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Bioinsecticidal activity of *Archidendron ellipticum* trypsin inhibitor on growth and serine digestive enzymes during larval development of *Spodoptera litura*

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Abstract

The roles of serine proteases involved in the digestion mechanism of the cutworm *Spodoptera litura* (Lepidoptera: Noctuidae) were examined (*in vitro* and *in vivo*) following feeding of plant protease inhibitors. A trypsin inhibitor from *Archidendron ellipticum* (AeTI) was purified by ammonium sulfate fractionation, ion-exchange chromatography and size-exclusion chromatography (HPLC) and its bioinsecticidal properties against *S. litura* were compared with Soybean Kunitz trypsin inhibitor (SBTI). AeTI inhibited the trypsin-like activities of the midgut proteases of fifth instar larvae of *S. litura* by over 70%. Dixon plot analysis revealed competitive inhibition of larval midgut trypsin and chymotrypsin by AeTI, with an inhibition constant (K_i) of 3.5×10^{-9} M and 1.5×10^{-9} M, respectively. However, inhibitor kinetics using double reciprocal plots for both trypsin and chymotrypsin inhibitions demonstrated a mixed inhibition pattern. Feeding experiments conducted on different (neonate to ultimate) instars suggested a dose-dependent decrease for both the larval body weight as well as % survival of larva fed on diet containing 50, 100 and 150 μM AeTI. Influence of AeTI on the larval gut physiology indicated a 7-fold decrease of trypsin-like protease activity and a 5-fold increase of chymotrypsin-like protease activity, after being fed with a diet supplemented with 150 μM AeTI. This study suggests that although the early (1st to 3rd) larval instars of *S. litura* are susceptible to the trypsin inhibitory action of AeTI, the later instars may facilitate the development of new serine proteases, insensitive to the inhibitor.
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Keywords: *Archidendron ellipticum*; Bioinsecticidal activity; Larval growth; *Spodoptera litura*; Trypsin inhibitor

1. Introduction

Plants upregulate novel phytotoxins to deter attacks by insect pests, in response to which the pests increase their detoxifying arsenal. The cutworm *Spodoptera litura* is one of the major pests of many important crop plants, whose larvae can defoliate many economically important crops (CAB International, 2002). Possessing a high dispersal capability, this pest has often generated high levels of agricultural losses. Consequently, introgression of pest resistance into crops has been one of the major priorities for plant biotechnologists against this particular pest. Research on host plant resistance to herbivores has established that plants have evolved serine protease inhibitors (PI) as one of the natural defensive strategies against various insect pests, including *Heliothis zea* (Broadway and Duffey, 1986), *S. litura* (Yeh et al., 1997), *Anthonomus grandis* (Franco et al., 2003, 2004; De Gomes et al., 2005) and *Ceratitis capitata* (Araujo et al., 2005).

PIs form an important component in the multi-mechanistic defensive strategy employed in many plant species — the rate of synthesis reaching a maximum within 12 h (Pearce et al., 1993) and resulting in maximum levels of accumulation of the proteins 24–48 h after attack by the insect. PI proteins in general are small stable defense molecules showing specificity for the major digestive proteases (serine, cysteine, and aspartyl proteases) utilized by herbivorous pests (Bode and Huber, 2000). Among the various inhibitor families, serine PIs have

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been implicated as regulators of endogenous proteolytic activity (Ryan, 1991) and components of programmed plant cell death mechanisms (Solomon et al., 1999). Mostly however, these proteinaceous inhibitors are among the defensive chemicals in plant tissues that affect growth and development of their pests.

Despite the success of PIs as an antifeedant against insect pests, the actual mechanism by which these proteins act is still under debate. The most widely accepted hypothesis is that the PIs act by attenuating enzyme function necessary for metabolic processes such as protein turnover or proteolytic digestion required for nutrient assimilation (Bolter and Jongsma, 1997; Shewry and Lucas, 1997; Pompermayer et al., 2001; Volpicella et al., 2003). Predictably, the larvae are arrested in development, and eventually die. Nonetheless, coevolutionary processes ensure the insect to overcome the rigidity of the PIs by switching over to the synthesis of new gut proteases. The new classes of proteases are either insensitive to the plant PIs (Broadway, 1995; Jongsma et al., 1995) or with a modified capability of acting upon a different substrate specificity class; for example, a chymotrypsin-like activity rather than trypsin-like (Gatehouse et al., 1997; Wu et al., 1997). Incidentally, a recent study by Oppert et al. (2005) also suggests compartmentalization of proteinases at specialized regions inside the insect guts that provide better stability and enhanced activity against the plant derived PIs.

