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Midgut proteases of the cardamom shoot and capsule borer *Conogethes punctiferalis* (Lepidoptera: Pyralidae) and their interaction with aprotinin

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Abstract

Protease inhibitors cause mortality in a range of insects, and transgenic plants expressing protease inhibitors have been protected against pest attack, particularly internal feeders that are not amenable to control by conventional means. A study of luminal proteases in *Conogethes punctiferalis* Guenée was performed to identify potential targets for proteinaceous biopesticides, such as protease inhibitors. The midgut protease profile of the gut lumen from *C. punctiferalis* was studied to determine the conditions for optimal protein hydrolysis. Optimum conditions for peptidase activity were found to be in 50 mM Tris-HCl, pH 10 containing 20 mM $CaCl_2$; incubation for 30 min at 40°C. Four synthetic substrates, i.e. benzoyl-arg-*p*-nitroanilide, benzoyl-tyr-*p*-nitroanilide, succinyl-ala-ala-pro-leu-*p*-nitroanilide (SAAPL*p*NA) and leu-*p*-nitroanilide were hydrolysed by *C. punctiferalis* gut proteases in Tris-HCl buffer pH 10. Trypsin and elastase-like chymotrypsin were the prominent digestive proteases, and age-related modulation of midgut proteases existed for trypsin, chymotrypsin, elastase-like chymotrypsin and leucine amino-peptidase. Serine protease inhibitors such as aprotinin, soybean trypsin inhibitor and phenylmethanesulfonyl fluoride inhibited peptidase activity. Some metal ions such as Ca^{2+}, Mg^{2+}, Pb^{2+} and Co^{2+} enhanced BA*p*NA-ase activity whereas others like Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+} and Hg^{2+} were inhibitory at 6 mM concentration. Trypsin and elastase-like chymotrypsin were significantly inhibited by 94% and 29%, respectively, by aprotinin (150 nM) under *in vitro* conditions. A possible incorporation of protease inhibitors into transgenic plants is discussed.

Keywords: cardamom borer, *Conogethes punctiferalis*, trypsin, chymotrypsin, leucine aminopeptidase, aprotinin

Introduction

Small cardamom *Elettaria cardamomum* Maton (Zingiberaceae) esteemed as the 'Queen of Spices' is a high value and remunerative spice crop native to the tropical rainforest of Western Ghats, India (Cardamom Hill Reserves). Though, more than 95% of cardamom is consumed in the domestic market, India exported nearly 1100 tonnes during 2001–2002 amounting to a foreign exchequer of US $ 12.6 million (Ravindran & Madhusoodanan, 2002). Cardamom is damaged by about 20 insect pests, of which the shoot and capsule borer *Conogethes punctiferalis* Guenée (Lepidoptera: Pyralidae) has become a major problem, infesting shoots, succulent panicles, racemes, immature capsules and stems

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causing typical 'dead hearts'. Furthermore, the late instar larvae thriving inside the stem are inaccessible to insecticidal sprays. In severely infested plantations a yield loss of up to 70–80% has been recorded (Varadarasan, 2001). It is a polyphagous pest infesting a wide range of commercial crops such as maize, castor, peach, sorghum, turmeric, ginger, durian, sunflower and cotton.

At present, the sole means of pest management of cardamom is application of insecticides at regular intervals. Extensive and exclusive use of chemical pesticides may result in rapid build-up of resistance to such compounds, but their non-selectivity affects the balance between pests and natural predators and generally favours the pests (Metcalf, 1986). Therefore, heavy reliance on this single strategy is highly unsafe, and the timely development of alternative or complementary methods to chemical control is advisable.

The use of genetic engineering to produce pest resistant transgenic plants represents one of many current approaches aimed at increasing agricultural productivity. Protease inhibitors are one group of candidate proteins being targeted for expression in cultivated plants for pest resistance. Protease inhibitors form stoichiometric complexes with specific proteolytic enzymes, thus preventing their catalytic function (Laskowski & Kato, 1980). Since protease inhibitors are primary gene products, they are excellent candidates for engineering pest resistance into plants. Moreover, protease inhibitors have been used because of their small size, abundance, specificity and stability. When expressed in plants, protease inhibitors can bind with key digestive proteases of insects feeding on the plants, disrupting their digestion and reducing growth and survival (Gatehouse *et al*., 2000). It might be possible to control larval stages of *C. punctiferalis* by producing cardamom cultivars that express insecticidal proteins such as protease inhibitors.

