel was rinsed in 0.2 M glycine-NaOH buffer (pH 10.0) for 5 min, and placed on another film with the second side of the gel in contact with the film. These X-ray films were placed in a tray containing hot water (60–70 °C) and shaken to facilitate removal of hydrolysed gelatin in the vicinity of proteinase activity. These X-ray films were developed and then contact printed. Two or three successively exposed films were overlapped carefully to match the profiles, to compare and confirm the mobility of activated proteinase bands on the X-ray film.

Factors affecting activation of *Helicoverpa armigera* gut pro-proteinases

The following discontinuous buffers were used for determination of activation of HaGPPs at various pH values: pH 3, 200 mM glycine-HCl; pH 4 and 5, 200 mM sodium acetate; and pH 6, 200 mM citrate-phosphate; pH 7, 200 mM phosphate buffer; pH 8, 100 mM Tris-Cl; pH 9, 10, and 11, 200 mM glycine-NaOH. *N*-α-benzoyl-L-arginine ethyl ether was used as a substrate for assessing the activity of trypsinogen isoforms of HaGPPs. For determination of

temperature optima of HaGPPs, gut extracts were co-incubated with azocasein and BAEE, respectively, for 30 min at 4, 10, 20, 30, 40, 50, 60, 70, and 80 °C.

Pro-proteinase and proteinase purification by affinity chromatography

A 1.6 × 13 cm bed size, with 20 ml bed volume, was used in a 1.6 × 30 cm column. Slurry of benzamidine-sepharose 4B was prepared in the pre-equilibrating buffer and poured into the column. The material was allowed to settle and the column was equilibrated with 0.25 mM Tris-Cl buffer, pH 7.2. For binding/washing the matrices, 0.05 M Tris and 0.5 M NaCl (pH 7.4) buffer was used, whereas 0.1 M Tris-Cl buffer (pH 8.0) was used for elution. The acetone precipitated and concentrated gut tissue extract proteins were reconstituted in 500 µl glycine-NaOH buffer, and the reconstituted samples were loaded in the column and allowed to settle for 10 min on the upper surface of the matrix bed. The column was then eluted (3× the column volume) using the elution buffer as mentioned earlier, at a flow rate of 1 ml per min, and 2-ml fractions were collected. After that, a buffer salt (1 N NaCl in the same elution buffer) gradient of five times the column volume was run. The 2-ml fractions were collected and their OD was measured at 280 nm. Affinity chromatography was carried out under cold conditions.

Inhibition of pro-proteinase and proteinase by synthetic inhibitors

Eight SIs, viz., antipain, aprotinin, chymostatin, E-64, EDTA-Na_2, leupeptin, pefabloc, pepstatin, and a cocktail of SIs (aprotinin, leupeptin, pefabloc, and pepstatin), were used in the range of ca. 1–30 mM (effective concentration per g diet for maximum inhibition of the proteinase activity). These inhibitors were initially dissolved in water (antipain, leupeptin, EDTA, and antipain), DMSO (chymostatin), methanol (pepstatin and pefabloc), or water/methanol (E-64 and the SIs cocktail), as per the manufacturer's instructions, and finally mixed in water to make up the volume required for diet preparation. In vivo effects of SIs on HaGP activity and activation of pro-proteinase were measured using azocasein (Brock et al., 1982) as a substrate. In vivo inhibition of HaGP and in vitro activation of HaGPP isoforms after native PAGE were carried out using the GXCP technique.

Table 1 In vivo effect of synthetic inhibitors (SIs) on mean (± SE; n = 3) *Helicoverpa armigera* gut proteinase activity and in vitro activation of pro-proteinase(s) using trypsin as an activator

Synthetic inhibitor	Effective concentration (per g diet)	% inhibition of proteinase activity	% activation of pro-proteinases
Antipain	284 mM	71.26 ± 2.99	11.91 ± 0.65
Aprotinin	2.1 mM	79.54 ± 1.44	21.20 ± 1.32
Chymostatin	238 mM	46.99 ± 3.91	16.47 ± 1.11
E-64	128 mM	14.46 ± 4.73	ND
EDTA-Na_2	1 M	26.8 ± 2.91	ND
Leupeptin	25 mM	81.82 ± 1.76	16.54 ± 2.11
Pefabloc	10 M	88.68 ± 1.09	23.76 ± 1.57
Pepstatin	10 mM	16.95 ± 4.94	ND
Cocktail of SIs	–	90.42 ± 0.43	33.27 ± 0.56

Inhibition of gut proteinase activity and activation of pro-proteinase were measured using azocasein as a substrate. ND, not detectable.

