In vivo and *in vitro* effect of *Acacia nilotica* seed proteinase inhibitors on *Helicoverpa armigera* (Hübner) larvae

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Acacia nilotica proteinase inhibitor (AnPI) was isolated by ammonium sulphate precipitation followed by chromatography on DEAE-Sephadex A-25 and resulted in a purification of 10.68-fold with a 19.5% yield. Electrophoretic analysis of purified AnPI protein resolved into a single band with molecular weight of approximately 18.6+1.00 kDa. AnPI had high stability at different pH values (2.0 to 10.0) except at pH 5.0 and are thermolabile beyond 80°C for 10 min. AnPI exhibited effective against total proteolytic activity and trypsin-like activity, but did not show any inhibitory effect on chymotrypsin activity of midgut of *Helicoverpa armigera*. The inhibition kinetics studies against *H. armigera* gut trypsin are of non-competitive type. AnPI had low affinity for *H. armigera* gut trypsin when compared to SBTI. The partially purified and purified PI proteins-incorporated test diets showed significant reduction in mean larval and pupal weight of *H. armigera*. The results provide important clues in designing strategies by using the proteinase inhibitors (PIs) from the *A. nilotica* that can be expressed in genetically engineered plants to confer resistance to *H. armigera*.

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1. Introduction

The cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), is a devastating pests in China, Australia and India (Liu *et al.* 2009), and it is able to adapt to various cropping systems because of its high fecundity and wide geographical range. Development of resistance in *H. armigera* due to the use of several synthetic chemicals used for its management was reported (Kranthi *et al.* 2002). Therefore, it is important to develop alternative methods of controlling this pest, includes novel biotechnological approaches like use of transgenics with *Bacillus thuringiensis* (Bt) toxin and/or proteinase inhibitors (PI) genes, vegetative insecticidal proteins (VIPs) and small RNA viruses (SRVs) (Arora *et al.* 2005). So, there is a need to explore the use of plant proteins like lectins, PIs and amylase inhibitors in the current scenario of pest control.

In general, PIs are low-molecular-weight proteins, stable and abundant, showing specificity for serine, cysteine, aspartic and metallo-proteinases. Serine proteinase inhibitors are the most studied class of PIs (Haq and Khan 2003), and they are mostly present in storage organs such as seeds (*Leguminosae* and *Graminae*) and tubers (*Solanaceae*) or even in leaves and fruits, which contain 1% to 10% of their total protein as PIs, which inhibit different types of enzymes (Ryan 1990).

Most Lepidopteran pests like *Helicoverpa armigera* and *Spodoptera litura* largely depend on serine proteinases for digestion of food proteins (Telang *et al.* 2003). The direct evidence for the involvement of PIs in the plant defense system has come from studies on transgenic plants. A cowpea protease inhibitor (CpTI) was shown, for the first time, to confer resistance to feeding by the tobacco budworm (*Heliothis virescens*) when the *CpTI* gene was expressed in transgenic tobacco (Hilder *et al.* 1987).

We demonstrated the effectiveness of non-host plant PIs from *Acacia*, an important genus from Mimosoideae subfamily, against *H. armigera*. The isolation and characterization of PIs were only described in a small number of its

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species, e.g. *Acacia elata* (Kortt and Jermyn 1981), *A. sieberana* (Joubert 1983), *A. confusa* (Lin *et al.* 1991) and *A. plumosa* (Lopes *et al.* 2009). In the present work, we studied the presence of serine proteinase inhibitors from *A. nilotica* and their biological functions against gut proteinases of *H. armigera* and their inhibitory effects on growth and development of *H. armigera*, which is the devastating pest of several economically important crops.

2. Materials and methods

2.1 *Materials*

Bovine trypsin and chymotrypsin were purchased from SRL (India). Standard substrates, viz *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BA*p*NA), *N*-α-benzoyl-DL-tyrosine-*p*-nitroanilide (BT*p*NA) and succinyl-alanyl-alanyl-prolyl-phenylalanyl-*p*-nitroanilide (SAAPF*p*NA), protein molecular weight markers and acrylamide were procured from Sigma Chemical Co. (St. Louis, Mo, USA). DEAE-Sephadex A-25 was obtained from Pharmacia Biotech, Sweden.

