Purification and characterization of protease inhibitors from rice bean 
(*Vigna umbellata*) and their efficacy against *Spodoptera litura* (Fabricius) 
gut proteinases

Kanika Sharma*

Department of Chemistry and Biochemistry, 
CSK Himachal Pradesh Krishi Vishwavidyalaya, Palampur-176 061, Himachal Pradesh, India.

Received: 27-04-2018  
Accepted: 01-06-2019  
DOI: 10.18805/IJARe.A-5023

**ABSTRACT**

A protease inhibitor from rice bean was isolated and purified to assess its potential against gut proteases of *Spodoptera litura*, a devastating pest of several economically important crops globally. The purified inhibitor showed a single band protein on SDS-PAGE corresponding to molecular weight of 24.0 kDa. The inhibitor was purified to near homogeneity with 181.55 fold purification and final yield of 29.18 per cent. The inhibitory potential of purified rice bean trypsin protease inhibitor on larvae of *Spodoptera* gut proteases was studied which showed maximum azocaseinolytic (1.93U), trypsin (0.66U) and chymotrypsin activity (0.37 U) in second larval instar which gradually decreased up to late fifth larval instar stage. Inhibitory assay revealed that rice bean trypsin inhibitors were strong inhibitor of *Spodoptera litura* and inhibited more than 80 per cent for trypsin and around 69 per cent for chymotrypsin activity. To further determine the efficacy of rice bean protease inhibitors, feeding assays were conducted by adding rice bean flour in larval artificial diet. High mortality rate was observed in *Spodoptera litura* larvae after 72 hrs when rice bean was given as sole diet (100% rice bean composition). The results obtained from present study provide important clues in designing strategies for sustainable use of rice bean protease inhibitors in developing insect-tolerant transgenic plants.

**Key words:** Chymotrypsin inhibition, Insect protease, Rice bean, *Spodoptera litura*, Trypsin.

**INTRODUCTION**

Insect pest menace is one of the major factors that destabilize crop productivity in agricultural ecosystems. They are responsible for severe reduction in crop yields, in spite of extensive use of chemical pesticides (Ferry et al. 2004). Plant traits that are important for resistance to insect pest attack are complex and operate on many spatial scales involving direct and indirect defenses. Protease inhibitors (PIs) are one class of plant defense proteins against insect pest infestation. PIs are usually present in plants storage organs, such as seeds and accumulate about 1 to 10% of the total soluble proteins of storage tissues. In plants, protease inhibitors have been detected in cereals such as rice, wheat, barley, rye, sorghum also in legumes such as chick pea, pigeon pea, kidney bean, cowpea and rice bean etc. Rice bean (*Vigna umbellata* (Thunb) Ohwi and Ohashi) is one of the promising pulses with high yield potential and optimum nutrition. Its seeds and storage organs have proven to be a very rich source of proteinase inhibitors, particularly the inhibitors of cysteine and serine proteinases (Kowalska et al. 2007).

Plant derived protease inhibitors inactivate proteases of animals and microbial origin while rarely, inhibiting endogenous enzymes is a compelling evidence for the current view that they are involved in the protection of plants against pests and possibly pathogens. They are one of the prime candidates with highly proven inhibitory activity against insect pests and also known to improve the nutritional quality of food (Charity et al. 1999). Efforts are being made to explore their use in developing insect resistance in susceptible crop plants. However, a thorough understanding of insect digestive enzymes is a prerequisite to plan strategies for successful and sustainable application of PIs.

PI-based approaches usually focus on the dominant mechanistic class of digestive protease in Lepidoptera. Lepidopteran insects have serine proteases (trypsin and chymotrypsin) as a major component of their digestive complement and are the most commonly found proteases (Srinivasan et al. 2006). Among these lepidopteran species *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) is a polyphagous insect which cause damage to numerous vegetables and field crops in many Asian countries. It has also proved to be an ideal model insect for the study of the basic functions of the digestive enzymes present in the midgut of insects due to its insatiable feeding habit.

