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EFFECT OF FRENCH BEAN SEED PROTEINASE INHIBITORS (FBPIS) ON TRYPSIN AND CHYMOTRYPSIN ACTIVITY, GROWTH AND DEVELOPMENT OF *HELICOVERPA ARMIGERA* (HUBNER)

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ABSTRACT

French bean proteinase inhibitors (FbPIs) were isolated by ammonium sulfate precipitation followed by chromatography on DEAE-Sephadex A-25 and resulted in a purification of 13.51 fold with a 11.34% yield. FbPIs had high stability at different pH values (2.0 to 10.0) except pH 5.0 and are thermolabile beyond 80°C for 10 minutes. FbPIs exhibited effective against total proteolytic activity and trypsin like activity, but did not show any inhibitory effect on chymotrypsin activity of midgut of *H. armigera*. The inhibition kinetics studies against *H. armigera* gut trypsin are of non-competitive type. FbPIs had high affinity for *H. armigera* gut trypsin. The purified PI proteins incorporated test diets showed significant reduction in mean larval and the 100% mortality in the total larval population of *H. armigera*. Bioassays suggested that FbPIs could be used as a tool in engineering crop plants, which might exhibit increased resistance against cotton bollworm.

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

(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, U.S.A.). DEAE-Sephadex A-25 was obtained from Pharmacia Biotech, Sweden.

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 8000-10,000 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 (F_{0-30}, F_{30-60} and F_{60-90}). The pellet was dissolved in minimal volume of extraction buffer and dialyzed overnight with the same extraction buffer at 4^0C and lyophilized. At each concentration, the proteinase inhibitory activity and protein content were estimated. The F_{30-60} 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} and 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 at 280nm and 410nm. A single broad peak with proteinase inhibitory activities was obtained. These fractions were separately pooled, dialyzed and lyophilized.

Preparation of enzymes 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 BA*p*NA as substrate. Different amounts of proteinase inhibitor were added to 20μg of Bovine trypsin in 200 μL of 0.01M Tris-HCl (pH 8.0) containing 0.02M $CaCl_2$ and incubated at 37°C in a water bath for 5-10 minutes. Residual trypsin activity was measured by adding 1mL of 1mM BA*p*NA in pre-warmed (37°C) buffer 0.01M Tris-HCl (pH 8.0) containing 0.02M $CaCl_2$ 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*-nitroaniline 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 BT*p*NA. One millimolar BT*p*NA was prepared in 0.01M Tris-HCl (pH 8.0) containing 40% ethanol.

Protein determination

Protein was determined according by 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 resultant 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 (BA*p*NA); *N*-α-benzoyl-DL-tyrosine-*p*-nitroanilide (BT*p*NA) 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 μmoles of *p*-nitroaniline hydrolyzed/min/mg protein. SAAPF*p*NA as a substrate was also 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 37ºC for 10 minutes, before addition of substrate to start the reaction. Residual activity was determined spectrophotometrically at 410nm and results were expressed as LC_{50} 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, K_m, V_{max} and K_i were calculated. The reaction velocity was expressed as 1/v (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

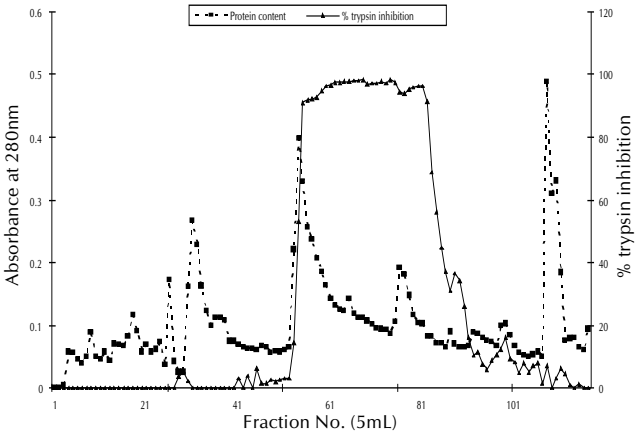

Figure 1: Elution profile on DEAE-Sephadex A-25 of F30-60, from French bean seeds. Approximately 100mg of protein was applied in the column equilibrated with 20mM Tris-HCl buffer, pH 8.0. and fractions were eluted and monitored at 280nm

(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%) and F_3 (60-90%)] were obtained. The F_2 protein showed strong inhibitory activity against trypsin, 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, 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 Pattabiraman, 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 60ºC for 10 minutes. Total loss of trypsin inhibitory activity was found when heated for 10 minutes at 100ºC (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