2.2 *Inhibitor isolation and purification*

Crude extract was obtained according to Hajela *et al.* (1999) with some modifications. Finely ground *Acacia nilotica* seed meal was extracted with 0.01 M sodium-phosphate buffer (1:10 w/v), pH 7.0, containing 0.15 M NaCl for 10–15 min and then stirred for 2 h at room temperature. The homogenized juice was centrifuged for 30 min at 8000–10,000 rpm at 4°C and to the supernatant (crude extract) solid ammonium sulphate was added to 30–60% saturation. The pellet was dissolved in minimal volume of extraction buffer and dialysed overnight with the same extraction buffer at 4°C and lyophilized, and then subjected to ion exchange chromatography on DEAE-Sephadex A-25 column (50 cm×2 cm column; flow rate of 15 mL h^{-1}), equilibrated with several bed volumes of 20 mM Tris–HCl buffer, pH 8.0. The column was first eluted with same buffer to wash out the unbound proteins. After washing, the bound proteins were eluted with a linear salt gradient of 0.1 to 0.4 M NaCl in 20 mM Tris–HCl pH 8.0. Fractions were collected and monitored for protein content at 280 nm and also analysed for trypsin inhibitory activity at 410 nm. A single peak with trypsin inhibitory activities was obtained. These fractions were separately pooled, dialysed and lyophilized.

2.3 *Inhibitory assay against serine proteinases*

Inhibitory activities of *A. nilotica* seeds towards two closely related serine proteinases were tested. The trypsin inhibitory assay was performed using BA*p*NA as substrate. Different volumes of PI were added to 20 µg of bovine trypsin in 200 µL of 0.01 M Tris–HCl (pH 8.0) containing 0.02 M $CaCl_2$ and incubated at 37°C in a water bath for 5–10 min. Residual trypsin activity was measured by adding 1 mL of 1 mM BA*p*NA in pre-warmed (37°C) buffer 0.01 M Tris–HCl (pH 8.0) containing 0.02 M $CaCl_2$ and incubated at 37°C for 10 min (Erlanger *et al.* 1961). Reactions were stopped by adding 200 µL of 30% glacial acetic acid. After centrifugation, the liberated *p*-nitroaniline in the clear solution was measured at 410 nm. All assays were performed in triplicate. The chymotrypsin inhibitor activity was also measured with the substrate BT*p*NA. Protein was determined according by the method of Lowry *et al.* (1951) where bovine serum albumin was used as a standard.

2.4 *Polyacrylamide gel electrophoresis*

A discontinuous buffer system of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), using a 4% stacking gel and a 10% resolving gel, was done by the method of Laemmli (1970) at room temperature.

2.5 *Thermal and pH stability of AnPI*

Thermal stability of purified AnPI was determined by using 0.1 M Tris–HCl, pH 8.0, incubated at various temperatures ranging from 20 to 100°C (+0.1°C) in a water bath for 30 min. After incubation at various temperatures, samples were cooled at 4°C for 10 min and centrifuged. The remaining proteinase inhibitor activity was measured. The effect of pH on inhibitory activities of AnPI was investigated at different pHs ranging from 2 to 10 using the following buffers at final concentrations of 0.1 M: Glycine-HCl for pH 2 and 3; Na-acetate-acetic acid for pH 4 and 5; phosphate buffer for pH 6 and 7; Tris–HCl for pH 8 and Glycine-NaOH for pH 9 and 10. After 24 h incubation at each pH at room temperature, residual trypsin inhibitory activities were measured as mentioned earlier.

2.6 *Preparation of insect gut proteinases and enzyme assays*

Gut enzyme extracts from final instar of *H. armigera* larvae was prepared according to the method of Johnston *et al.* (1991) with some modifications. Trypsin, chymotrypsin and total proteolytic activities in *H. armigera* larvae were estimated using the chromogenic substrates BA*p*NA and BT*p*NA and sodium caseinate according to modified protocol of Erlanger *et al.* (1961) and Lee and Anstee (1995). The proteinase enzyme activity was expressed as micromoles of *p*-nitroaniline hydrolyzed/min/mg protein. SAAPF*p*NA as a substrate was also used for measuring the chymotrypsin activity.