*Corresponding author’s e-mail and address: s.kanika7@gmail.com  
Hans Raj Mahila Mahavidyalaya, Jalandhar-144 008, Punjab, India.*
The insect has host range of more than 120 host plants including crops, vegetables, weeds and ornamental plants. In recent times, it also became resistant to many commonly used insecticides, particularly pyrethroids and carbamates, resulting in failure of effective controls (Ahmad et al. 2007). Although much has been understood and studied about this phytophagous lepidopteran species, the overwhelming complexities of proteases and their interaction with different types of protease inhibitors leave much to be explored. In the present work, efforts were made to purify protease inhibitor from rice bean seeds and its inhibitory potential against Spodoptera litura gut proteases was studied.

MATERIALS AND METHODS

Synthetic substrates Azocasein, N-α-Benzoyl-DL-Arginine-paranitroanilide (BApNA), N-α-Benzoyl-DL-tyrosine-paranitroanilide (BTpNA), DEAE-Sepharose, Sephadex G-75 and Bovine trypsin were obtained from Sigma Chemical Co, St Louis, MO, USA.

Extraction of rice bean protease inhibitor: Seeds of rice bean genotype BRS-2 were procured from the Department of Crop Improvement, CSKHPKV, Palampur. Dry seeds were ground to fine powder, defatted and depigmented with several washes of hexane and acetone. Sample was homogenized with 0.1 M phosphate buffer pH 7.5 for 4h, filtered and then centrifuged at 10,000g for 15min. The supernatant obtained was precipitated overnight with 80 per cent ammonium sulphate. The precipitates were dissolved in 0.1 M phosphate buffer pH 7.5 and dialyzed thoroughly against 0.05 M phosphate buffer pH 7.5 for 24 h. The final supernatant was freeze-dried and lyophilized for purification.

Purification of rice bean protease inhibitor: The concentrated extract was loaded into a DEAE-Sepharose column (2x21cm), equilibrated with the 0.1 M phosphate buffer pH 7.5. Stepwise elution was carried out with a linear gradient of 0.1-0.5M NaCl in 0.1M phosphate buffer (pH 7.5). Fractions (3ml) were collected with flow rate of 0.5ml/min. The eluted fractions were analyzed for protein content and trypsin inhibitory activity. The active fractions with high trypsin inhibitory activity were pooled and used for carrying out electrophoresis. Freeze dried sample pooled from DEAE-Sepharose column was dissolved in phosphate buffer 0.1M pH 7.5 and applied to a Sephadex G-75 column (1.5x22.5 cm) for gel permeation chromatography. The active fractions were further purified by trypsin-Sepharose affinity chromatography for which column was prepared with a slight modification in the method given by Paiva et al. (1992) and Chang and Tsen (1981). TI sample pooled from Sephadex G-75 column was applied to CNBr activated Sepharose column (0.9 cm x 14 cm). Fractions of 1.5 ml/tube were eluted with 0.05N Tris buffer pH 7.8 and collected using a flow rate of 0.5 ml/min. The active fractions with trypsin inhibitory activity were pooled and the purity of the final trypsin inhibitor protein obtained was checked by SDS-PAGE.

Determination of protein content and trypsin inhibitory activity: Protein concentration during the trypsin inhibitor purification steps was determined using the method of Lowry et al. (1951). The trypsin inhibitory assay was done with a slight modification in the method described by Chitra and Sadasivam (1986). Absorbance was recorded at 410 nm. The trypsin inhibitor activity is expressed as trypsin inhibitor units (TIU) per gram sample or per mg protein.

Electrophoretic analysis: The purity of the final trypsin protease inhibitor protein obtained after the course of purification was checked by polyacrylamide gel electrophoresis using 12.5 per cent acrylamide gels with a trivial modification in the method given by Lamelli (1970).

Collection of experimental insects: Spodoptera litura larvae from first to fifth instar stage were collected from infected plants of Solanum lycopersicum (tomato) and Capsicum annum (Capsicum) plants from Biological Control Lab CSK HPKV, Palampur during the month of June-July. The population was maintained on castor (Ricinus communis) and capsicum leaves in optimum rearing conditions (27±1°C, 60±5% RH).

