EFFECT OF FRENCH BEAN SEED PROTEINASE INHIBITORS (FBPIs)
ON TRYPsin AND CHYMOTRYPSIN ACTIVITY, GROWTH AND
DEVELOPMENT OF HELICOVERPA ARMIGERA (HUBNER)

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INTRODUCTION
Development of insect resistance by incorporating genes that express insecticidal proteins in crop plants has been considered as a novel approach (Hilder and Boulter, 1999). Use of plant proteins like lectins, proteinase inhibitors and amylase inhibitors are some of the best alternate approaches in the current scenario of pest control (Gatehouse and Gatehouse, 1998). In nature, plants can protect themselves against pests by synthesizing specific macromolecules derived from secondary metabolic pathways. The promising molecules that confer resistance against insects are the proteinase inhibitors (PIs) that are present in storage organs like seeds and tubers. Proteinase inhibitors are small molecular weight proteins that are quite common in nature. In plants, different roles for proteinase inhibitors have been suggested, including their action as storage proteins, as regulators of endogenous proteolytic activity (Ryan, 1990) as participants in many developmental processes, including programmed cell death and as components associated with the resistance of plants against insects and pathogens They may be synthesized constitutively during normal development or may be induced in response to herbivory or wounding (Koiwa et al., 1997).

Among different types of inhibitors, serine PIs are most studied class of PIs because they are ubiquitous in plants (Mello et al., 2002; Haq and Khan, 2003) and most Lepidopteran pests like Helicoverpa armigera and Spodoptera litura largely depend on serine proteinases for digestion of food proteins (Telang et al., 2003). Serine proteinase inhibitors in general are small, stable and abundant proteins showing specificity to trypsin and/or chymotrypsin (Bode and Huber, 1992). Most of these inhibitors bind to cognate enzymes according to a common substrate-like standard mechanism. 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). Support for a defensive role of plant PIs initially came from studies of insects raised on artificial diets containing PIs and in vitro inhibition assays of insect gut proteases with purified PIs from various plant sources (Ryan, 1990). The results of these studies strongly implicate plant PIs to interfere with the growth and digestive proteinases of many phytophagous insects.

Due to the complexity of enzyme/inhibitor interactions, the choice of an efficient inhibitor will determine the success of pest a control strategy. In this study, proteinase inhibitors from French bean seeds were purified, characterized and the biological effects on the growth and digestive proteinases of H. armigera larvae, to identify candidate genes for deployment through transgenic plants for controlling this pest.

MATERIALS AND METHODS
Bovine trypsin and chymotrypsin were purchased from SRL
Inhibitor isolation and purification

Crude extract was obtained according to Hajela et al., 1999 with some modifications. Finely ground French bean seed meal was extracted with 0.01M sodium phosphate buffer (1:10 w/v), pH 7.0, containing 0.15M NaCl for 10-15 minutes and then stirred for 2h at room temperature. The homogenized juice was centrifuged for 30 minutes at 8000-10000 rpm at 4°C. Solid ammonium sulfate was added to the supernatant (crude extract) to obtain a precipitate formed at 0-30, 30-60 and 60-90% saturation with respect to this salt. The pellet was collected in all fractions (F0, F30-60, F60-90) and lyophilized. At each concentration, the proteinase inhibitory activity and protein content were estimated. The F0,60 fraction, which corresponds to a 30-60% saturation range, showed a high level of inhibitory activity against trypsin. This fraction was applied to a DEAE-Sephadex A-25 column (50cm x 2cm column), equilibrated with several bed volumes of 20mM Tris-HCl buffer, pH 8.0. Clear supernatant obtained after centrifugation, was applied to the column and fractions of 5mL were collected at an initial flow rate of 15 mL h⁻¹. The column was washed with 20mM Tris-HCl buffer, pH 8.0, with a flow rate of 30mL h⁻¹ eluted by a linear gradient system in which a NaCl concentration was increased up to 0.4M in 20mM Tris-HCl pH 8.0, the chromatography was monitored in which a NaCl concentration was increased up to 0.4M in 20mM Tris-HCl buffer, pH 8.0, the chromatography was monitored.