Statistical analysis

All experiments were carried out in three replicates. Standard error was calculated for the pH and temperature optima, and for HaGP and HaGPP activity (Table 1) using Genstat 10.1 software.

Results

Helicoverpa armigera gut proteinase profile and in-gel activation of pro-proteinase(s) by trypsin

Total HaGP activity was distributed in at least 10 isoforms, of which four were major proteinases (HaGPs 2, 5, 7, and 9), four were less abundant, and two were minor (Figure 1A). Concentrations of HaGPs 5 and 7 were highest, HaGPs 4 and 8 had moderate concentrations, and that of HaGP 10 was lowest. *Helicoverpa armigera* gut proteinases 1 and 2 exhibited very low mobility – in fact, HaGP 1 remained in the stacking gel – whereas HaGPs 9 and 10 were the fast moving bands. By increasing the overlay time of gel on X-ray film (35–40 min, at 37 °C), HaGPs 8 and 10 could be detected; by this time, however, the other proteinases having high activity merged into one band. On the basis of substrate specificity, inhibition by SIs, and their molecular weight and position of proteinase(s) in the gel, the HaGPs were classified into two major serine proteinase families. *Helicoverpa armigera* gut proteinases 1–4 belonged to the chymotrypsin proteinase family, whereas those having comparatively low mobility and molecular weight (HaGPs 5–10) showed trypsin-like activity. Activity of proteinase isoforms decreased as the incubation period increased. No new proteinase(s) or autoactivation of proteinase isoforms was observed on the X-ray film.

The HaGP activity was detected in 10 isoforms, but six inactive isoforms of proteinase(s) were observed upon activation on X-ray film (Figure 1B). *Helicoverpa armigera* gut pro-proteinases I, II, III, IV, V, and VI were inactive isoforms of the HaGPs 2, 3, 4, 5, 7, and 9, respectively. Pro-proteinase isoforms showed less mobility compared with their active isoforms (Figure 2). A mixture of SIs of

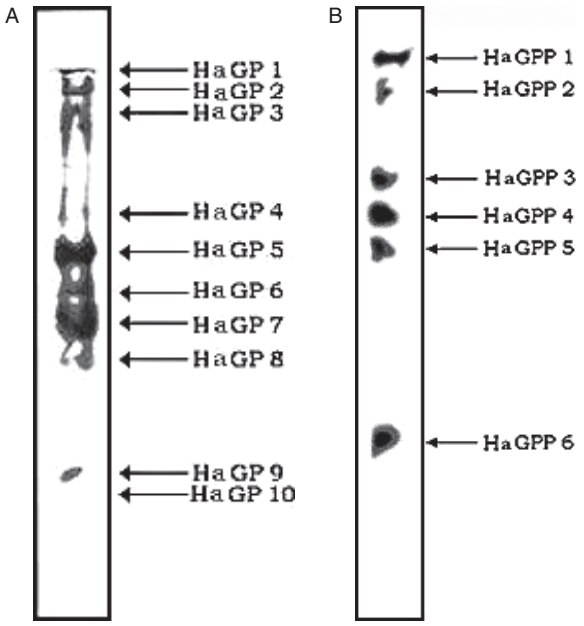

Figure 1 (A) Gut proteinase profile of *Helicoverpa armigera* fed on chickpea-based artificial diet. *Helicoverpa armigera* gut proteinase (HaGP; 0.02 U activity) was separated on 10% native-polyacrylamide gel electrophoresis (PAGE) and the same gel was processed for X-ray film contact print technique to visualize proteinase activity bands. (B) Visualization of in-gel activation of *H. armigera* gut pro-proteinases (HaGPPs). Larvae fed on a cocktail of synthetic inhibitors incorporated into the artificial diet were separated on 10% native-PAGE, and HaGPPs activation was visualized by gel X-ray film contact prints on treatment with bovine trypsin. High *N*-α-benzoyl-L-arginine ethyl ether (BAEE) activity units of HaGPPs gut extract were used for visualization of activation of pro-proteinases. For HaGP and HaGPP, profiling gels were exposed to X-ray films for 30 min.