Table 1: Purification of French bean proteinase inhibitors (FbPIs)

Step	Total protein (mg)	Total Trypsin Inhibitory	Specific activity Unit (TIU)	Fold purification (TIU/mg protein)	% Recovery
Crude extract[1]	6,022.26	2,74,400	45.50	1	100
$F_{30\text{-}60\%}$ $(NH_4)_2SO_4$	198.5	56,170	282.9	6.21	20.47
DEAE-Sephadex A-25	50.6	31,124	615.1	13.51	11.34

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

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

Substrate	Inhibitor source	Amount of inhibitor	Specific activity	% Relative activity [a]
BA*p*NA(Trypsin)	French bean(FbPIs)	5.00 μg	0.15 $\pm$ 0.005* (μmol pNA released/min/mg protein)	88.10
	SBTI	5.00 μg	0.08 $\pm$ 0.010(μmol pNA released/min/mg protein)	93.65
SAAPF*p*NA(Chymotrypsin)	French bean(FbPIs)	5.00 μg	0.20 $\pm$ 0.173 (μmol pNA released/min/mg protein)	50.03
	SBBI	5.00 μg	0.17 $\pm$ 0.025(μmol pNA released/min/mg protein)	56.84
Na-Caseinate(General protein substrate)	French bean(FbPIs)	5.00 μg	5180 $\pm$ 385.74 (Units/min/mg protein)	80.72
	SBTI	5.00 μg	3313 $\pm$ 206.98(Units/min/mg protein)	87.67

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

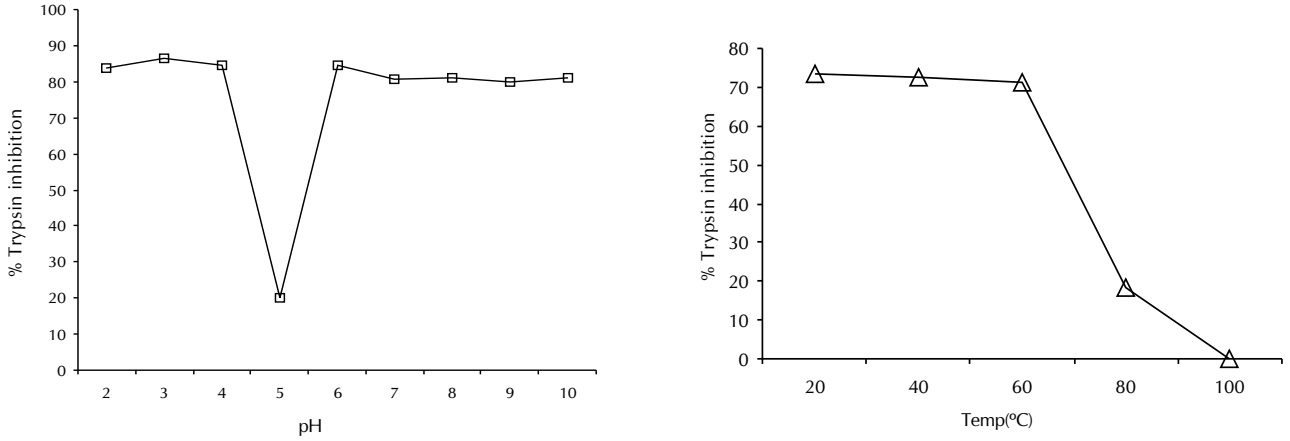

Figure 2: Stability of FbPI (A) pH stability of FbPIs after incubation at the indicated pH at 37°C. (B) Temperature stability of FbPIs inhibitor activity after incubation for 30min. at the indicated temperature