Preparation of enzyme and substrates

Ten mg of each enzyme (Trypsin and chymotrypsin) were weighed and dissolved in 1mM HCl and stored in small aliquots at 4°C and used within 2 weeks. All substrates were prepared at a final concentration of 1mM in 5-10% of dimethyl sulfoxide (DMSO) or dimethyl formamide (DMF).

Inhibitory assay against serine proteinases

Inhibitory activities of French bean seeds towards two closely related serine proteinases were tested. The trypsin inhibitory assay was performed using BApNA as substrate. Different amounts of proteinase inhibitor were added to 20μg of Bovine trypsin in 200μL of 0.01M Tris-HCl (pH 8.0) containing 0.02M CaCl₂ and incubated at 37°C in a water bath for 5-10 minutes. Residual trypsin activity was measured by adding 1mM BApNA in pre-warmed (37°C) buffer 0.01M Tris-HCl (pH 8.0) containing 0.02M CaCl₂ and incubated at 37°C for 10 minutes (Erlanger et al., 1961). Reactions were stopped by adding 200μL of 30% glacial acetic acid. After centrifugation, the liberated p-nitroanilide in the clear solution was measured at 410nm. Only 20μg of trypsin in 200μL of buffer without crude extract was considered as control. Inhibitor activity was calculated by the amount of crude extract required to inhibit 50% of trypsin activity, which is considered as one unit of trypsin inhibition and expressed as trypsin inhibitor units per mg seed protein. All assays were performed in triplicate.

The chymotrypsin inhibitor activity was also measured in a similar way except that the substrate used was BTPNA. One millimolar BTPNA was prepared in 0.01M Tris-HCl (pH 8.0) containing 40% ethanol.

Protein determination

Protein was determined according to the method of Lowry et al., 1961 where bovine serum albumin was used as a standard.

Thermal and pH stability of FbPIs

Thermal stability of purified French bean Proteinase Inhibitors (FbPIs) was determined by using 0.1M Tris-HCl pH 8.0 incubated at various temperatures ranging from 20 to 100°C (±0.1°C) in a water bath for 30 minutes. After incubation at various temperatures, samples were cooled at 4°C for 10 minutes and centrifuged. The remaining proteinase inhibitor activity was measured.

The effect of pH on inhibitory activities of FbPIs 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 4 and 5; phosphate buffer for 6 and 7; Tris-HCl for 8 and Glycine-NaOH for 9 and 10. After 24h incubation at each pH at room temperature residual trypsin inhibitory activities were measured as mentioned earlier. All experiments were carried out in triplicate.

Preparation of insect gut proteinases

Gut enzyme extracts from final instar of H. armigera larvae was prepared according to the method of Johnston et al., 1991 with some modifications. The midguts were homogenized in ice-cold 0.2M Glycine-NaOH buffer, pH 10.0 containing 2mM DTT and 10% PVP (5guts/ mL buffer). The homogenates were kept for 2h at 10°C for 15 minutes and centrifuged at 8,000 rpm for 15 minutes at 4°C. The salt supernatant was used as a source of gut proteinases and stored at –20°C.

Enzymatic assays

Trypsin, chymotrypsin and total proteolytic activities in H. armigera larvae were estimated using the chromogenic substrates N-α-benzoyl-DL-arginine-p-nitroanilide (BApNA); N-α-benzoyl-DL-tyrosine-p-nitroanilide (BTPNA) and sodium caseinate according to modified protocol of Erlanger et al., 1961; Lee and Anstee, 1995. Rate of proteolysis of sodium caseinate was expressed in units (1,000 x OD) of trichloroacetic acid soluble peptides released/min/mg protein. The proteinase enzyme activity was expressed as pmoles of p-nitroaniline hydrolyzed/min/mg protein. SAAPFpNA as a substrate was used for measuring the chymotrypsin activity.