2.7 *AnPI inhibitory assay against gut extracts from Helicoverpa armigera larvae*

Three to four different doses of PIs from *Acacia nilotica* (AnPI), standard Soybean Kuntiz type Trypsin Inhibitor (SBTI) and standard Soybean Bowman Birk type Inhibitor (SBBI) were used to determine the 40–50% inhibition of proteinases of *H. armigera* midgut extract. All the inhibitors were mixed with 10 µL of *H. armigera* gut extract. It was incubated at 37°C for 10 min, before addition of substrate to start the reaction (Lee and Anstee 1995). Residual activity was determined spectrophotometrically at 410 nm and results were expressed as IC_{50} or % inhibition relative to controls without inhibitor. The enzyme activity was expressed as micromoles of *p*-nitroaniline released/min/mg protein. All *in vitro* assays were carried out in triplicates.

2.8 *Kinetics of inhibitory activity against Helicoverpa armigera from AnPI*

The mechanism of inhibition (competitive or non-competitive) against gut enzymes of *H. armigera* was determined at different substrate concentrations and at a fixed concentration of the inhibitor. We used Lineweaver-Burk plots, in which the inverse of the initial velocity was plotted against the inverse of the substrate concentration (0.2, 0.4, 0.6, 0.8 and 1 mM) (Macedo *et al.* 2004). In the absence of inhibitor and in the presence of inhibitor, K_m, V_{max} and K_i were calculated. The reaction velocity was expressed as $1/v$ (µM substrate hydrolysed/min/ml) $^{-1}$.

2.9 *Bioassays with H. armigera larvae fed on diet containing A. nilotica PI*

For feeding studies, the PIs from *Acacia nilotica* partially purified by ammonium sulphate saturation (at 30–60%) were incorporated into the artificial diet at different concentrations (w/w) of 0.1, 0.5 and 1% as suggested by Johnston et al. (1991), while diet without added PIs was used as control diet. Starved third instar larvae were released into the rearing trays containing either control diet (or) inhibitor containing diet. Data on pupal weight, malformed pupae, malformed adults and fecundity were recorded. Each larva served as a replication and data was analysed statistically. To test the effectiveness of PI, AnPI purified on DEAE-Sephadex, they were incorporated separately in the artificial diet of *H. armigera*. The feeding experiments started with release of 50 neonates on each of the test diets in three replicates in rearing cups. They were maintained up to 5 days. Cumulative mortality in the first 5 days was noted. Surviving larvae were transferred to rearing trays containing the respective test diets and reared individually to monitor growth. Larval weight was taken on every alternate day. After the 13th day, which is the end of feeding period of larvae growing on normal diet, all the surviving larvae in the test diets were transferred to normal diet (without inhibitor). The recovery of larvae was monitored by recording their weight at regular intervals. Pupal weight and adult emergence were measured at the end of the experiment (Broadway and Duffey 1986; McManus and Burgess 1995).

3. Results and discussion

Serine proteinase inhibitors (trypsin and chymotrypsin) have been purified and characterized from a variety of plant sources (Fan and Wu 2005). Various studies have shown that PIs are active against the enzymes of different insect species both in *in vitro* and *in vivo* bioassays (Lawrence and Koundal 2002). In this study, related PI from *A. nilotica* seeds was purified and characterized, and its *in vitro* and *in vivo* potential insecticidal activity against *H. armigera* was examined.

3.1 *Inhibitor isolation and AnPI purification*

Crude soluble protein extract obtained from the mature *A. nilotica* seeds was initially precipitated at 30%, 60% and 90% saturation with ammonium sulphate and three protein fractions were obtained. The F_2 protein obtained showed strong inhibitory activity against trypsin (more than 85% inhibition), while the other fractions exhibited low inhibitory activity. The F_2 protein was then applied to an ion exchange chromatography, DEAE-Sephadex A-25 column and the retained broad peak was assayed against trypsin. This purification procedure of trypsin inhibitor from *Acacia nilotica* seeds was observed by SDS-PAGE (figure 1.) and resulted in a purification of 10.68 fold with a 19.5% yield (table 1). Odei-Addo (2009) demonstrated the specific activity of the purified fraction was 7.20 times that of the crude extract with a yield of 8% yield by the use of affinity and RP-HPLC. These results showed low recovery percentage and purification level compared to purification of PIs achieved from other plant species. Similar results were obtained while working on *Terminalia arjuna* (Rai *et al.* 2008). This may be due to interferences from high levels of phenols and mucilaginous polysaccharides during purification. Low levels of purification achieved may also be due to a high concentration of the inhibitor in the seeds of Indian red wood as suggested by Prabhu and Pattabiraman (1980).