serine protease inhibitors, such as antipain, leupeptin, and aprotinin, totally inhibited the activity of HaGPs (data not shown). Activation of trypsinogen and chymotrypsinogen revealed the active form of each proteinase band (24 and 25.7 kDa, respectively). The apparent molecular masses of HaGPs were 61.8, 54.5, 46.9, 43.1, and 40.8 kDa, whereas the HaGPPs had molecular weights of 47.6 and 42.1 kDa.

Factors affecting activation of *Helicoverpa armigera* gut pro-proteinase

Maximum pro-proteinase activation was obtained at pH 8 and 10 (Figure 3A). At pH 7, activation was 50%. Proteinases were not stable at pH 12 as they degraded quickly (Ahmad et al., 1980). When BAEE was used as a substrate, at °C, activation of trypsinogen isoforms was only 10%. Activation increased with an increase in temperature and declined with a further temperature increase; maximum activation occurred at 40 °C (Figure 3B).

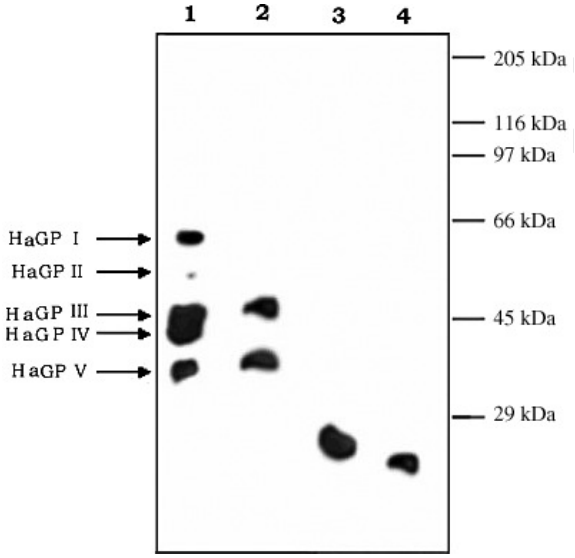

Figure 2 *Helicoverpa armigera* gut pro-proteinase and proteinases (HaGPPs and HaGPs), and proenzymes separated on denaturing gel 12% sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis under non-reducing conditions. After electrophoresis, the gel was washed with 2.5% Triton X-100 to remove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

Purification and characterization of pro-proteinase and proteinases on affinity column chromatography

The major unbound inactive and active proteinase isoforms were eluted with 0.1 M Tris-Cl buffer, pH 8.0, in the first pool (fractions 1–24), whereas in the second pool (fractions 25–70) trypsin isoforms bound to the column were eluted by a 0–1 M NaCl gradient in the same buffer (Figure 4A). There were two major peaks of unbound proteins, the first between fractions 6–10, the second between fractions 13–21. Fraction 8 showed maximum proteinase activity in the first peak, fraction 17 in the second. When the NaCl gradient was used for elution of bound trypsin isoforms of *H. armigera*, trypsin activity was first detected in fraction 41, and maximum activity appeared in fraction 51.

The HaGPP isoforms activation was observed in the same eluted fraction in the activation buffer (Figure 4B). The first pool of fractions showed two peaks of activation of pro-proteinases; fraction 8 showed maximum activation of pro-proteinase isoforms in the first peak, fraction