FbPIs inhibitory assay against gut extracts from H. armigera larvae

Three to four different doses of proteinase inhibitors from French bean (FbPI), 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 10μL of *H. armigera* gut extract. It was incubated at 37°C for 10 minutes, before addition of substrate to start the reaction. Residual activity was determined spectrophotometrically at 410nm and results were expressed as LC50 or % inhibition relative to controls without inhibitor. The enzyme activity was expressed as μmoles of p-nitroaniline released/min/mg protein. All in vitro assays were carried out in triplicates.

**Kinetics of inhibitory activity against *H. armigera* from FbPIs**

The mechanism of inhibition (competitive or non-competitive) against gut enzymes of *H. armigera* was determined at different substrate concentrations and at fixed concentration of inhibitor. Using 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 1mM) (Macedo et al., 2004). In the absence of inhibitor and in the presence of inhibitor, \( V_{\text{max}} \), \( V_{\text{in}} \) and \( K \) were calculated. The reaction velocity was expressed as \( 1/ν \) (μM pNA released/min/mg protein).

**Bioassays with *H. armigera* larvae fed on diet containing French bean PI**

For feeding studies and to test the effectiveness of proteinase inhibitor, FbPI purified on DEAE-Sephadex, they were incorporated separately in the artificial diet of *H. armigera*. Diet not containing the inhibitor was used as control. 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 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 interval. Pupal weight and adult emergence was measured at the end of the experiment (Broadway and Duffey, 1986; McManus and Burgess, 1995). Each larva served as a replication and data was analyzed statistically.

**Statistical analysis**

Results were expressed as means ± SEM. The comparisons of the means of the larval weight and other parameters were made by using analysis of variance (ANOVA) at a 5% level of probability.

**RESULTS AND DISCUSSION**

Serine proteinase inhibitors (trypsin and chymotrypsin) have been purified and characterized from a variety of plant sources.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total Trypsin Inhibitory</th>
<th>Specific activity (TIU)</th>
<th>Fold purification (TIU/mg protein)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract(^1)</td>
<td>6,022.26</td>
<td>2,74,400</td>
<td>45.50</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>F(_{200}) ((\text{NH}_4)_2\text{SO}_4)</td>
<td>198.5</td>
<td>56,170</td>
<td>282.9</td>
<td>6.21</td>
<td>20.47</td>
</tr>
<tr>
<td>DEAE-Sephadex A-25</td>
<td>50.6</td>
<td>31,124</td>
<td>615.1</td>
<td>13.51</td>
<td>11.34</td>
</tr>
</tbody>
</table>

\(^a\) One Inhibition Unit is defined as the amount of the inhibitor required to inhibit 50% of trypsin activity, under the trypsin inhibition assay.

(Fan and Wu, 2005). Crude soluble protein extract obtained from the mature French bean seeds was initially precipitated at 30%, 60% and 90% saturation with ammonium sulfate and three protein fractions \( [F_1 (0-30%), F_2 (30-60%) \text{ and } F_3 (60-90%)] \) were obtained. The \( F_1 \) protein showed strong inhibitory activity against trypsin, while the other fractions exhibited low inhibitory activity. The \( F_1 \) protein was then applied to an ion exchange chromatography, DEAE-Sephadex A-25 column, which yielded a broad peak as shown in Fig. 1A major broad peak denominated as FbPIs showed high inhibitory activity against bovine pancreatic trypsin, while no inhibitory activity was found in the other peaks (data not shown). The specific activity of the purified fraction was 13.51 times that of the crude extract with an 11.34% yield (Table 1.). These results showed low recovery percentage and purification level compared to purification of proteinase inhibitors achieved from other plant species. Similar results were obtained while working on *T. 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 Pattabhiraman, 1980.