Despite the adaptive strategy adopted by the insect pests, PI families among plant species unrelated and unexposed to pests may provide new and more potent inhibitors of insect proteases (Jongsma et al., 1995). Thus, seldom exposure of *Archidendron ellipticum* (Mimosoideae) to common crop pests, an infrequently occurring tree in forest gaps of Great Nicobar Island, makes its inhibitor an ideal candidate for the development of transgenic crops against insect pests (Bhattacharyya et al., 2006). The present paper describes the purification and bioinsecticidal activity of a strong trypsin inhibitor from *A. ellipticum* (AeTI) towards the growth and development of the polyphagous insect *S. litura*.

2. Materials and methods

2.1. Materials

Bovine trypsin, Soybean Kunitz trypsin inhibitor (SKTI) and the substrates tosyl arginyl methyl ester hydrochloride (TAME) and *N*-benzoyl-L-tyrosine ethyl ester (BTEE) were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals were obtained from Sigma/Merck, unless otherwise noted.

2.2. Purification of AeTI

AeTI was purified as described previously by Bhattacharyya et al. (2006) with some minor modifications. Two cuts of $(NH_4)_2SO_4$ precipitations were done, 0–30% and 30–65%. The latter fraction was preferred for ion-exchange chromatography on DEAE-52. Fractions eluted with 0.2 M NaCl were submitted to size exclusion chromatography on a Bio-select SEC 125-5 column (300 × 7.8 mm). Size exclusion HPLC was performed as described earlier (Bhattacharyya et al., 2006). The position of the AeTI peak was detected at 280 nm. The peak obtained was pooled, concentrated, and then submitted to *S. litura* midgut antitryptic assays.

2.3. Estimation of proteins

Protein contents were estimated by a dye-binding method (Bradford, 1976) and from the absorbance at 280 nm. BSA (1 mg/mL) was used as the standard protein.

2.4. Insect rearing

Initially, *S. litura* (Lepidoptera: Noctuidae) collected from the Division of Entomology, Indian Agricultural Research Institute (IARI) were reared on fresh leaves of *Ricinus communis* (L.), before transferring them to artificial diet. The composition of the artificial diet was similar to that followed by Telang et al. (2003), and chiefly comprised of (for 0.5 L distilled water) 55.0 g of wheat germ, 8.5 g of agar-agar, 55.0 g of kidney bean flour, 20.0 g of dried yeast powder, 2.5 g casein, 3.5 g of ascorbic acid, 0.35 g of methyl-*p*-hydroxybenzoate, 0.75 g of sorbic acid, 80 mg of streptomycin sulfate, 0.25 g of cholesterol, 0.7 mL multivitamin drops and vitamin B complex (~0.2 g). Rearing was done at room temperature (28 °C±2 °C). Normally, over 80% of the pupae molted into the adult stage.

2.5. Preparation of larval midgut homogenates

To assess the potency of AeTI on proteases of *S. litura,* midgut proteases were extracted from the digestive tract of the final instar larvae. To compare the levels of gut proteases qualitatively and quantitatively, it was essential to have physiologically synchronized larvae. Since the ultimate (5th) instar larvae harness a repertoire of different proteolytic enzymes in sufficiently extractable amounts they were considered for experimental purposes. Larvae were chilled on ice, decapitated, and the midguts and contents dissected over ice. Replicated sets of five guts were maintained and each gut was transferred into an Eppendorf tube kept on ice at 4 °C containing 40 μL of 1 mM HCl. Since trypsin can be stored frozen in 1 mM HCl, where it is stable almost indefinitely, the same was preferred as the extractant for the larval gut serine proteases (Walsh, 1970). It was ensured that the inhibitory assay was performed in a buffer capable of neutralizing the acid. The presence of Ca^{2+} (>10 mM) retards trypsin autolysis and maintains the stability of trypsin in solution. The midguts were homogenized with a glass rod at 4 °C. Homogenates were then centrifuged at 12,000 ×*g* for 10 min at 4 °C. Supernatants were used as the crude enzyme (trypsin-like activity) extract and were stored at −20 °C.

2.6. Protease and protease inhibitor assay

The crude enzyme extract was diluted in the ratio of 1:12.5 using 46 mM Tris buffer containing 11.5 mM $CaCl_2$ (pH 8.1).

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Trypsin and chymotrypsin inhibitory assays were carried out by estimating the remaining esterolytic activity of trypsin and chymotrypsin towards the substrate tosyl arginyl methyl ester hydrochloride (TAME) and *N*-benzoyl-L-tyrosine ethyl ester (BTEE), respectively, following Birk (1976). Stock solutions of TAME (10 mM in Milli Q water) and BTEE (1.07 mM in 50% aqueous methanol) were used. Bovine trypsin and chymotrypsin (25 μL and 30 μL from stock 1 mg/mL) prepared in 1 mM HCl were used as reference standards. Reactions were carried out in a UV–VIS spectrophotometer, set at 247 nm for trypsin and 256 nm for chymotrypsin. One trypsin/ chymotrypsin unit is defined as 1 μmol of substrate hydrolyzed per minute of reaction. One inhibition unit is defined as unit of enzyme inhibited.