3.2 *Electrophoresis analysis of AnPI*

Electrophoretic analysis of more active 30–60% ammonium sulphate fraction on 10% SDS-PAGE resolved into protein

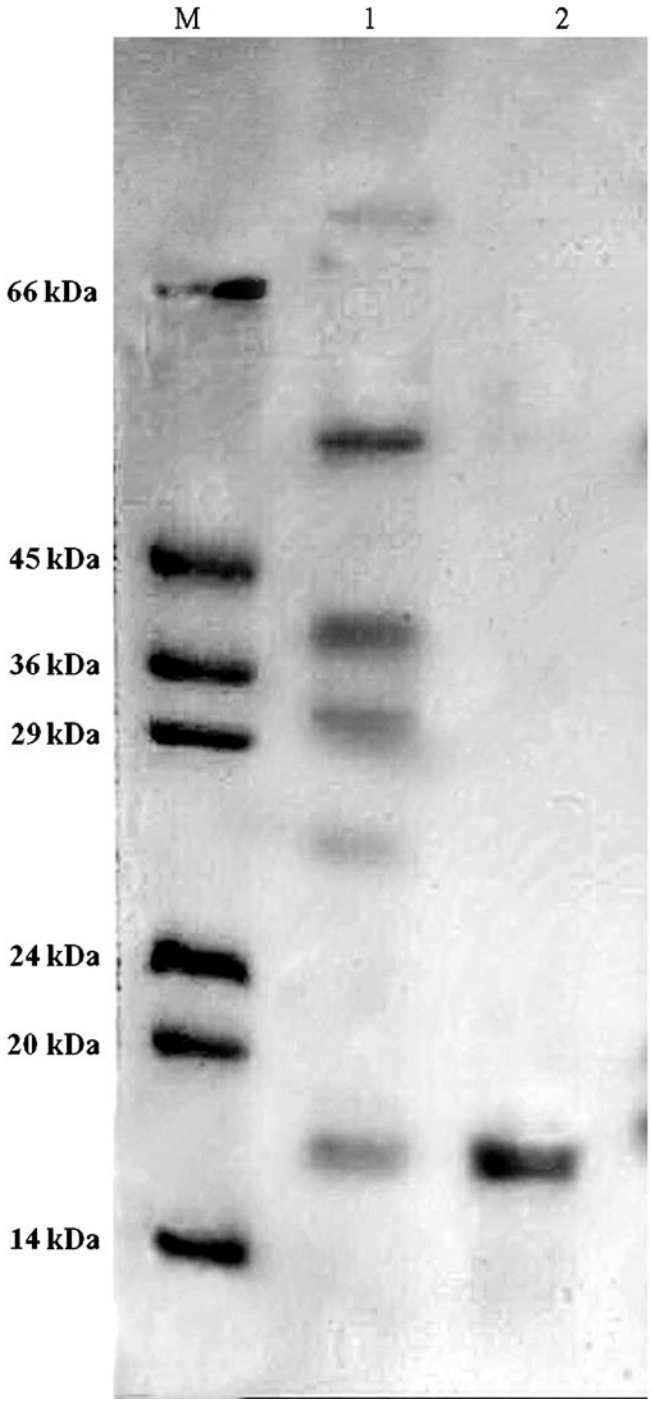

Figure 1. SDS-PAGE analyses of AnPI fraction stained with coomassie blue. M – Molecular weight markers 1 – Ammonium sulfate (30-60%) precipitated fraction, 2 – Fraction purified on DEAE-Sephadex A-25.

bands ranging from approximately lower than 20+ 1.00 kDa to more than 66+1.00 kDa (figure 1). Fraction of AnPI obtained by Ion exchange chromatography was also subjected to 10% SDS-PAGE, resolved into a single protein band corresponding to a molecular weight of approximately 18.6+1.00 kDa. PIs demonstrate a range of molecular masses from 13 to 21 kDa, e.g. *A. victoriae* (Ee *et al.* 2009); *A. sieberana* ~ 19 kDa (Joubert 1983); *A. plumosa* ~ 20 kDa (Lopes *et al.* 2009); *A. elata* ~ 20 kDa (Kortt and Jermyn 1981) and *A. confusa* ~ 21 kDa (Lin *et al.* 1991).