Preincubation of the inhibitor in the pH range (2.0 to 10.0) did not affect trypsin inhibitory activity but at pH 5.0, FbPIs lost their trypsin inhibitory activity of 90% (Fig. 2A). The study on the temperature effect on FbPI showed that the inhibitory activity was stable at 60°C for 10 minutes. Total loss of trypsin inhibitory activity was found when heated for 10 minutes at 100°C (Fig. 2B). Stability at acidic and alkaline condition shows the high intrinsic stability of the purified inhibitors in this study. Wide range of stability in pH values reveals that purified PIs were effective for the control of insect pests, which are having...
variation in their gut environment, for example; acidic condition in Homoptera and Coleoptera and alkaline condition in Lepidoptera. Its high degree of thermal and pH stability was also similar to other proteinase inhibitors (Franco et al., 2004). Lopes et al., (2009) demonstrated that the inhibitors were thermally stable up to 65ºC but underwent abrupt denaturation with a midpoint at 75ºC for isoinhibitors of other tree legumes. The internal disulfide bridges that are present in all three isoforms structures support this high thermal stability.

To assess potential effects of French bean proteinase inhibitors on the digestive proteinases of *Helicoverpa* larvae, the midguts of final instar larvae was dissected. By using synthetic substrates (BApNA, BTPNA and SAAPPNA), the presence of serine proteinases (trypsin and chymotrypsin) were detected in midgut extracts of *H. armigera*. Trypsin was assayed with specific substrate, BApNA. This substrate is specific for the determination of trypsin activity (Christeller et al., 1992). The evidence for chymotrypsin like activity in the alimentary tracts of Lepidoptera is less clear than that for trypsin like activity. Chymotrypsin activity could not be assayed with BTPNA as a standard substrate. However, chymotrypsin like activity was found in *H. armigera* by using SAAPPNA as the substrate in this study. Inhibitory assays using FbPIs showed that the inhibitors showed high inhibitory activity towards trypsin, total gut proteolytic enzymes followed by chymotrypsin (Table 2).

**Table 2: Inhibition of *H. armigera* larval gut protease activities by FbPIs**

| Substrate | Inhibitor source | Amount of inhibitor | Specific activity | % Relative activity *
<table>
<thead>
<tr>
<th></th>
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<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>BApNA(Trypsin)</td>
<td>French bean(FbPIs)</td>
<td>5.00 μg</td>
<td>0.15 ± 0.005* (μmol pNA released/min/mg protein)</td>
<td>88.10</td>
</tr>
<tr>
<td></td>
<td>SBTI</td>
<td>5.00 μg</td>
<td>0.08 ± 0.010(μmol pNA released/min/mg protein)</td>
<td>93.65</td>
</tr>
<tr>
<td>SAAPPNA(Chymotrypsin)</td>
<td>French bean(FbPIs)</td>
<td>5.00 μg</td>
<td>0.20 ± 0.173 (μmol pNA released/min/mg protein)</td>
<td>50.03</td>
</tr>
<tr>
<td></td>
<td>SBBI</td>
<td>5.00 μg</td>
<td>0.17 ± 0.025(μmol pNA released/min/mg protein)</td>
<td>56.84</td>
</tr>
<tr>
<td>Na-Caseinate(General protein substrate)</td>
<td>French bean(FbPIs)</td>
<td>5.00 μg</td>
<td>5180 ± 385.74 (Units/min/mg protein)</td>
<td>80.72</td>
</tr>
<tr>
<td></td>
<td>SBTI</td>
<td>5.00 μg</td>
<td>3313 ± 206.98 (Units/min/mg protein)</td>
<td>87.67</td>
</tr>
</tbody>
</table>

*values are mean ± Standard error for at least three replications (p<0.05); *100% Activity is enzyme and buffer alone reacting with substrate; Values < 100 indicate inhibition of enzyme activity.