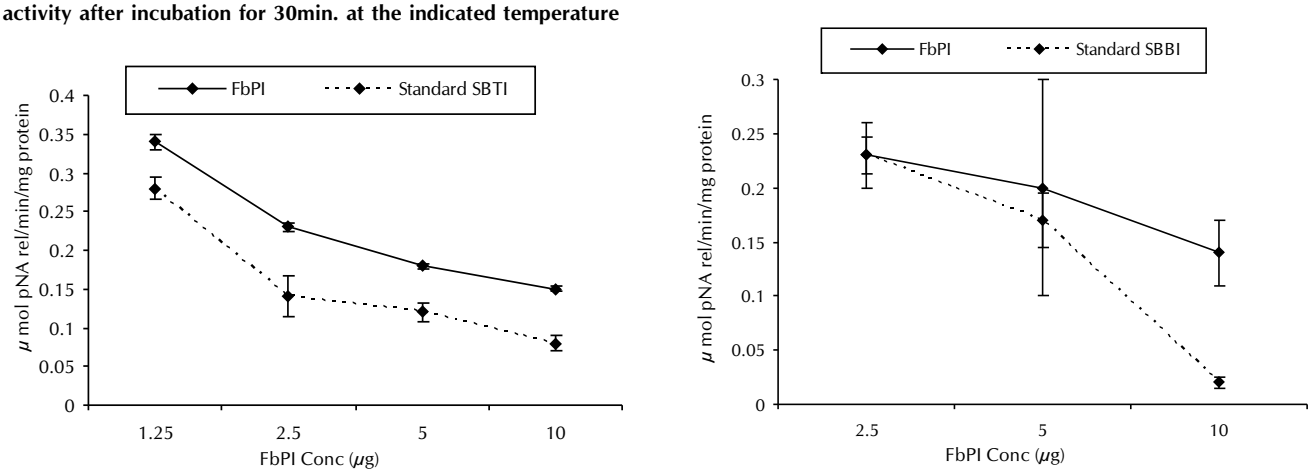

Figure 3: Inhibitory activity of FbPIs against (A) midgut trypsin (B) and chymotrypsin of *Helicoverpa armigera* different concentrations of purified FbPI were incubated with crude midgut extracts showing trypsin and chymotrypsin activity at 37°C for 10 min. Each mean represent 3 replicates $\pm$ standard error

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 65°C but underwent abrupt denaturation with a midpoint at 75°C 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 (BA*p*NA, BT*p*NA and SAAPF*p*NA), the presence of serine proteinases (trypsin and chymotrypsin) were detected in midgut extracts of *H. armigera*. Trypsin was assayed with specific substrate, BA*p*NA. 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 BT*p*NA as a standard substrate. However, chymotrypsin like activity was found in *H. armigera* by using SAAPFpNA 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).

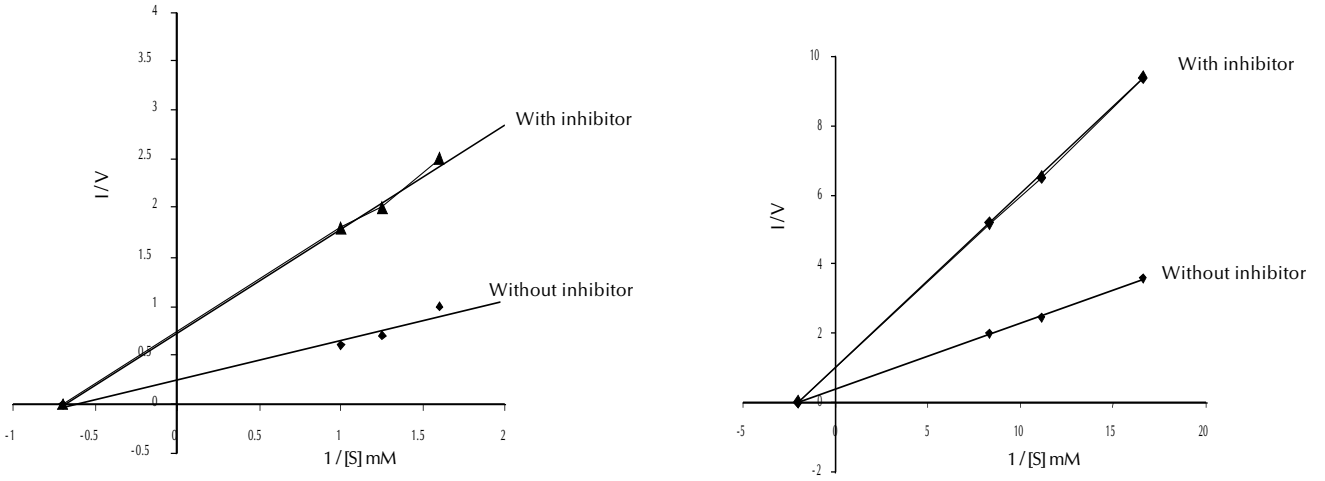

Figure 4: Inhibition of midgut trypsin (A) and chymotrypsin (B) activity of *H. armigera* by inhibitor FbPIs, kinetic mechanism data are illustrated by linewearver-Burk duble-reciprocal plots. Enzyme activity was evaluated using several concentrations of substrates in the absence or in the presence of the concentration of the FbPIs. ordinate: reciprocal of BApNA concentration (mM)