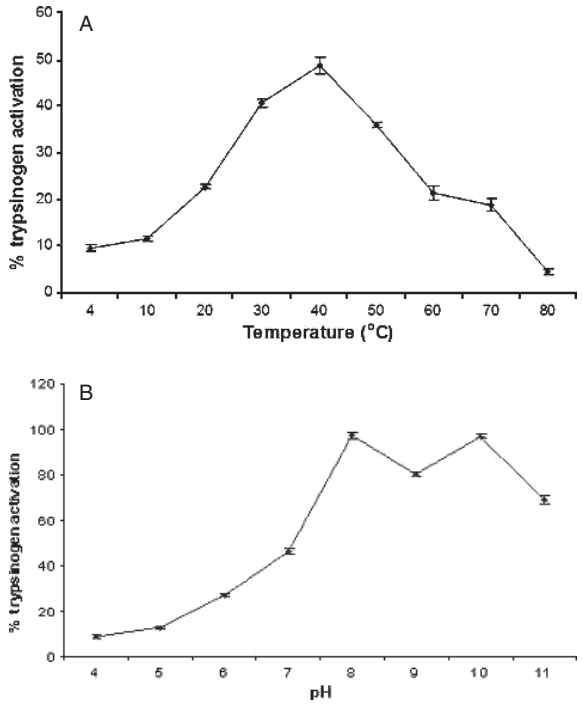

Figure 3 Optimal mean (± SE) (A) pH and (B) temperature of *Helicoverpa armigera* gut pro-proteinases (HaGPPs). Trypsinogen activation of *H. armigera* gut isoforms was estimated at a range of pH and temperatures, using the synthetic substrates *N*-α-benzoyl-L-arginine ethyl ether (BAEE). Maximum trypsinogen of HaGPPs in larvae fed on a cocktail of synthetic inhibitors was considered 100%, and percentage activity and activation values were calculated accordingly.

17 in the second. Fractions eluted by NaCl gradient did not show any activation (Figure 4B). Eluted fractions contained protein bands with corresponding molecular mass of 23–70 kDa (data not shown), although SDS-PAGE analysis on X-ray film indicated that pro-proteinases and proteinases were separated within the same polypeptide range (Figure 2).

Inhibition of *Helicoverpa armigera* pro-proteinases activation by synthetic inhibitors

In vivo studies revealed the specificity of HaGP in inhibiting the activation of pro-proteinases. Larvae fed on SIs incorporated into chickpea-based artificial diet inhibited activity similar to serine proteinases. A cocktail of synthetic protease inhibitors resulted in 90.4% inhibition, whereas pefabloc, leupeptin, aprotinin, and antipain resulted in 88.7, 81.8, 79.5, and 71.3% inhibition to HaGPs, respectively (Table 1). Chymostatin (50.0%) and EDTA-Na_2 (26.8%) caused partial inhibition, but pepstatin (16.9%) and E-64 (14.5%) resulted in very low inhibition of gut proteinase activity. In vitro activation of pro-proteinases revealed apparently low levels of activation. A cocktail of SIs resulted in 33.3% activation of pro-proteinases, followed by pefabloc (23.8%) and aprotinin (21.2%) (Table 1). Chymostatin and leupeptin resulted in similar activation of pro-proteinases (16.5%), whereas with E-64, pepstatin, and EDTA-Na_2 no activation of pro-proteinases was observed.

Visualization of in vivo activation of pro-proteinase on X-ray film revealed that serine proteinase inhibitors were potent inhibitors of HaGPs as well as HaGPPs (Figure 5). Pefabloc and leupeptin resulted in total inhibition of HaGPs (Figure 5A; lanes 1 and 6); antipain, aprotinin, and chymostatin did not result in substantial inhibition of major proteinase activity (Figure 5A; lanes 2, 3, 7, and 8). In gel activation of HaGPPs led to major as well as minor proteinase activity bands, as visualized on X-ray film. Pefabloc resulted in activation of six HaGPPs, whereas other serine protease inhibitors, leupeptin, showed activation of two HaGPPs (Figure 5B; lanes 8 and 3). Aprotinin and antipain showed the activation of HaGPs 6, 7, 8, and 9 (Figure 5B; lanes 6 and 7). The *H. armigera* larvae fed on SIs showed low to moderate levels of pro-proteinases after 1 day, but 2 days feeding resulted in maximum pro-proteinase amounts (Figure 6). However, larvae fed on diets with SIs for more than 2 days showed retarded growth, and pro-proteinase and proteinase levels were also reduced significantly.