Figure 2: Stability of FbPI (A) pH stability of FbPIs after incubation at the indicated pH at 37ºC. (B) Temperature stability of FbPIs inhibitor activity after incubation for 30min. at the indicated temperature

Figure 3: Inhibitory activity of FbPIs against (A) midgut trypsin (B) and chymotrypsin of *Helicoverpa armigera* different concentrations of purified FbPI were incubated with crude midgut extracts showing trypsin and chymotrypsin activity at 37ºC for 10 min. Each mean represent 3 replicates ± standard error
for at least three replications ($p < 0.05$).

100 indicate inhibition of enzyme activity; * values are mean ± Standard error.

FbPIs for midgut trypsin was 0.5 μg/mL and for midgut chymotrypsin of (5 μg/mL) inhibited approximately 88% of midgut trypsin and activity of the gut enzymes of $H. \text{armigera}$ FbPIs showed equally effective for inhibiting trypsin activity of concentration (Table 3). In case of trypsin inhibitory activity, compared to FbPIs against gut enzymes at the equal inhibitor Standard proteinase inhibitors were highly effective when 2.5 to 10.0 μg) ($p < 0.05$; Fig. 3B) was observed in this study.

2.5 to 10.0μg in a dose dependent manner (Fig. 3A) similarly, decreased specific activity of midgut trypsin enzyme from 0.34 to 0.15 μmol pNA released/min/mg protein in a dose dependent manner (i.e. Dose: from 1.25 to 5.0μg) ($p < 0.05$; Fig.3A) similarly, decreased specific activity of midgut chymotrypsin enzyme from 0.23 to 0.14 μmol pNA released/min/mg protein in a dose dependent manner (i.e. Dose: from 2.5 to 10.0μg) ($p < 0.05$; Fig.3B) was observed in this study. Standard proteinase inhibitors were highly effective when compared to FbPIs against gut enzymes at the equal inhibitor concentration (Table 3). In case of trypsin inhibitory activity, FbPIs showed equally effective for inhibiting trypsin activity of $H. \text{armigera}$ larvae when compared to standard SBTI. FbPIs also showed equally effective by inhibiting about 42-88% activity of the gut enzymes of $H. \text{armigera}$ larvae. As the FbPIs (5μg/mL) inhibited approximately 88% of midgut trypsin and 50% of midgut chymotrypsin of $H. \text{armigera}$ larvae confirmed this inhibitor is a trypsin-chymotrypsin like serine proteinase inhibitor (Table 3). This result was confirmed by inhibition of bovine trypsin and chymotrypsin (data not shown). The $I_{C_{50}}$ of FbPIs for midgut trypsin was 0.5μg/mL and for midgut chymotrypsin was 7.5μg/mL (Table 4). FbPIs had required 3 to 4 times more for inhibition of gut enzymes when compared to the standard inhibitors. Similarly, SBTI had showed the lower $I_{C_{50}}$ value when compared to other inhibitors against $S. \text{littoralis}$ (Lee and Anstee, 1995).

Figure 4A and B shows the Lineweaver-Burk double reciprocal plots for the inhibition of trypsin and chymotrypsin by FbPIs. The inhibition was of the non-competitive type for both the gut enzymes of $H. \text{armigera}$, in which there was a decrease in $V_{max}$ with no change in $K_m$ compared to the reaction in the absence of inhibitor. The $k_i$ value for the FbPIs inhibitor was determine using a double reciprocal plot of data, where the $k_i$ value was found to be 0.150 nmol and 0.409 nmol for midgut trypsin and chymotrypsin, respectively. This inhibitor showed high affinity towards both the midgut serine proteinase enzymes of $H. \text{armigera}$. Similarly, soybean trypsin inhibitor was showing effective inhibition against $H. \text{armigera}$ gut protease activity with $k_i$ value of 1.4 nmol. However, pigeonpea trypsin inhibitor was not effective against the gut enzyme of $H. \text{armigera}$ and the $k_i$ value was in the range of 100 nmol (Godbole et al., 1994).