It is well established that proteolytic enzymes in insect guts are primarily responsible for the digestion of plant proteins. Proteins are digested in the insect gut by enzymes that are active in fairly alkaline (Lepidoptera) to slightly acidic pH (Coleoptera) (Applebaum, 1985). Protein breakdown in lepidopteran guts is mediated by the concerted action of digestive enzymes, particularly trypsin-like and chymotrypsin-like proteases, which are the primary digestive proteases in this insect order (Terra *et al*., 1996). Lepidopteran larvae have been extensively studied because of their overwhelming impact as pests on economically important plants. The digestive enzymes of these larvae are of interest first, as a target for insect pest management and second, due to their unusual ability to function in an alkaline environment in lepidopteran guts at pH 10–12 (Christeller *et al*., 1992) in which serine proteases and metallo exopeptidase are most active. Serine proteases have been identified from the digestive tracts of insects from many families and many of these enzymes are inhibited by protease inhibitors. Additionally, serine protease inhibitors have an anti-nutritional effect on several lepidopteran insect species (Ussuf *et al*., 2001).

The most effective protease inhibitor tested to date in terms of retardation of growth of insects is aprotinin or bovine pancreatic trypsin inhibitor, which inhibits serine proteases such as trypsin, chymotrypsin, plasmin and kallikrein (Zhong *et al*., 1999). Aprotinin was identified independently by Kraut *et al*. (1930) and by Kunitz & Northrop (1936) from bovine pancreas and was the first protease inhibitor to be extensively studied. The other distinguishing features of aprotinin are high stability due to its internal disulphide cross-links (Kassell & Laskowski, 1965) and a high binding constant to trypsin (Gebhard *et al*., 1986). Aprotinin, when included in artificial diet, reduced the growth rate and increased the mortality of economically important insect pests (Burgess *et al*., 2002; Christeller *et al*., 2002). The growth and development of larvae of the porina moth *Wiseana cervinata* Walker (Lepidoptera: Hepialidae), a major pest of New Zealand pastures (Burgess *et al*., 1993) were decreased by aprotinin. Larval growth rate of tomato fruit worm *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) was significantly reduced by an artificial diet spiked with 0.3% aprotinin and on a diet of leaves of *Lotus corniculatus* (Fabaceae) painted with aprotinin (Burgess *et al*., 1993). Aprotinin also inhibited the growth of the Argentine stem weevil, *Listronotus bonariensis* Kuschel (Coleoptera: Curculionidae) when administered continuously in artificial diets to neonate or one-week-old larvae (Todd *et al*., 2002).

Since proteolysis is an essential part of food digestion in insects, studies on insect proteases are important. Moreover, disruption of protein digestion by protease inhibitors represents an alternative approach to pest management in a world dominated by chemical pesticides or Bt toxin for pest management. This approach requires a thorough understanding of the biochemical properties of the proteases from the gut homogenate, characterization of these proteases in relation to developmental stages and an understanding of the way they interact with various protease inhibitors so as to evolve strategies on the development of transgenic cardamom using protease inhibitors. Cardamom plantations in high ranges are subjected to heavy applications of pesticides as well as fertilizers containing a high proportion of heavy metals. In the present study the luminal protease profile of *C. punctiferalis* at different stages of the final instar larvae is reported for the first time. Enzyme assay conditions were optimized and the effect of metal ions and inhibitors on peptidase activity of the crude midgut homogenate from *C. punctiferalis* was determined.

Materials and methods

Chemicals and equipment

All substrates and protease inhibitors were obtained from Sigma-Aldrich (St Louis, Missouri, USA). Spectrophotometric measurements were recorded using a Shimadzu UV-VIS 1601 double beam spectrophotometer.

Insect source and rearing conditions

Field strains of *C. punctiferalis* were collected from the research farm of Cardamom Research Station, Kerala Agricultural University, Pampadumpara, Kerala, India, situated at an elevation of 1100 m above sea level. This population has been maintained on cardamom shoots in optimum rearing conditions of $26 \pm 2^{\circ}$C, 60–70% rh and photoperiod of 14 h light and 10 h dark as per Kumaresan *et al*. (1988). One-day-old (40–50 mg), three-day-old (110–120 mg) and five-day-old (160–170 mg) fifth-instar larvae used in the investigation were assigned as early, mid and late stages coinciding with the physiological age of development before the larval–pupal transformation. Biochemical studies for the determination of digestive enzyme activities were conducted at the Centre for Biotechnology, Southern

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Petrochemical Industries Corporation (SPIC) Science Foundation, Chennai, India.