Discussion

Proteinases are the major digestive enzymes in the insect gut, where they are responsible for a continuous supply of essential amino acids and energy for development (Tamhane et al., 2005). Most of the midgut proteolytic enzymes in lepidopteran larvae have been shown to be extracellular serine proteases, exhibiting high pH optima, well suited for the alkaline conditions of the midgut. Johnston et al. (1991) observed two major bands of BA*p*NAase activity in *H. armigera* gut extract, but Harsulkar et al. (1998) observed six HaGPs, and three bacterial proteinases in *H. armigera*. Using the GXCP method, we observed 10 proteinases in *H. armigera* gut extract, of which four were chymotrypsin-like and six trypsin-like proteinases. Six distinct pro-proteinase bands from the gut of *H. armigera* larvae were observed after native PAGE, of which two showed detergent stable activity with molecular masses of 47.6 and 42.1 kDa at pH 10.0. Based on substrate specificities, these pro-proteinases appeared to be serine pro-proteinases.

Estébanez-Perpiñá et al. (2001) reported the three-dimensional crystal structure of procarboxypeptidase from

LOW RESOLUTION FIG

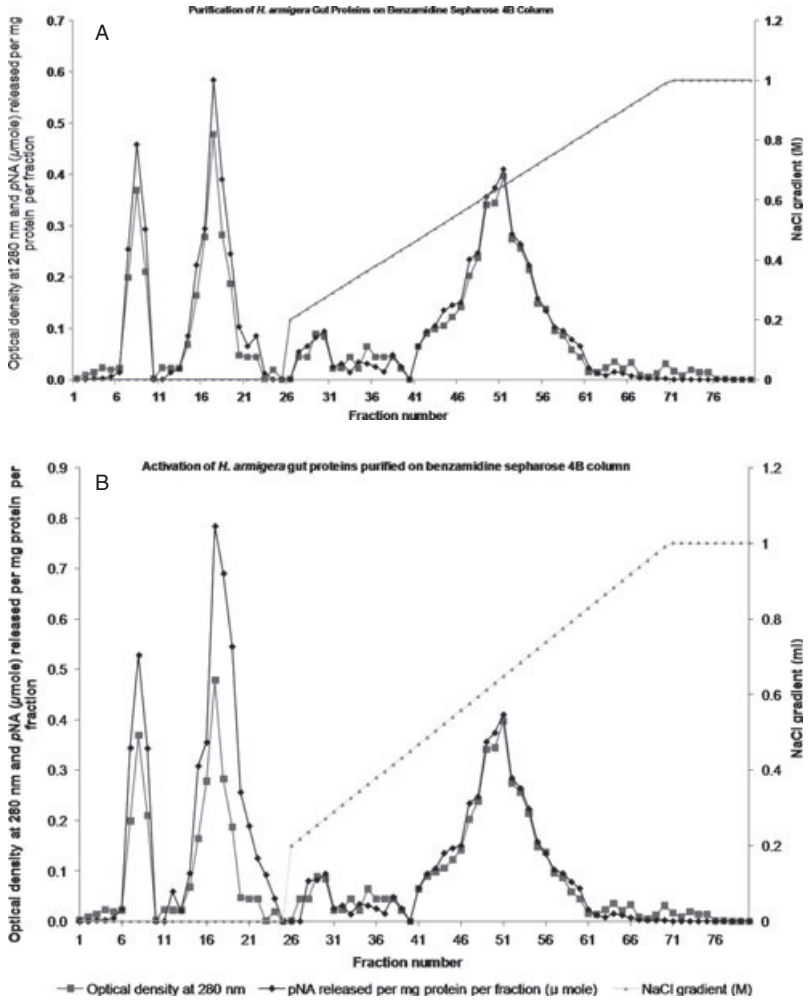

Figure 4 (A) Benzamidine-sepharose 4B column (fast flow) affinity chromatography of acetone-precipitated proteins from *Helicoverpa armigera* larval gut and (B) activation of *H. armigera* gut pro-proteinases. The protein content of fractions eluted by NaCl gradient was measured by absorbance at 280 nm, and enzyme activity by BA*p*NAase assay. Buffers 0.25 mM Tris-Cl (pH 7.2) and 0.05 M Tris-Cl, 0.5 M NaCl (pH 7.4) were used for equilibration, binding, and/or washing the matrices, and 0.1 M Tris-Cl (pH 8.0) buffer was used for elution. Fractions (2 ml per tube) were collected at a flow rate of 1 ml per min. 12