Purified FbPIs was incorporated separately in the artificial diet at 0.25% (w/w) and fed to $H. \text{armigera}$ larvae. When neonates were used in the assay, there was 24% of larval mortality in the diet containing 0.25% FbPI. Neonate larvae might be more susceptible to the effects of protease inhibition than older larvae (McManus and Burgess, 1995). It is remarkable to note that the larvae growing on test diet could not attain the body weight comparable to that of control in the first 11 days. While

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**Table 3: Inhibition of trypsin and chymotrypsin activities of midgut of $H. \text{armigera}$**

<table>
<thead>
<tr>
<th>Proteinase class</th>
<th>Amount of inhibitor (μg)</th>
<th>Inhibitor and% inhibition *</th>
<th>FbPI</th>
<th>Standard SBTI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine (Trypsin-like)</td>
<td>1.25</td>
<td>73.0 ± 1.15</td>
<td>77.77 ± 1.73</td>
<td></td>
</tr>
<tr>
<td>2.50</td>
<td>80.15 ± 1.15</td>
<td>88.88 ± 1.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.75</td>
<td>85.71 ± 1.15</td>
<td>90.47 ± 1.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.00</td>
<td>88.10 ± 1.15</td>
<td>93.65 ± 1.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serine (Chymotrypsin-like)</td>
<td>2.50</td>
<td>42.47 ± 0.57</td>
<td>13.67 ± 1.45</td>
<td></td>
</tr>
<tr>
<td>5.00</td>
<td>50.03 ± 1.15</td>
<td>56.84 ± 1.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.00</td>
<td>64.55 ± 2.31</td>
<td>94.13 ± 0.57</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 100% Activity is enzyme and buffer alone reacting with substrate; Values < 100 indicate inhibition of enzyme activity; * values are mean ± Standard error for at least three replications ($p < 0.05$).

These results suggested that higher activity towards general proteolysis could be explained as the inhibitor being able to inhibit proteinase, other than those, which possess trypsin-chymotrypsin like activity. Similarly, proteinase inhibitor 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). FbPI decreased specific activity of midgut trypsin enzyme from 0.34 to 0.15 μmol pNA released/min/mg protein in a dose dependent manner (i.e. Dose: from 1.25 to 5.0μg) ($p < 0.05$; Fig.3A) similarly, decreased specific activity of midgut chymotrypsin enzyme from 0.23 to 0.14 μmol pNA released/min/mg protein in a dose dependent manner (i.e. Dose: from 2.5 to 10.0μg) ($p < 0.05$; Fig.3B) was observed in this study.

**Table 4: $I_{C_{50}}$ (μg) values for FbPIs and standard PIs**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Inhibitor source</th>
<th>$I_{C_{50}}$ (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>French bean (FbPIs)</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Standard SBTI</td>
<td>0.13</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>French bean (FbPIs)</td>
<td>7.50</td>
</tr>
<tr>
<td></td>
<td>Standard SBTI</td>
<td>2.00</td>
</tr>
</tbody>
</table>

$I_{C_{50}}$ Concentration of inhibitor, which reduces the enzyme activity to 50% of the activity in the absence of inhibitors.
The control larvae attained a body weight of 199 mg on D11 indicates an increase of 195 mg after 6 days of feeding, the larvae feeding on test diets gained only 3.11 mg during the same 6 days of feeding time (Fig. 5). 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. The mortality was found to be 100% in the PI-fed larvae from D5 to D13 as compared to the control.

Results from both in vivo and in vitro studies unequivocally demonstrated that the proteinase inhibitory proteins isolated from the seeds of French bean are very effective in inhibiting the development of Helicoverpa armigera and also its gut proteases. These results indicate that transgenic crops expressing FbPI gene(s) could probably present an enhanced resistance against this bollworm. These results also indicate that FbPIs may be an effective bioinsecticide in the protection of H. armigera.

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REFERENCES