The trypsin inhibitory potential of AeTI and Soybean Kunitz Trypsin Inhibitor (SKTI) were monitored after mixing variable concentrations of the inhibitors respectively, with the crude enzyme extract. SKTI was used as a reference standard for assessing the inhibitory potential of AeTI. Both larval midgut extract and bovine trypsin were pre-incubated with AeTI and SKTI for 5–10 min at 25 °C, in 1 mL Tris buffer (46 mM) containing 11.5 mM $CaCl_2$, pH 8.1. As a control, only the crude gut extract (without inhibitor) was used in the enzymatic assays. 5 replicates were used for all the tests.

2.7. Effect of AeTI on larval growth and development

The activity of AeTI and SKTI (as a reference standard) were assessed *in vivo* by feeding the different instars (1st to 5th) of *S. litura* on artificial diet supplemented with ~85% pure protease inhibitors. Ion-exchanged AeTI and SKTI were incorporated into the larval base diet at concentrations of 50, 100 and 150 μM. A set of controls was maintained where the five different instars were fed on base diet only. In all treatments the larvae were allowed to develop into pupae. Ten larvae were used for each treatment. For each replicate, survival and larval instar were recorded daily throughout the trail, and from day 3 onwards the larval weights were noted at an interval of three days. The efficacy of the inhibitor was determined by changing of the body weight and the number of larvae reaching pupal stage in comparison to the control.

2.8. Determination of larval growth rate

The relative growth rate indexed the rate of change in larval weight (in units of g/g/day) every third day throughout the different instar stages. This was determined as the slope of the relationship between log (weight) and time; more precisely, [log (wt at end of interval) − log (wt at start of interval)] / number of days in interval. The mean relative growth rate of larvae in each treatment was calculated for each 3-day interval (0–3, 3–6, 6–9, and 9–12) as the average of the rates for all individuals considered in the treatment. Following McManus and Burgess (1995), residual maximum likelihood analysis (REML) (Thompson, 1980; Verbyla, 1990) was used to compare growth rates in the different treatments within each time interval. For an accurate display of the REML analysis, standard errors (SE) were assumed, to be a constant proportion of the weight.

2.9. Kinetic measurements

Kinetic analyses over a range of AeTI concentrations were used to more accurately determine the inhibition constant (K_i) value using a Dixon plot (Segel, 1975). Since the crude extract could possess more than one type of proteolytic enzyme, the K_i value is apparent. Enzyme inhibition was characterized with TAME (0.005 mM and 0.01 mM) and BTEE (0.53 mM and 1.07 mM), respectively. Samples were prepared to achieve inhibitor concentrations (nM) of 4, 5, 6.6 and 10. The initial slope v was determined for each inhibitor concentration. The reciprocal velocity ($1/v$) versus [PI], for each substrate concentration, [S_1] and [S_2], was plotted. A single regression line for each [S] was obtained, and the K_i was calculated from the intersection of the two lines. The velocity of the reaction was expressed as $1/v$ (OD_{247} mM/min/mL)$^{-1}$. The mechanism of inhibition (competitive, noncompetitive, or uncompetitive) was determined using Lineweaver–Burk plots, in which the inverse of the initial rate was plotted against the inverse of the substrate concentration in the absence or presence of AeTI.

2.10. Effect of AeTI on larval endogenous proteolytic activity

To determine digestive protease activity in larvae following feeding trials at the conclusion of each instar stage, midguts at each instar stage were dissected, homogenized and tested for trypsin-like activity as described previously. A control without the presence of AeTI in the larval diet was maintained. Ten larvae each per instar stage were used to determine the proteolytic activity. The mean (±SE) was calculated.

2.11. Statistical treatment of data

Statistical analyses were carried out using GraphPad Prism 4 (GraphPad software version 4.0, San Diego, CA, USA). To detect significant differences in mean larval weights, the pooled treatment data was subjected to two way repeated measures ANOVA followed by Bonferroni post tests. Comparisons were made for all parameters within the amounts of inhibitor used as well as the different days of larval development. Simple comparisons, where appropriate, were made using unpaired *t*-tests. The acceptance level of statistical significance was $P<0.05$.