the *H. armigera* gut extract. The carboxypeptidase was expressed as a recombinant proprotein in the yeast *Pichia pastoris* (Guillierm.) Phaff, and could be activated by treating with bovine trypsin. Degradation of bound pro-region, rather than cleavage of pro-region from mature protein, was the rate-limiting step in activation (Bown & Gatehouse, 2004). In the present study, we observed that bovine trypsin can activate trypsinogen- and chymotrypsinogen-like pro-proteinases from the *H. armigera* gut extract in the solution assays as well as by the in-gel activation method. In our earlier efforts, we observed that low concentrations of partially purified HaGPs treated with gut extract of larvae fed on potent protease inhibitors showed that of 10 proteinase isoforms, HaGPs 5 and 9 were activators of pro-proteinases (Parde et al., 2010). The HaGPs showed stability and activity over a wide range of pH, the pro-proteinase pH maxima was at pH 8–10. Gut proteinase activity was detected at high temperatures, but activation of pro-proteinase(s) declined due to degradation of activated proteinases.

Insects are known to make use of various enzymes, such as cysteine proteinases, aminopeptidases, serine proteinases, carboxypeptidases, aspartyl-proteinases, and metalloproteinases, for digestion of food (Murdock & Shade, 2002; Ferry et al., 2004). However, presence of such a wide range of proteinase(s) in the insect gut implies multiple roles for these enzymes in insect development and interaction with food ingredients (Bown et al., 1997; Ferry et al.,

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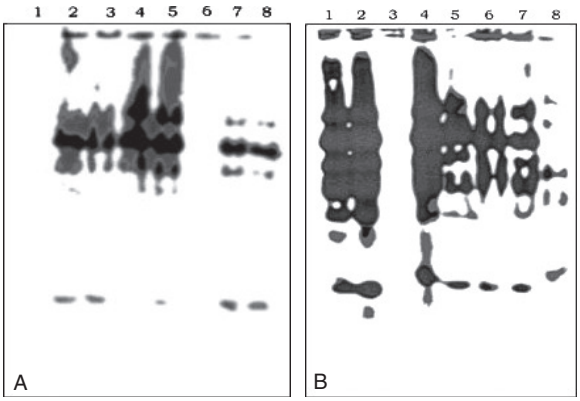

Figure 5 In vivo inhibition of (A) *Helicoverpa armigera* gut proteinase (HaGP) isoforms by synthetic inhibitors, and (B) *H. armigera* gut pro-proteinase (HaGPP) isoforms by bovine trypsin. APMSF/pefabloc (lane 1), antipain (lane 2), aprotinin (lane 3), E-64 (lane 4), EDTA-Na_2 (lane 5), leupeptin (lane 6), chymostatin (lane 7), and pepstatin (lane 8) on 10% native-polyacrylamide gel electrophoresis (PAGE). (B) The *H. armigera* larvae fed on artificial diet containing the synthetic inhibitors pepstatin (lane 1), chymostatin (lane 2), leupeptin (lane 3), EDTA-Na_2 (lane 4), E-64 (lane 5), aprotinin (lane 6), antipain (lane 7), and APMSF/pefabloc (lane 8) on 10% native-polyacrylamide gel. Inhibition of HaGPs and activation of gut pro-proteinase isoforms were visualized by the gel X-ray film contact print technique.

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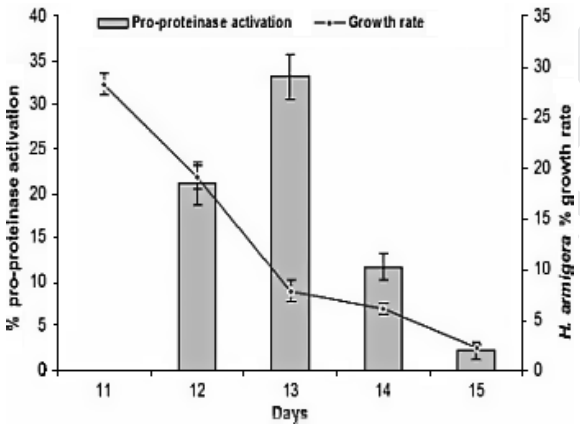

Figure 6 Total pro-proteinase activation and growth rate of *Helicoverpa armigera* determined by larvae fed on a cocktail of synthetic inhibitors. Pro-proteinase activation was measured using azocasein as a substrate in activation buffer, whereas growth rate was determined of larvae feeding from the 11th day onwards on a cocktail of synthetic inhibitors. Means (± SE) are based on three independent experiments conducted in duplicate.