Preparation of gut extracts

Fifth-instar larvae of appropriate age were selected for gut extraction. Individual guts of cold-anesthetized larvae were dissected out, cleaned with tissue paper to remove foodstuff, weighed in Eppendorf tubes and stored at −20°C. Guts were homogenized in a plastic homogenizer in 150 µl of ice-cold 20 mM Tris-HCl buffer (pH 8.0). Homogenates were clarified to remove particulate matter by centrifugation at 12,000 × *g* for 15 min at 4°C. Supernatants were transferred to clean tubes, clarified again by the same procedure, and stored at −20°C for use in activity assays.

Standardization of peptidase assay conditions

The peptidase assay conditions for the crude gut extract were standardized using Tris-HCl buffer pH range (8–11), temperature (32–50°C) and incubation time (0–50 min) using benzoyl-arg-*p*-nitroanilide (BA*p*NA) as substrate (1 mM). Assays were performed according to Burgess *et al*. (2002) with slight modifications in a reaction volume of 300 µl (containing 20 mM $CaCl_2$) using 2 µl (13 µg protein) of crude gut extract (mid fifth instar). The reaction was stopped by the addition of 30 µl acetic acid (30%) after the required period of incubation. All assays were carried out in triplicate and blanks were used to account for spontaneous breakdown of substrates. Controls were incubated similarly, but acetic acid was added at the beginning of each assay. The peptidase activity was determined by the amount of *p*-nitroaniline (*p*NA) released from the substrate (BA*p*NA) and was measured at 405 nm (Thangam & Rajkumar, 2002). The activity was expressed as nmoles of *p*-nitroaniline released per minute per gram of the gut tissue. Total protein in the crude gut extract was determined according to the method of Bradford (1976) using bovine serum albumin as standard. Specific activity was represented as activity per mg protein.

Determination of midgut protease profile

Activities of serine-type endopeptidases, particularly trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), elastase-like chymotrypsin and one exopeptidase, leucine aminopeptidase (LAP) were assayed using the substrates, benzoyl-arg-*p*-nitroanilide (BA*p*NA), benzoyl-tyr-*p*-nitroanilide (BT*p*NA), succinyl-ala-ala-pro-leu-*p*-nitroanilide (SAAPL*p*NA) and leu-*p*-nitroanilide (L*p*NA), respectively. Assays were performed according to Burgess *et al*. (2002) with slight modification. The assay conditions were 50 mM Tris-HCl pH 10, 20 mM $CaCl_2$, 2 µl (13 µg protein) of crude gut extract in a reaction volume of 300 µl containing 0.33 mM respective substrate. The reaction mixture was incubated for 30 min at 40°C and the product formed was measured as previously described.

Influence of protease inhibitors

Different amounts of protease inhibitors were added to make final concentrations of 0–3.33 nM for aprotinin, 0–1.67 nM soybean trypsin (Kunitz) inhibitor (SBTi) and 0.04–1.00 mM phenylmethanesulfonyl fluoride (PMSF) in a reaction mixture containing 2 µl (13 µg protein) crude gut extract of early and mid stages of the test insect. Enzyme and inhibitor are incubated together for 10 min before the addition of substrate. All assay conditions were maintained as mentioned above. The peptidase activity using BA*p*NA (0.33 mM) was determined at 405 nm.

Effect of metal ions

The effect of various metal ions (6 mM) on the peptidase activity of crude midgut homogenate was investigated. Assay buffer conditions for the control contained 50 mM Tris-HCl, pH 10.

In vitro *inhibition of midgut proteases by aprotinin*

The effect of aprotinin (150 nM) on the activities of trypsin, chymotrypsin, elastase-like chymotrypsin and leucine aminopeptidase from midguts of early fifth-instar larvae was evaluated.

Statistical analysis

All data sets were analysed for significant differences either by Duncan's multiple range test (DMRT) if there were more than two mean values or by Student's *t*-test when only two sets of mean values were available for comparison, both at a probability level of $P = 0.05$ (Gomez & Gomez, 1984). Triplicate measurements of unique pool of gut extracts were used in all assays and independent replication from different gut extracts were used to compare proteolytic activities at different stages (fig. 2).

Results

Standardization of peptidase assay

A quantitative summary of the standardization of peptidase assay protocol on crude gut extract of mid fifth-instar larvae of *C. punctiferalis* is shown in fig. 1. Although the crude gut enzyme was active over a broad pH range (8–11), the peak activity was found at pH 10 (fig. 1a). The optimum temperature for the crude gut enzyme was found to be 40°C and thereafter the enzyme was rapidly inactivated at higher temperatures (fig. 1b). The enzyme retained only about 6.3% of the specific activity at 50°C. Specific activity was found to be linear with time and after 30 min of incubation, no significant enhancement in specific activities was recorded (fig. 1c). It is therefore suggested that the optimum conditions for peptidase assay with respect to the crude gut extract are 50 mM Tris-HCl, pH 10 containing 20 mM $CaCl_2$ and incubated for 30 min at 40°C.