3. Results and discussion

3.1. Assessment of AeTI purity by inhibition of midgut trypsin-like activity

The inhibitory levels complementing with each purity level of the inhibitor have been detailed in Table 1. The protein eluted with 0.2 M NaCl demonstrating a substantial inhibition (~72%) of midgut trypsin-like activity (Table 1) was pooled, lyophilized and subsequently loaded on size exclusion HPLC on Bio-select SEC 125-5 column (300×7.8 mm). AeTI was eluted out as a single protein peak slightly before chymotrypsin (9.23 *versus* 10.46 min, by internal Waters peak-detect software) and showed ~80% trypsin inhibition (Table 1).

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Table 1
Purification chart of a trypsin inhibitor from *Archidendron ellipticum* (AeTI) based on trypsin-like activity of midgut proteases of *S. litura*

Step/characteristics	Total protein (mg)	% Inhibition	Specific activity TIU/A_{280}	% Recovery	Purification factor
Crude inhibitor	675.5	43.7	0.04	100	1.0
Ammonium sulfate precipitation (30–65%)	155.8	61.3	1.4	23.0	35
Ion-exchange on DEAE-52 (0.2 M NaCl fraction)	78.5	71.3	3.8	11.6	95.0
Size-exclusion chromatography (HPLC-system)	12.3	78.4	5.7	1.8	142.5

TIU — Trypsin inhibitory units; A_{280} — Absorbance at 280 nm.
Note: One trypsin unit is defined as 1 μmol of substrate (TAME) hydrolyzed per minute of reaction. One inhibition unit is defined as unit of trypsin inhibited. Specific activity is defined as inhibition units per absorbance unit, at 280 nm, of the inhibitor.

3.2. Proteolytic activity during larval development and PI assay

Trypsin-like serine proteases from either whole body (first and second instars) or larval guts (third to fifth instars) were used to monitor the digestive proteolytic activity of *S. litura* using the substrate TAME (Fig. 1). Maximum activity was recorded for the third instar stage corresponding to 8th and 9th day of larval development. Proteolytic activity overlapped with the changes in gut protein content until the fourth instar. During the final instar the fall in tryptic activity was slightly more ($P<0.05$) than the gut protein concentration. Proteolytic studies on the midguts of *S. litura* larval instars showed high trypsin-like activity that closely reflected the protein content among the different instar stages. Such an observation suggested that the changes in protein concentration through the larval instar stages might govern the corresponding changes in midgut protease activity. Previously, similar observations have also been noted in larval *S. litura* (Ahmad et al., 1976) and many other lepidopteran larvae (Christeller et al., 1992; Johnston et al., 1995; McManus and Burgess, 1995; Gatehouse et al., 1999; Araujo et al., 2005). Earlier we have shown that AeTI was highly effective at inhibiting the midgut tryptic activity (~80%) (Bhattacharyya et al., 2006). Similar results have been obtained with other Kunitz inhibitors on lepidopteran larvae (De Leo et al., 2001; Macedo et al., 2004; Araujo et al., 2005). In fact, SKTI showed a strong *in vitro* inhibitory activity against gut proteases from larval *S. litura* (Christeller et al., 1992; McManus and Burgess, 1995; Yeh et al., 1997).

3.3. Effects of AeTI in diet on larval growth and survival

The potential insecticidal effects of AeTI towards the weight, growth rate and proportion of survivors of *S. litura* larvae respectively, at different developmental stages were assessed by incorporating different concentrations of the inhibitor in the larval base diet (Fig. 2A, B and C). Larvae fed on base diet showed better development, followed by those consuming 50 μM AeTI. At moderate (100 μM) and high (150 μM) concentrations of AeTI, there was a marked reduction in weight (~43% and 61%, respectively) of the early instars (1st to 3rd) (Fig. 2A). Although initially (0–3 larval development days) there was no change in the difference among means of the various treatments ($P<0.05$), the reduction in larval weight varied significantly ($P<0.001$) between different treatment groups during their remaining growth period (Table 2A). The reduction in larval weights was particularly severe during 3–6 larval development days (F-value>13), among all the dosage