3.3 *Thermal and pH stabilities of AnPI*

Preincubation of the inhibitor in the pH range (2.0 to 10.0) did not affect trypsin inhibitory activity, but at pH 5.0, AnPI lost their trypsin inhibitory activity of 90%. A study on the temperature effect on AnPI showed that the inhibitory activity was stable at 60°C for 10 min. Total loss of trypsin inhibitory activity was found when heated for 10 min at 100°C. Similarly, Lopes *et al.* (2009) demonstrated that the inhibitors were thermally stable up to 65°C but underwent abrupt denaturation with a mid-point at 75°C for isoinhibitors of *A. plumosa*. The internal disulphide bridges that are present in all three isoforms structures support this high thermal stability. Additionally, inhibitors are highly stable in a pH range from 2.0 to 12.0. Wide range of stability in pH values reveals that purified PIs were effective for the control of insect pests that had variation in their gut environment, e.g. acidic condition in Homoptera and Coleoptera and alkaline condition in Lepidoptera.

3.4 *In vitro inhibitory activity of AnPI*

The presence of serine proteinases (trypsin and chymotrypsin) was detected in midgut extracts of *H. armigera*. Inhibition assays using crude midgut protease of *H. armigera* and purified AnPI along with standard, SBTI, demonstrated that at higher concentration of 5 µg/mL inhibitor, inhibition of trypsin (93.65+3.00) was significantly higher in SBTI when compared to purified AnPI . PI isolated from *Acacia nilotica* (AnPI) was not effective against *H. armigera* gut chymotrypsin (data not presented) but was effective only against bovine chymotrypsin. AnPI inhibited total gut proteolytic activity of *H. armigera* to a maximum of 86.25%. These results also indicated that AnPI exhibited high affinity towards trypsin enzyme (76.19% inhibition of gut trypsin) and was also effective against other midgut proteases except chymotrypsin in *H. armigera*. Inhibitors from capsicum demonstrated promising *in vitro* inhibition of gut proteinase activity of *H. armigera* larvae, exhibiting more affinity towards trypsin-like proteinases than chymotrypsn/elastase-like proteinases (Tamhane *et al.* 2005). Similarly Parde *et al.* (2010) reported that *A. nilotica* inhibited 72% and 54%

Table 1. Purification steps of trypsin inhibitor from *Acacia nilotica* seeds

Step	Total protein (mg)	Total trypsin[a] inhibitory unit (TIU)	Specific activity (TIU/mg protein)	Fold purification	% Recovery
Crude extract	1,59,814.5	41,56,250	26.01	1	100
$F_{30\text{-}60\%}$ $(NH_4)_2$ SO_4	1,69,31.5	18,41,085	108.7	4.18	44.30
DEAE-Sephadex A-25	2,905.9	8,07,141.70	277.7	**10.68**	**19.50**

[a] One inhibition unit is defined as the amount of the inhibitor required to inhibit 50% of trypsin activity, under the trypsin inhibition assay.

of proteinase and trypsin activity, respectively, in *H. armigera*. Red gram PIs were marginal towards the midgut trypsin (21%), while black gram PIs showed moderate to low inhibitory activity towards the midgut trypsin-like proteinses of *H. armigera* (48%) (Prasad *et al.* 2010). The IC_{50} of standard SBTI was 0.1 µg/mL, whereas it was 8.7 times more for AnPI.

PI in chickpea exhibited better inhibition of total gut proteolytic activity and trypsin-like activity. It also did not possess any chymotrypsin inhibitory activity (Srinivasan *et al.* 2005). Similarly, we observed in the present study that AnPI was effective against total proteolytic activity and trypsin-like activity, but did not show any inhibitory effect on chymotrypsin activity of *H. armigera*. This higher inhibitory activity towards general proteolysis would give more pronounced effects on larval growth and physiology. *In vitro* inhibition of digestive proteinases is not enough to determine an efficient resistance factor against insect/pest. Pests are able to adapt to the presence of inhibitors, modifying the composition of their digestive proteinases, altering their concentrations or inducing the expression of novel proteinases (Jongsma *et al.* 1995). It also been reported that strong inhibitors of gut proteinases, *in vitro*, do not necessarily retard larval growth and development. In order to measure the AnPIs inhibitory efficiency, insect feeding tests were performed to assess the antibiosis exerted on *H. armigera*.