Spectrum of midgut proteases

Specific activities of the midgut protease profiles for trypsin, chymotrypsin, elastase-like chymotrypsin and leucine aminopeptidase at three different stages of development of the *C. punctiferalis* are presented in fig. 2. It is interesting to note that all the four enzymes tested in the midgut proteases exhibited hydrolytic activity, though to varying degrees. Highest protease activity was found during early fifth-instar for all four digestive enzymes evaluated. Trypsin and elastase-like chymotrypsin were prominent digestive proteases, wherein the specific activities at early

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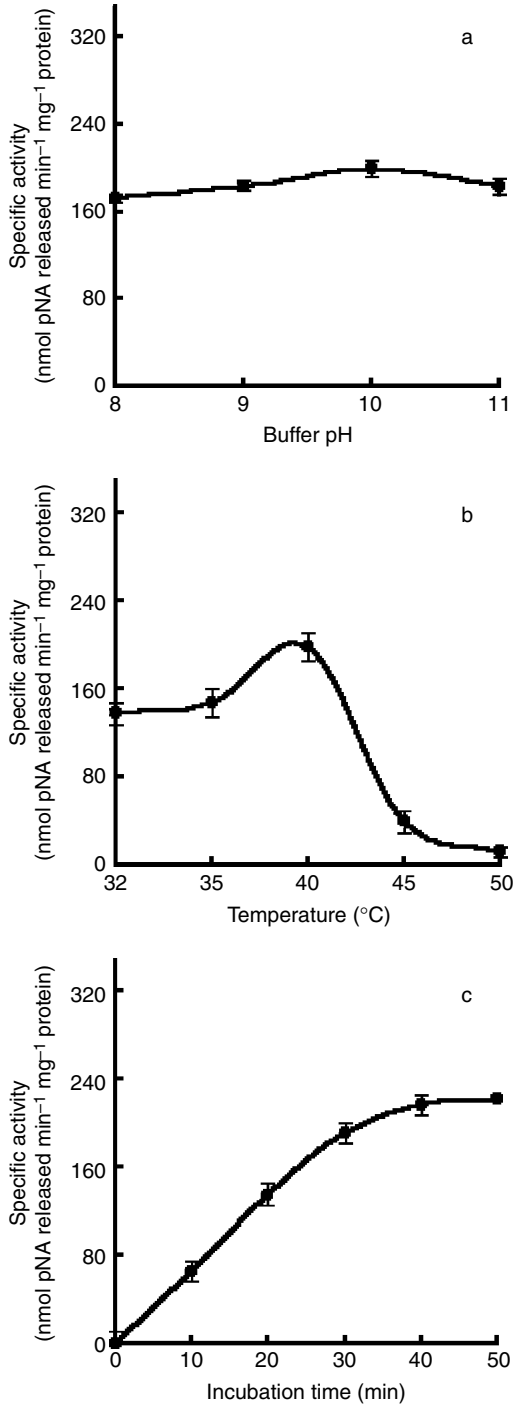

Fig. 1. Influence of buffer pH (a), temperature (b) and incubation time (c) on peptidase activity of crude gut extract of *Conogethes punctiferalis* (mid fifth instar). (a, 50 mM Tris + 20 mM Ca^{2+}; b and c, + 50 mM Tris pH 10 + 20 mM Ca^{2+}).

fifth-instar exceeded 320 nmole of *p*NA released $min^{-1}\,mg^{-1}$ protein. Further, there were significant differences in the midgut protease profile for each stage, except for chymotrypsin. BA*p*NA-ase (trypsin) activity was highest in early