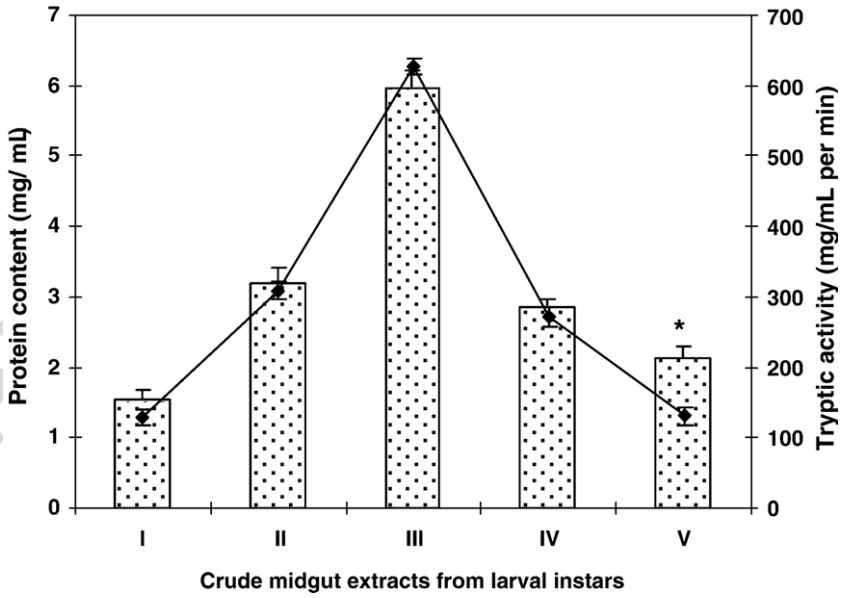

Fig. 1. Variation in the total protein content and trypsin-like protease activity (TAME substrate) in gut extracts from different larval instars of *S. litura*. The trypsin-like activity and protein concentration are indicated in bars and line, respectively. Both protein and tryptic activities overlap each other, and increase with time due to growth of the insect. * Significant difference at $P<0.05$ when compared between the protein content and inhibitor concentration.

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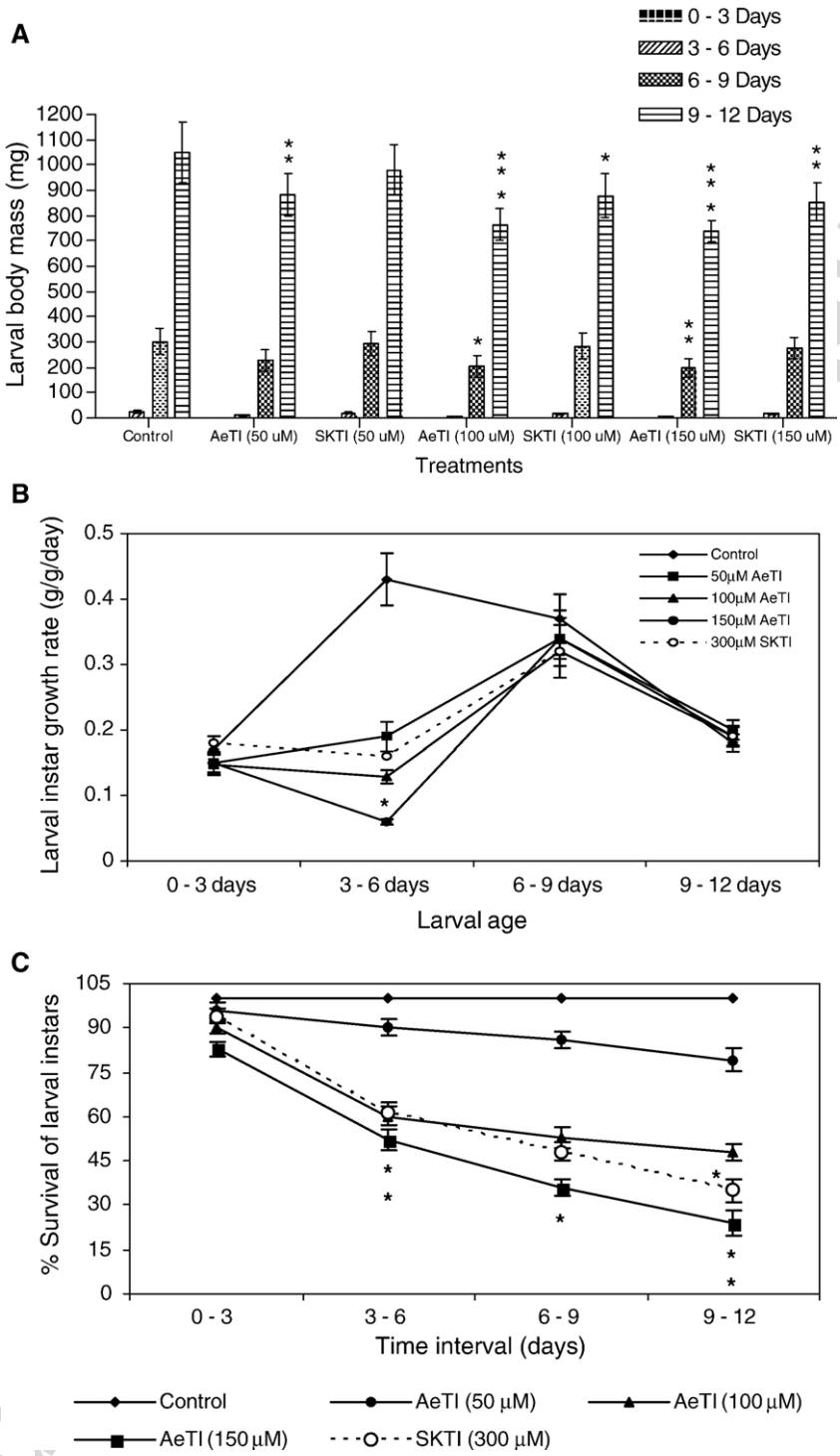