An inhibition kinetics study against *H. armigera* midgut trypsin was of non-competitive type (figure 2). Non-competitive inhibition was characterized by no changes in K_m value but a decrease of V_{max}, when compared to the reaction in the absence of the inhibitor. SBTI contained low k_i value when compared to k_i value of AnPI, suggesting that it has high affinity for *H. armigera* gut trypsin.

3.5 *In vivo assay of proteinase inhibitor*

Based on *in vitro* enzymatic studies, we have demonstrated effect of *A. nilotica* PIs on growth and development of *H. armigera*, in which three different doses of inhibitor were added per gram of the control diet. The mean weight of larvae fed on standard chickpea diet was significantly greater than the mean weight of larvae fed on diet containing soybean trypsin inhibitor (McManus and Burgess 1995). These results confirm the observations made in the present study. Some deformations could be caused by the absence of certain proteins necessary for metamorphosis. In *in vivo* studies, after day 10 the presence of high levels of inhibitors in the diets affecting larval growth. Statistical analysis indicates that 0.5% and 1%, diets significantly ($P<0.05$) reduced the mean larval weight when compared with that observed with the control diet. Larval period was extended by 1 day due to feeding on test diets containing 0.5 and 1% PI. The mean pupal weight was significantly reduced in test diets, 0.5% and 1% compared to that of the control. The presence of higher concentrations of PI in the diet caused several deleterious effects at all stages of *H. armigera* development, including pupae and adult emergence. Around 10–20% malformed pupae and malformed adults (larval-pupal

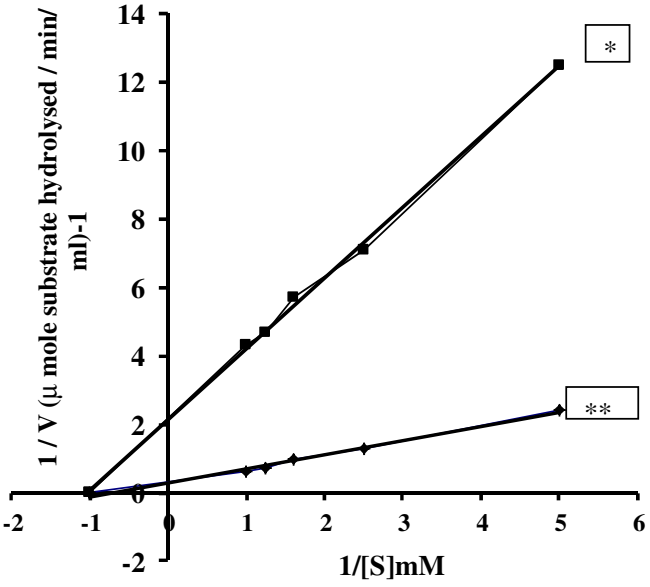

Figure 2. Inhibition of midgut trypsin activity of *H. armigera* by inhibator AnPI. Kinetic mechanism data are illustrated by Lineweaver-Burk double-reciprocal plots. Trypsin activity was evaluated using several concentrations of BA*p*NA in the absence or in the presence of the concentration of the AnPI.

Table 2. Effect of *H. armigera* larvae fed on diets incorporated with *A. nilotica* obtained by ammonium sulfate (30–60%) saturation

Partially purified PI	Pupation %	% adult emergence	Mean Fecundity $n=3$
Control (without inhibitor)	100[a]	100[a]	600[a]
A. nilotica 0.1%	100[a]	100[a]	352[b]
A. nilotica 0.5%	80[b]	80.73[b]	168[c]
A. nilotica 1%	70[b]	84.44[b]	34[c]

Means in a column followed by the same letter are not significantly different by DMRT ($P<0.05$).