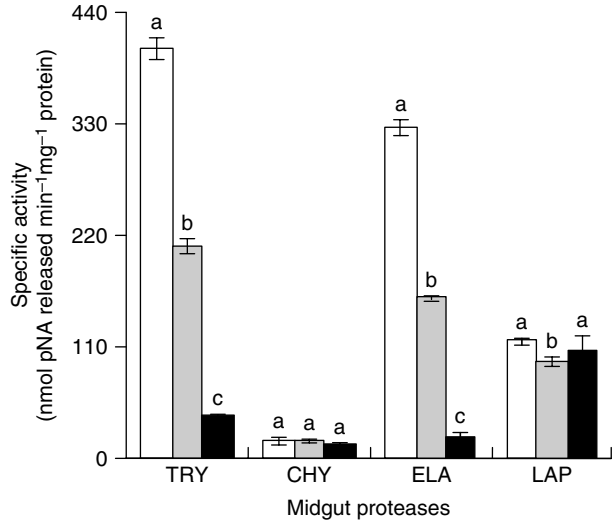

Fig. 2. Midgut protease profile of *Conogethes punctiferalis* at three different stages (□, early; ▨, mid; ■, late) during the fifth instar. Buffer composition includes 50 mM Tris-HCl, pH 10 + 20 mM. Mean ± SD (error bar) values indicated by the same letter are not significantly different for each enzyme (Duncan's multiple range test, $P < 0.05$). (TRY, trypsin; CHY, chymotrypsin; ELA, elastase; LAP, leucine aminopeptidase.)

fifth-instar (402 nmole of *p*NA released $min^{-1}\,mg^{-1}$ protein) and lowest in late fifth-instar (40 nmole of *p*NA released $min^{-1}\,mg^{-1}$ protein), showing a 10-fold decrease in the specific activity as the insect advanced towards pupation. BT*p*NA-ase (chymotrypsin) activity for the three different stages ranged from 11–16 nmole of *p*NA released $min^{-1}\,mg^{-1}$ protein and was found to be similar for the three stages studied. SAAPL*p*NA-ase (elastase-like chymotrypsin) activity was found to be lowest for late fifth-instar, approximately increased 8-fold for mid fifth-instar and doubled again for early fifth-instar. L*p*NA-ase (leucine aminopeptidase) activity was comparable between early and late stages and was found significantly lower in the mid stage of fifth-instar larvae.

Effect of metal ions

Divalent metal ions such as Ca^{2+}, Mg^{2+}, Pb^{2+} and Co^{2+} exhibited stimulatory effects on peptidase activity, whereas Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+} and Hg^{2+} were inhibitory and the effect of other ions such as Na^{+}, K^{+}, Li^{+}, Rb^{+} and Cs^{+} were only marginal (table 1).

Serine protease inhibitor

In order to elucidate the nature of the crude gut extract from larval guts of early and mid stages, the effect of various serine protease inhibitors, i.e. aprotinin, soybean trypsin inhibitor and phenylmethanesulfonyl fluoride on BA*p*NA-ase activity was investigated (fig. 3). The inhibition profiles show that all the inhibitors tested inhibited peptidase activity. Aprotinin at 3.33 nM showed 7-fold and 8-fold decrease in specific activities for early and mid stages of the *C. punctiferalis*, respectively. Specific activities were reduced by 11- and 10-fold, respectively for the same stages with SBTi

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Table 1. Influence of metal ions on peptidase activity of a midgut extract of fifth instar larvae of *Conogethes punctiferalis*

Metals ions (6 mM)	Relative activity (%)
Tris-HCl (control)	100 ± 1.29
Lithium	102 ± 1.67
Potassium	92 ± 3.89
Sodium	98 ± 2.54
Rubidium	101 ± 0.45
Caesium	100 ± 2.05
Calcium	178 ± 6.89
Magnesium	163 ± 4.45
Manganese	52 ± 3.63
Mercury	12 ± 0.67
Lead	140 ± 7.25
Copper	22.4 ± 0.56
Zinc	37.8 ± 2.67
Iron	12.1 ± 0.67
Cobalt	129 ± 5.78

Buffer composition was 50 mM Tris-HCl pH10 with BA*p*NA (0.33 mM) as substrate.

(1.67 nM). However, phenylmethanesulfonyl fluoride reduced the activities by only 3 and 2-fold, respectively at a higher concentration of 1 mM for the same stages. It is therefore suggested that nanomolar concentrations of soybean trypsin inhibitor and aprotinin as well as phenylmethanesulfonyl fluoride at millimolar concentrations are needed to inhibit trypsin.

Aprotinin sensitivity on midgut proteases

The inhibition profile of midgut proteases of early fifth-instar larvae of *C. punctiferalis* due to aprotinin is shown in fig. 4. Trypsin and elastase-like chymotrypsin were drastically and significantly inhibited by 94% and 29%, respectively by aprotinin (150 nM) under *in vitro* conditions. In contrast, leucine aminopeptidase levels were largely unaffected, as the increase was found to be non-significant (t-test, $P < 0.05$). The chymotrypsin activity was too low to demonstrate meaningful inhibition by aprotinin.