Fig. 2. Dose-dependent effect of AeTI on the growth and digestive physiology of larval *Spodoptera litura*. Larvae of *S. litura* were reared on a defined diet supplemented with increasing doses of AeTI (50, 100 and 150 μM) and the weights (A), growth rate (B) and % survival (C) of 5th instar larvae was noted. SKTI was used as an inhibitor reference against the effects of AeTI on larval digestive physiology. Each measurement was done in triplicate. The vertical lines represent the confidence intervals ($n=10$). * Significant difference at $P<0.05$, ** Significant difference at $P<0.01$ and *** Significant difference at $P<0.001$ when compared between the control and inhibitor fed diet.

treatments. Notably, AeTI was comparatively superior to SKTI ($P<0.01$) in lowering of the larval body weights among the early instars. In fact, the decrease in body weight at the later instar stages (4th and 5th) was stabilized for SKTI, unlike AeTI, whose deleterious effects persisted among the ultimate instars (Fig. 2A). There was a decrease of ~30% larval body weight on consumption of high doses of AeTI.

Similar to the larval body weight, retardation in the growth rate with increasing time was also apparent among the control and AeTI fed larvae (Fig. 2B). Notably, growth rates for the

Table 2A
Statistical comparison of AeTI/SKTI (50, 100, 150 and 300 μM) sensitivities of *S. litura* larvae between four treatment groups by using larval weight as outcome parameter

Larval body mass (mg)		Control vs		
		AeTI (50 μM)/SKTI (50 μM)	AeTI (100 μM)/SKTI (100 μM)	AeTI (150 μM)/SKTI (150 μM)
3–6 days	*P*-value	8.57E−05	5.18E−07	4.72E−08
	F-value	13.51	25.94	33.6
6–9 days	*P*-value	0.03	4.30E−03	0.0017
	F-value	3.87	6.712	8.165
9–12 days	*P*-value	0.039	1.56E−03	1.34E−05
	F-value	3.65	12.34	17.49

control and AeTI exposed larvae were much conspicuous through the 3–6 days of larval development ($P<0.001$). During this period, growth rate of larvae on experimental diet (150 μM AeTI) fell over 85% as compared to larva growing under control conditions (Table 2B). The differences in the mean larval growth rates among the different treatments thereafter were not significant in any of the following 3-day intervals. Predictably, effect of AeTI on larval growth rate pattern was dose-dependent; least exposed recovered the fastest while those exposed maximum showed a much-delayed response of overcoming the adverse effects. So it seems that impediment of the growth rate rather than any other detrimental effects of AeTI patterns the larval body weight.

A trend analogous to the growth rate patterns was also noted on the survival patterns of the larvae exposed to AeTI. At lower dosage, AeTI had no adverse effect on larval survival rates (~80%) (Fig. 2C). However, moderate to high doses of AeTI induced high to severe mortality rates. In fact, larva fed on 150 μM AeTI exhibited as high as 76% mortality by the final instar stage (Fig. 2C). Additionally, AeTI containing diet caused higher mortality among the larval instars compared with a diet supplemented with SKTI (Fig. 2C).

Our results on the larval growth and development closely follow the studies performed by McManus and Burgess (1995) and Gatehouse et al. (1999) for SKTI against larval *S. litura* and *Lacanobia oleracea*, respectively. Overall, it is worth noting that the deleterious effects of AeTI exceeded that of SKTI significantly ($P<0.01$) as exhibited by the differences in mean weight, growth rates and survival patterns among the larval instars fed on diet with both the inhibitors respectively (Fig. 2). This can be explained by the fact that wild and unexposed legume plants, like *A. ellipticum* provide new protease inhibitors, predators of whose seeds are yet to adapt to the inhibitory mechanisms.

3.4. Kinetics of midgut protease inhibition

To determine the mechanism of inhibition and inhibition constant of AeTI against *S. litura* midgut trypsin-like and chymotrypsin-like activities, kinetic analyses were carried out with midgut extracts of 5th instar larvae. The trypsin-like (TAME hydrolyzing) units used for the inhibition kinetics were ~4.5 μmol/min/mL extract. The apparent K_i of AeTI against larval gut trypsin-like activity was $\sim 3.5\times10^{-9}$ M (Fig. 3A) whereas the apparent K_i value for larval gut chymotrypsin corresponded to 1.5×10^{-9} M (Fig. 3B). Similar K_i values have been reported for other related Kunitz-type inhibitors like, *Prosopis juliflora* trypsin inhibitor (Oliveira et al., 2002), *Adenanthera pavonia* trypsin inhibitor (Macedo et al., 2004) and *Tamarindus indica* trypsin inhibitor (Araujo et al., 2005). Although Dixon plot analysis (Fig. 3A and B) showed competitive type of inhibition, the initial rates of reaction in the presence or absence of AeTI followed the Michaelis–Menten equation, and Lineweaver–Burk double reciprocal plots generated thereafter indicated a classical illustration of the mixed nature of inhibition for both trypsin-like and chymotrypsin-like midgut proteases (Fig. 3C and D).