Table 3: Inhibition of trypsin and chymotrypsin activities of midgut of *H. armigera*

Proteinase class	Amount of inhibitor(μg)	Inhibitor and% inhibition [a] FbPI	Standard SBTI
Serine (Trypsin-like)	1.25	*73.0 ± 1.15	77.77 ± 1.73
	2.50	80.15 ± 1.15	88.88 ± 1.15
	3.75	85.71 ± 1.15	90.47 ± 1.15
	5.00	88.10 ± 1.15	93.65 ± 1.73
Serine (Chymot-rypsin-like)	2.50	42.47 ± 0.57	13.67 ± 1.45
	5.00	50.03 ± 1.15	56.84 ± 1.45
	10.00	64.55 ± 2.31	94.13 ± 0.57

[a] 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. 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. armigera* larvae when compared to standard SBTI. FbPIs also showed equally effective by inhibiting about 42-88% activity of the gut enzymes of *H. armigera* larvae. As the FbPIs (5μg/mL) inhibited approximately 88% of midgut trypsin and 50% of midgut chymotrypsin of *H. 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 IC_{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_{50} value when compared to other inhibitors against *S. littoralis* (Lee and Anstee, 1995).

Table 4: IC_{50} (μg) values for FbPIs and standard PIs

Enzyme	Inhibitor source	LC_{50} (μg)$_a$
Trypsin		
	French bean (FbPIs)	0.50
	Standard SBTI	0.13
Chymotrypsin		
	French bean (FbPIs)	7.50
	Standard SBBI	2.00

[a]LC_{50} = Concentration of inhibitor, which reduces the enzyme activity to 50% of the activity in the absence of inhibitors

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. 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. armigera*. Similarly, soybean trypsin inhibitor was showing effective inhibition against *H. 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. 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. 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

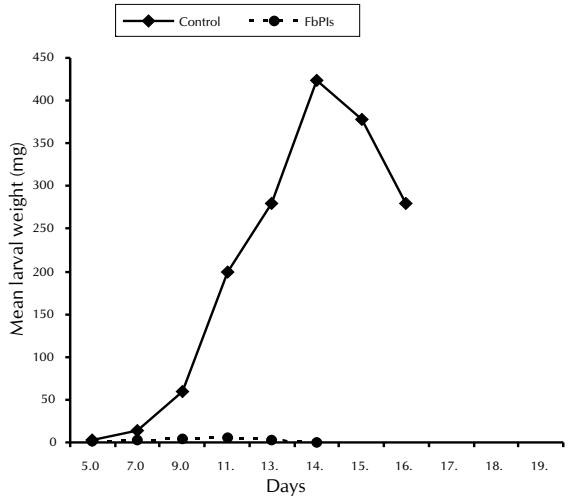

Figure 5: *In vivo* bioinsecticidal activities of FbPIs Effect of dietary purified FbPIs on growth of *H. armigera* larvae

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. Larval survival varied from 49% in larvae reared on diet impregnated with SBTI to 90% in larvae reared on the control diet at 5 days after initiating the experiment (Sonali Shukla *et al*., 2005). Significant reduction in growth and development of the larvae of *H. zea* and *S. exigua*, when larvae were fed with soybean trypsin inhibitor and potato proteinase inhibitor II (Broadway and Duffey, 1986). These results are confirming the present investigation. The presence of inhibitors in the diet significantly reduced the average weight of larvae at moult. The mode of action of proteinase inhibitors is to cause the pernicious hyper production of trypsin. This coupled with insufficient dietary availability of sulphur-containing amino acids (methionine) needed for enzyme synthesis, results in inhibition of growth. Mode of action of PIs suggests that they act in a straightforward way by significantly reducing protein digestion (Broadway, 1997). Telang *et al*., 2003 reported similar effect on *H. armigera* and *S. litura* by using PIs from non-host source such as bitter gourd in the diet. Bitter gourd proteinase inhibitors (BGPIs) affect fertility and fecundity for the above said 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).

These *in vivo* assays results clearly indicated that French bean PIs affect the *H. armigera* growth and development significantly.

Results from both *in vivo* and *in vitro* studies unequivocally demonstrate 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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