Discussion

The gut proteolytic profiles of most lepidopteran species comprise trypsin, chymotrypsin, and elastase-like proteinases, amino-peptidases and carboxy-peptidases (Terra & Ferreira, 1994). A few of these enzymes have been isolated and characterized in detail (Lam *et al*., 1999; Zhu & Baker, 1999; Oppert *et al*., 2002; Anwar & Saleemuddin, 2002; Volpicella *et al*., 2003). In the present investigation the interaction of aprotinin with digestive midgut proteases from the gut extract of *C. punctiferalis* was reported. The data also suggest that *C. punctiferalis* primarily digests proteins via serine proteases, since the pH optima for the hydrolysis for BA*p*NA, BT*p*NA and SAAPL*p*NA were in the alkaline range and also specific inhibition of these proteolytic activities by serine protease inhibitors, especially aprotinin was observed.

Gaining an insight into the proteolytic properties of the digestive enzymes of *C. punctiferalis* is critical for developing appropriate and effective pest management strategies through protease inhibitors. Results from these studies

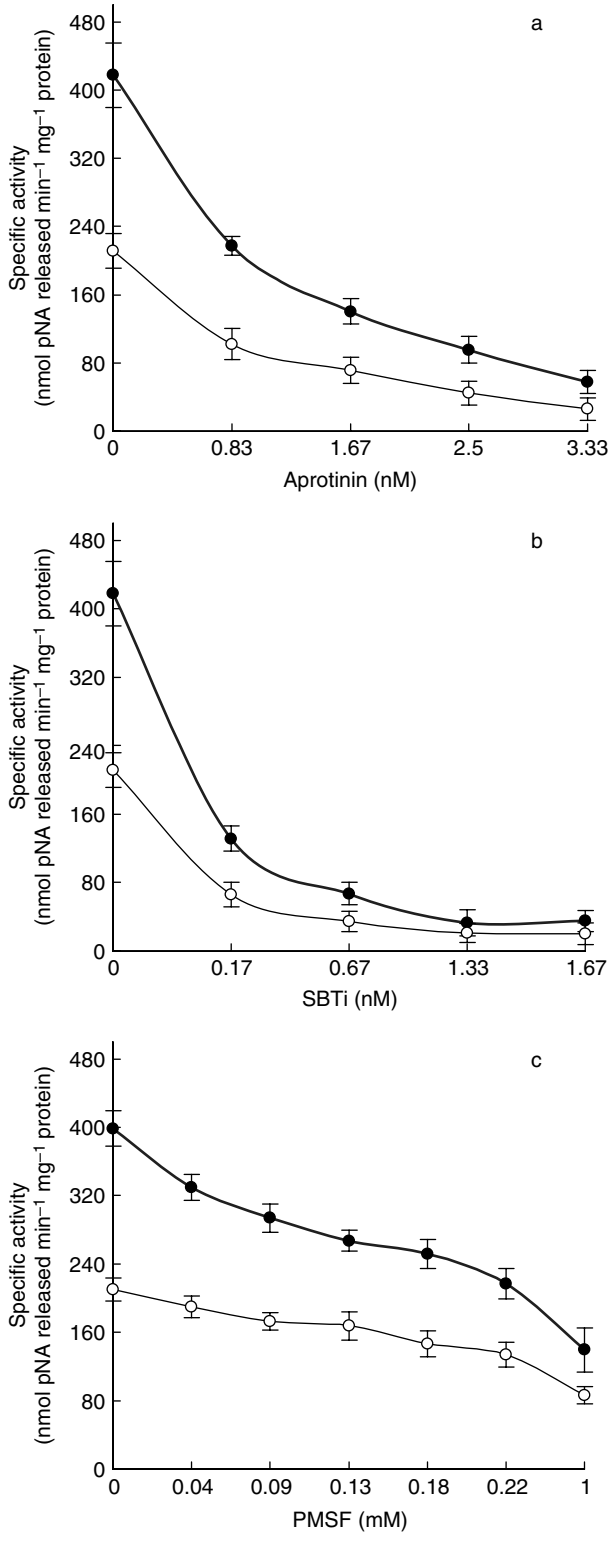

Fig. 3. Influence of aprotinin (a), SBTi (b) and phenylmethanesulfonyl fluoride (c) on peptidase activity of crude gut extract of early (●) and mid (○) fifth instar larvae of *Conogethes punctiferalis*. Buffer composition includes 50 mM Tris-HCl, pH 10 + 20 mM Ca^{2+}.