3.5. Effects of ingestion of AeTI on larval digestive proteases

Following the standard insect feeding trials we examined the antagonistic effects of AeTI on the endogenous level of protease activity in fifth instar larvae of *S. litura*. Noticeably, there was a significant decrease ($P<0.001$) in the trypsin-like activity (when expressed as per individual insect gut) in those larvae fed with 150 μM AeTI in their diet as compared to those fed control diet only (Table 3). In fact, an overall decline of ~7-folds was recorded in the tryptic activity of AeTI fed fifth instar larvae as compared to fifth instar larvae reared on control diet. Intriguingly, a 5-fold increase was recorded in the levels of chymotrypsin-like activity from the AeTI treated *S. litura* guts.

This hyper-reduction in the endogenous levels of larval midgut proteases under the influence of AeTI was in contrast to the otherwise abnormally high protease production under the influence of PIs (Bolter and Jongsma, 1995; Broadway, 1995, 1997; Bown et al., 1997; Gatehouse et al., 1997; Overney et al., 1997; Wu et al., 1997; Rivard et al., 2004). To date, it appeared

Table 2B
Statistical comparison of AeTI/SKTI (50, 100, 150 and 300 μM) sensitivities of *S. litura* larvae between four treatment groups by using growth rate as outcome parameter

		Control vs			
		AeTI (50 μM)	AeTI (100 μM)	AeTI (150 μM)	SKTI (300 μM)
Treatment	*P*-value (*t*-distribution)	0.00014	3.31E−04	3.14E−06	1.60E−05
	F-value	35.67			

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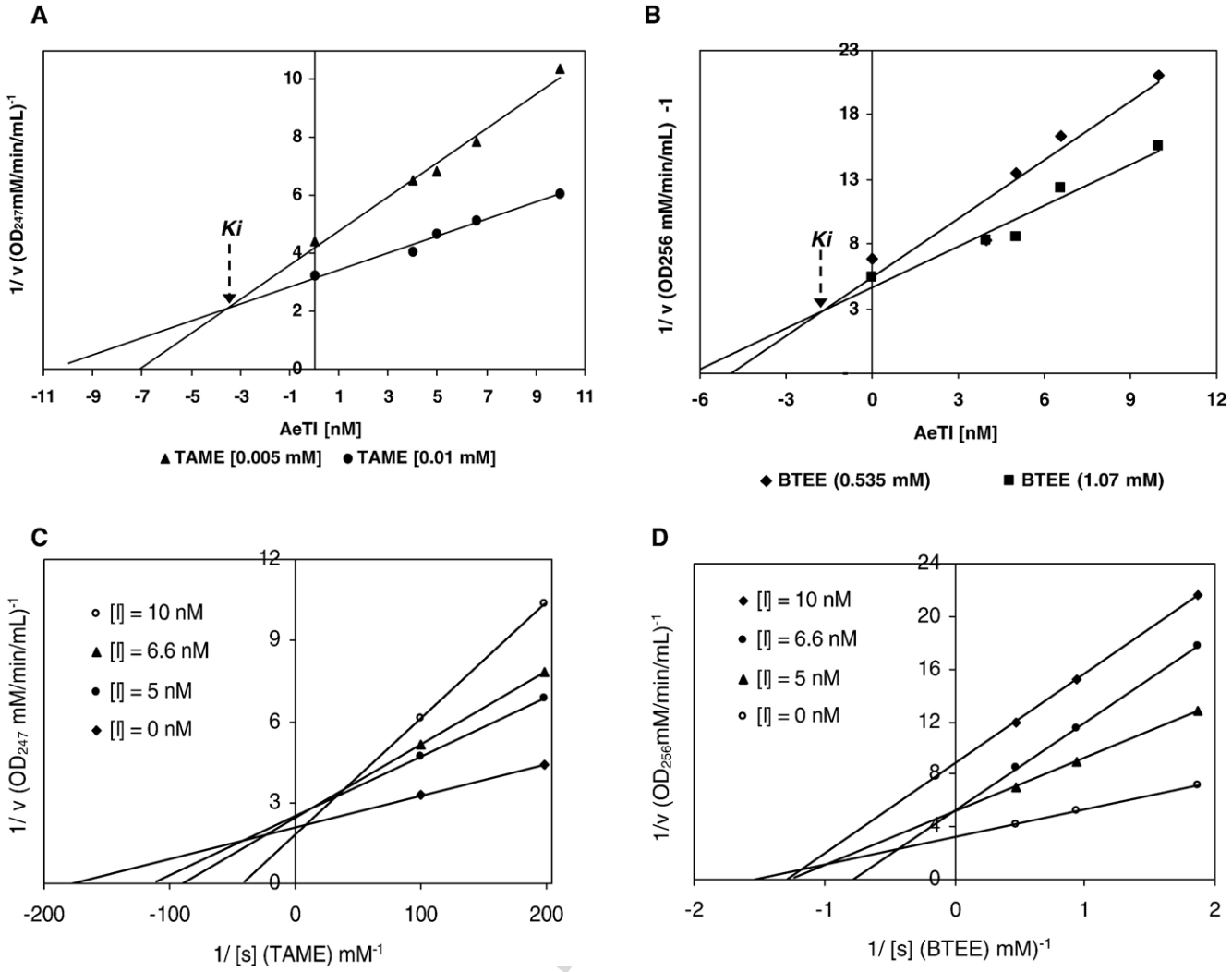