2004; Telang et al., 2005). Although several trypsins, chymotrypsins, and carboxypeptidases have been characterized at the molecular level in *H. armigera* (Bown et al., 1997; Bown & Gatehouse, 2004; Bayés et al., 2005), the biochemical properties of these enzymes have not been described in detail due to lack of appropriate expression systems.

Insect gut proteinases have been purified and characterized using chemical inhibitors, substrates of different specificities, and other activators or stabilizers (Christeller et al., 1989; Johnston et al., 1991; Patankar et al., 2001). In the present studies, pro-proteinases, especially trypsinogen-like isoforms from the midgut extract of *H. armigera*, were partially purified by affinity column chromatography, and characterized for substrate specificities. In the affinity column chromatography, benzamidine-sepharose 4B bound or inhibited the total activity of *H. armigera* gut trypsin-like proteinases in the column, and pro-proteinases eluted into two major pools. The HaGPP isoforms activation was observed in the same eluted fraction in activation buffer. Partially purified HaGPs and HaGPPs activities were corroborated and analysed with their substrate specificities. In *H. armigera*, the 20–70 kDa polypeptides were likely the serine pro-proteinases present in fractions with low BA*p*NAase activation.

Several groups of the major part of *H. armigera* gut protease activity can be blocked by a number of inhibitors (Johnston et al., 1991; Harsulkar et al., 1999). The efficient inhibitor SKTI, which inhibits maximum trypsin activity in gut extracts, did not affect the larval development on artificial diet (Bown et al., 1997). This may be because of adaptation of *H. armigera* to protease inhibitors, due to their ability to alter the complement of proteolytic activity in the gut. However, synthetic protease inhibitors showed maximum inhibition of *H. armigera* gut digestive enzymes and retarded the growth of the larvae when incorporated into artificial diet. In the present studies, the protease inhibitors resulted in inhibition of HaGPPs activation. Larvae fed on a cocktail of synthetic PIs showed more than two- to three-fold total as well as trypsinogen-like pro-proteinase activity compared with larvae fed on the individual protease inhibitors. High HaGPPs activity was observed in larvae fed on a cocktail of SIs, because of inhibition of total proteinase activity. Bown et al. (1997) observed that serine PIs antipain, leupeptin, and aprotinin inhibited maximum activity of HaGP.

The relative effectiveness of SIs and/or their cocktail in vivo indicated that these inhibitors were interfering with normal proteolysis, effectively starving the larvae of protein or, more likely, of essential amino acids. In vivo studies revealed that long-term exposure of larvae to synthetic protease inhibitors resulted in lower amounts of pro-proteinases in the gut. For obtaining adequate amounts of pro-proteinases, *H. armigera* larvae were fed on synthetic protease inhibitors for 1 or 2 days. Pepstatin, E-64, and EDTA-Na_2 are aspartic-, cysteine-, and metallo-proteinas-

es; but these did not inhibit pro-proteinase activation, because they are relatively non-specific for inhibition of target activator proteinases. Therefore, it is essential to study the composition of insect mid-gut to plan protease inhibitor-based strategies to develop plants with resistance to insect pests (Terra & Ferreira, 1994; Murdock & Shade, 2002; Giri et al., 2003; Ferry et al., 2004; Tamhane et al., 2005). The cotton bollworm has the capacity to alter its midgut contents within the same generation, thus neutralizing the effect of protease inhibitors. The nature of gut proteinase activity together with the activity induced upon ingestion of protease inhibitors is important for selecting protease inhibitors, or combinations of protease inhibitors, for developing transgenic plants with resistance to *H. armigera*. Synthetic inhibitors completely inhibited all the proteases, and they were also the inhibitors of zymogen activators' proteases. This strategy would be useful for designing protease inhibitors that reduce the growth of *H. armigera*.

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