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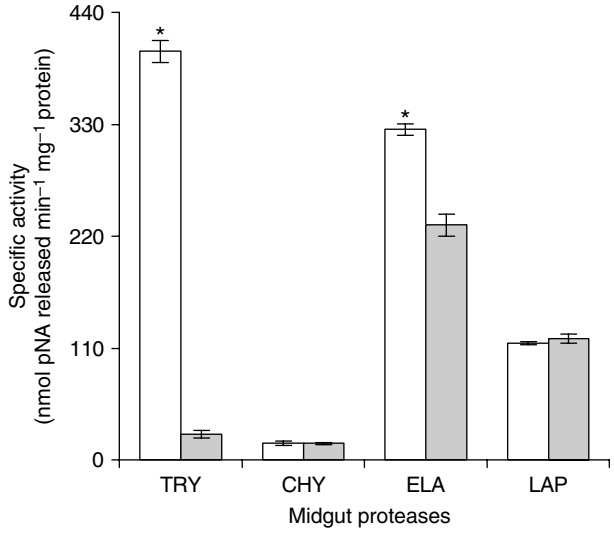

Fig. 4. Influence of aprotinin on midgut proteases of early fifth instar of *Conogethes punctiferalis*. Buffer composition was 50 mM Tris-HCl, pH 10 + 20 mM Ca. *significantly higher than treatment (n = 10, t-test, $P < 0.05$). (▣, Aprotinin 150 nM; □, control; TRY, trypsin; CHY, chymotrypsin; ELA, elastase; LAP, leucine aminopeptidase.)

suggest that protein digestion in *C. punctiferalis* is primarily due to serine proteases that are sensitive to the serine protease inhibitors tested. Digestion of food by serine proteases is the preferred mode in lepidopteran insects. Targeting these enzymes may be a good strategy for the development of effective bio-pesticides. It is now well established that a number of proteases present in lepidopteran guts have alkaline pH optima. These include those from *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae), pH 10 (Ishaaya *et al*., 1971), *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae), pH 9, 10.5 and 11 (Ahmad *et al*., 1976, 1980), *Heliothis zea* Boddie (Lepidoptera: Noctuidae), pH 10 (Klocke & Chan, 1982), *Galleria mellonella* Linnaeus (Lepidoptera: Pyralidae), pH 10.5 and 11.2 (Hamed & Attias, 1987), *H. armigera*, pH 9.5 and 10 (Johnston *et al*., 1991), *Phthorimaea operculella* Zeller (Lepidoptera: Gelechiidae), pH 9 (Christellar *et al*., 1992), *Manduca sexta* Linnaeus (Lepidoptera: Sphingidae), pH 8.5 (Samuels *et al*., 1993), *Spilosoma obliqua* Walker (Lepidoptera: Arctiidae), pH 10 (Anwar & Saleemuddin, 2002) and *Heliothis virescens* Fabricius (Lepidoptera: Noctuidae), pH 10–11 (Johnston *et al*., 1995).

It was reported that a midgut extract obtained from different larval instars of *S. littoralis* exhibited changes in gut protease profile with each larval stage (Keller *et al*., 1996). There existed differential effects of various inhibitors such as E-64 and lima bean inhibitor on trypsin-like proteinase activity of midgut extracts from different larval instars of *Sesamia nonagrioides* Lefèbvre (Lepidoptera: Noctuidae) (Novillo *et al*., 1999). This agrees with the findings of age-related modulation of the digestive protease profile for three different stages of *C. punctiferalis* and the differential inhibition pattern of aprotinin, which specifically targets trypsin and elastase-like chymotrypsin, the major proteases of the test insect. Serine proteases in the midgut of lepidopteran species, especially chymotrypsin activity, as indicated by BT*p*NA hydrolysis, were reported to be rather low relative to trypsin activity (Johnston *et al*., 1991; Christeller *et al*., 1992). No chymotrypsin amidase like (BT*p*NA-hydrolysing) was detected in midgut protease of *S. litura* (McManus & Burgess, 1995). In more recent studies, the use of N-terminally extended substrates such as N-succinyl-$(Ala)_2$ ProPhe-*p*NA or N-succinyl-$(Ala)_2$ ProLeu-*p*NA, has revealed substantial chymotryptic activities in midgut of *S. littoralis* (Lee & Anstee, 1995), *M. sexta* (Peterson *et al*., 1995) and *H. virescens* (Johnston *et al*., 1995). Furthermore, SAAPL*p*NA, a substrate for insect chymotrypsin/elastase is not hydrolysed by trypsin, whereas, BA*p*NA, a trypsin substrate, is not hydrolysed by chymotrypsin/elastase (Christeller *et al*., 2002).