Fig. 3. Kinetic studies on the inhibition of trypsin-like and chymotrypsin-like activity of *S. litura* larval guts by AeTI. Dixon plots for the determination of the inhibition constant (K_i) of AeTI against gut trypsin (A) and chymotrypsin (B) and Lineweaver–Burk plots for gut trypsin-like (C) and chymotrypsin-like (D) activities from 5th instar larvae of *S. litura*. Enzyme assays were carried out with gut trypsin and chymotrypsin-like protease in the presence of increasing concentrations of AeTI at two different concentrations of substrate TAME and BTEE respectively. For Dixon plots the reciprocals of velocity were plotted against the AeTI concentrations while for the Lineweaver–Burk plots the reciprocals of velocity were plotted against the reciprocals of the substrate concentrations in the presence and absence of AeTI.

that on consumption of PIs there is an over-production of the sulfur deficient primary larval proteolytic enzyme which is detrimental to larval health (Broadway and Duffey, 1986). However, a significant decrease in tryptic activity in digestive tract extracts from *Helicoverpa armigera* larvae fed with a diet containing SKTI observed in early 1990's by Johnston et al. (1993) paved the beginning of yet another strategy adopted by insects to counter the deleterious effects of a new PI.

A recently emerging trend emphasizes that leaf chewing lepidopterans induce not only over-production of PI insensitive proteases belonging to same class as that of the inhibitor (Jongsma et al., 1995; Markwick et al., 1998) but may also switch over to alternate pathways involving enzymes within the same mechanistic class, but with a swapped substrate specificity class (Gatehouse et al., 1997; Wu et al., 1997). Similar responses of switching of proteases of same class but with different substrate specificity have also been observed for coleopteran insects (Bolter and Jongsma, 1995; Girard et al., 1998). So it may be possible that the down-regulation of tryptic activity by AeTI is compensated by an up-regulation of yet another class of serine protease. Also, since AeTI is effective

Table 3
Protease activity in digestive tract extracts from different instars of larval *S. litura* fed on diet containing AeTI

Enzyme activity	Treatment	Instars (μmol/min/mL extract)		
		3	4	5
Trypsin-like	Control	5.08±0.17	4.31±0.16	4.14±0.14
(TAME-hydrolyzing)	100 μM AeTI	0.11±0.01	0.1±0.001	0.1±0.001
	150 μM AeTI	0.07±0.0	0.06±0.0	0.06±0.0
Chymotrypsin-like	Control	1.66±0.07	1.67±0.04	1.62±0.08
(BTEE-hydrolyzing)	100 μM AeTI	6.82±0.22	7.24±0.31	7.35±0.33
	150 μM AeTI	7.05±0.32	7.42±0.34	7.6±0.37

Authors

against both trypsin and chymotrypsin (Bhattacharyya et al., 2006), it could generate better approach to combat the overall growth and developmental physiology of larval *S. litura*. Previous studies on insect protease–PI interactions have reiterated in the fact that best inhibitory effects are obtained with an inhibitor with multiple inhibitory activities (Christeller et al., 1992). Finally, in spite of possessing close sequence homology among them, PIs can still vary considerably in their potential to bind and inhibit a cognate protease. Minor variations near the inhibitor active site may potentially alter their biological property and specificity. This variation intensifies the inhibiting ability of PI against a repertoire of proteases present in the insect's (*S. litura*) gut, and also safeguards it against proteases, which have the capability to degrade the inhibitor proteins.

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