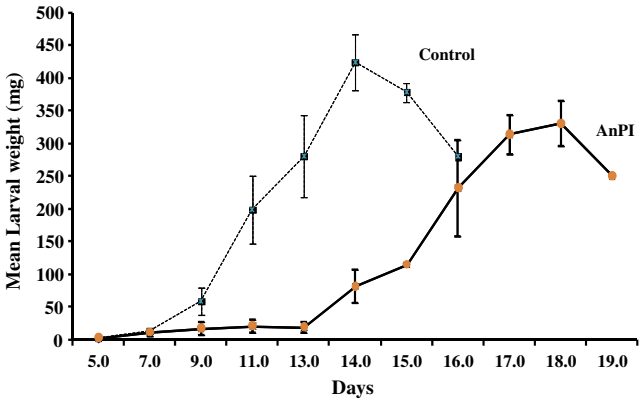

Figure 3. *In vivo* bioinsecticidal activities of AnPI. Effect of dietary of *H. armigera* larvae. Each mean represent 3 replicates± standard deviation.

intermediate and pupal-adult intermediate) were observed on the larvae fed on test diets (0.5% and 1%) and were statistically significant. It is interesting to observe that the mean fecundity was drastically affected in moths emerged from diet containing 0.5% and 1% PI diet (table 2). Starvation and added stress on gut proteinase expression system to synthesize new and higher amounts of proteinases could be the possible reasons for arrested growth and mortality of *H. armigera* larvae. Fecundity of *H. armigera* was severely affected by 0.33% concentration of winged bean PI in the diet (Gupta *et al.* 2002). Telang *et al.* (2003) reported a similar effect on *H. armigera* and *S. litura* by using PI from non-host source such as bitter gourd in the diet. Bitter gourd proteinase inhibitors (BGPIs) affect fertility and fecundity for the aforementioned lepidopteran insects. They further reported that ingestion of BGPIs adversely affected protein uptake, at the larval stage, which caused developmental abnormalities and also reduced fertility and fecundity of the adult. Thus, our observations are in agreement with these recent studies that accumulation of proteins during the larval stage is critical to vitellogenesis (Telang *et al.* 2000).

Purified AnPI was incorporated separately in the artificial diet at 0.25% (w/w) and fed to *H. armigera* larvae. It is remarkable to note that the larvae growing on test diet could not attain the body weight comparable to that of the control in the first 11 days. While the control larvae attained a body weight of 199 mg on day 11 indicates an increase of 195 mg after 6 days of feeding, the larvae feeding on test diets gained only 3–14 mg during the same 6 days of feeding time. This decimal growth rate is suggestive of imminent mortality. Therefore, the surviving larvae were shifted to the normal diet (without any PI protein) for possible recovery. Larvae fed on AnPI diet had extended larval period by 3 to 4 days. They attained comparable pupal weight as in the control. Lowest pupation percentage was recorded in test diet containing AnPI (figure 3). Fecundity data could not be obtained, as adult emergence was poor and prolonged in the treatment (AnPI).

Most plants produce PIs for insect protection, but insects can adapt to PI ingestion by overproducing PI-sensitive proteases (Bonade-Bottino et al. 1998), and/or up-regulating the expression of proteases that are insensitive to the PIs produced by that plant (Bolter and Jongsma 1997), or inducing the production of PI-degrading enzymes. Dunse *et al.* (2010) demonstrated that the combined inhibitory effect of NaPI (inhibitor from *Nicotiana alata*) and StPin1A (inhibitor from *Solanum tuberosum*) on *H. armigera* larval growth in the laboratory was reflected in the increased yield of cotton bolls in field trials of transgenic plants expressing both inhibitors. Better crop protection is thus achieved using combinations of inhibitors in which one class of PI is used to match the genetic capacity of an insect to adapt to a second class of PI.

Results from both *in vivo* and *in vitro* studies unequivocally demonstrate that the proteinase inhibitory proteins isolated from the seeds of *A. nilotica* are very effective in inhibiting the development of *H. armigera* and also its gut proteases. The study also suggests that the AnPIs gene(s) could be potential targets for future studies in developing insect-resistant transgenic plants against *H. armigera*. However, we must consider the complexity of *H. armigera* gut proteinases and attention must be paid to select appropriate PIs genes with the gene-pyramiding strategy for inhibition of a diverse range of proteinases from the gut complex of lepidopteran pests.

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