In the present study, chymotrypsin activity, as indicated by BT*p*NA hydrolysis was the lowest and elastase-like chymotrypsin activity, as indicated by SAAPL*p*NA hydrolysis was the highest (20-fold) in early fifth-instar larvae of *C. punctiferalis*. This enzyme has been shown to be a major protease in several lepidopteran insects including the noctuid, *Chrysodeixis eriosoma* Doubleday (Lepidoptera: Noctuidae) (Christeller *et al*., 1992; McManus *et al*., 1994). However, the highest and significant specific activity was observed for trypsin (402 ± 11 nmole of *p*NA released $min^{-1}\,mg^{-1}$ protein) as indicated by BA*p*NA hydrolysis at *p*H 10 and incubated at 40°C.

This study also demonstrated that, *in vitro*, soybean trypsin inhibitor, aprotinin and phenylmethanesulfonyl fluoride were effective at retarding trypsin-like (BA*p*NA hydrolysing) activity extracted from the digestive tract of *C. punctiferalis*. For each enzyme, the interaction with aprotinin was examined, *in vitro*. As expected, aprotinin was particularly effective at inhibiting the trypsin-like activity. However, the inhibition was also seen against elastase-like chymotrypsin, although not to the same extent as that observed for trypsin. Nevertheless, the observed inhibition of the elastase-like chymotrypsin activity in this study, however limited, is not unexpected since kinetic evaluation of aprotinin reveals that it does have some chymotrypsin inhibitory activity (Zhong *et al*., 1999). Further, enzymes that can hydrolyse SAAPL*p*NA are part of the chymotrypsin super-class of serine proteinases (Barrett, 1986). In contrast, this investigation revealed that aprotinin was ineffective against the endopeptidase chymotrypsin (BT*p*NA hydrolysing) activity as well as the exopeptidase, leucine aminopeptidase, *in vitro*. Protease activity was found to be highest for early fifth-instar larvae with respect to the four digestive enzymes evaluated. Together, these results suggest that, in addition to proteinase inhibitor specificity, the developmental stage of *C. punctiferalis* and the concentration of the inhibitor applied may also be important factors in determining the efficacy of the inhibitor against trypsin and elastase activities. A significant amount of inhibitors may have to be ingested during the early and active feeding stages of the test insect coinciding that with highest levels of activity of digestive proteases. This is because of reduced activities of midgut proteases when the insect approached pupation. As the larvae approached pupation, lower levels of proteolytic activity are present in the insect guts, concomitant with decreased feeding activity.

Some metal ions such as Ca^{2+}, Mg^{2+}, Pb^{2+} and Co^{2+} enhanced BA*p*NA-ase activity whereas others like Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+} and Hg^{2+} were inhibitory at 6 mM concentration. The relative activity of two ions, *i.e.* Ca^{2+}

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and Mg^{2+} were found to be above 150% suggesting their possible role as cofactors for trypsin-like enzymes of *C. punctiferalis*. Accumulation of heavy metals due to excessive application of fertilizers and pesticide molecules could possibly alter the peptidase activity of C. *punctiferalis* leading to desensitization and adaptive behaviour.

Adaptive response by insects to ingested protease inhibitors may complicate inhibitor based control strategies. A typical insect midgut contains an estimated 1020 different proteases (Bown *et al*., 1997), which are differentially regulated and all cannot be inhibited by protease inhibitors. Insects also appear to adapt to protease inhibitors by compensatory mechanisms (Oppert, 2000). These include an increased production of inhibitor-sensitive proteases or synthesis of novel inhibitor-insensitive proteases. Successful development of protease inhibitors that can be used as pest control agents requires knowledge of the inhibitory pattern of these inhibitors against each class of proteases within the family of insect species (Zhu & Baker, 1999). Some coleopteran larvae have demonstrated resistance to protease inhibitors by resorting to inhibitor proteolysis (Girard *et al*., 1998). In this context, every protease inhibitor should be bio-assayed for the effect on larval growth and survival before developing strategies for insect pest management using protease inhibitors (Oppert *et al*., 2002). In order to employ an effective pest control strategy, it is very important to achieve multiple inhibitor expressions in a concerted manner.

While pest-resistant transgenic plant cultivars currently available commercially employ only Bt toxin genes, the development of transgenic plants expressing protease inhibitors has emerged in recent years as an additional strategy for pest management (Hilder *et al*., 1987). Protease inhibitor derived genes are found to have the advantage of efficient expression in transgenic plants (Ussuf *et al*., 2001). Although the exact mode of action of protease inhibitors is complex, it is fundamentally different from that by which Bt toxins operate. Transgenic plants expressing protease inhibitors may therefore be a useful alternative or adjunct to the use of Bt as a biopesticide. Thus it seems likely that a transgenic plant expressing aprotinin or an equivalent protease inhibitor could be protected from attack by a susceptible pest species.

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