Preparation of gut luminal enzyme extract of Spodoptera litura: Larvae at first to fifth instar stage were anesthetized by exposing them to diethyl ether for few seconds. They were carefully dissected out and 8-10 clean midguts / larval instar stage were extracted by homogenizing the tissue with its contents in equal volume (5ml) of ice cold distilled water. The gut luminal contents were removed by centrifugation at 10,000 rpm for 20 min at 4°C. The resultant supernatant was then filtered, frozen in aliquots and was used to study the metabolic interaction of gut proteases with protease inhibitors.

Protease and Protease inhibitor activity assays: Protease activity was measured by slight modification in the method given by Telang et al. (2005). Total gut protease activity was determined using chromogenic substrate azocasein at a final concentration of 2 per cent. Activity was estimated by measuring the OD at 450 nm. Specific gut protease trypsin and chymotrypsin activity were measured with BApNA and BTpNA as synthetic substrate. The reaction was terminated by addition of 750 µl of 30% acetic acid and O.D was measured at 410 nm.

Assays were run in triplicates with appropriate blanks. Proteinase activity unit was calculated in terms of micromoles of paranitroaniline produced per minute. The molar extinction coefficient (M-1 cm-1) for paranitroanilide (pNA) at 410 nm equals to 8800 (Erlanger et al. 1961) was taken in to account to calculate trypsin and chymotrypsin-
like activity (BApNA/ BTpNA units /mg protein) using the formula:

\[ \text{Activity units} = \frac{\text{Abs}_{410}/\text{min} \times 1000 \times \text{ml of reaction mixture}}{\text{Extinction coefficient of chromagen - mg protein in reaction mixture}} \]

For inhibitory activity, 1.35ml purified fraction of rice bean protease inhibitor was added to 30µl of gut extract for 10 min at 37°C. 200 µl of substrate BApNA (8mM) and BTpNA (1mM) was added to reaction mixture as described earlier by Shorey and Hale (1965). For feeding studies the artificial diet was supplemented with rice bean flour in plastic cages. The diet was prepared with four treatments and a control. The final composition of diet was made 25%, 50%, 75% and 100% (w/w) with rice bean flour and a positive control of artificial diet (i.e. without PIs). The culture was routinely maintained on rice bean flour based artificial diet at standard conditions, of 27±5ºC, 75±5 % relative humidity. The cages were cleaned and shaken everyday to improve aeration and to prevent attack of unwanted micro organisms. The experiment was conducted for 96 hrs (4 days) and was started by rearing 20 insects on each diet preparation.

**Data collection and statistical analysis:** Data was collected included the number of live and alive insects per day and per cent larval mortality was calculated. Data were analyzed using SAS version 9.3 software. Means of three independent determinations was tested by one way ANOVA followed by post hoc testing using Duncan’s multiple range test. A significant level of 0.05 was used for all statistical tests, \( p = 0.05 \).

**RESULTS AND DISCUSSION**

**Extraction of rice bean protease inhibitor:** Rice bean genotype BRS-2 was extracted with different extraction media among which maximum trypsin inhibitory activity was observed when the rice bean seed flour was extracted with 0.1 M phosphate buffer (pH 7.5). Therefore, in all the subsequent experiments 0.1 M phosphate buffer (pH 7.5) was used for extraction. Final purification of protease inhibitor from BRS-2 was carried out with 0.1 M phosphate buffer as inhibitor was found to be stable and could be extracted well in this media.

**Purification of rice bean protease inhibitor:** The protease inhibitor from rice bean was purified to apparent homogeneity with 181.55 fold purification and 29.18 per cent yield after extraction, \((\text{NH}_4)_2\text{SO}_4\) precipitation, ion exchange chromatography on DEAE-Sepharose, gel permeation chromatography through Superdex-75 and finally

---

**Table 1:** Purification of trypsin protease inhibitor from rice bean flour.

| Step                      | Volume (ml) | TUI  | Protein (mg) | Specific activity | Fold purification | Yield (%) |
|---------------------------|-------------|------|--------------|-------------------|-------------------|-----------|
| Crude extract             | 100         | 42806| 4811         | 8.89              | 1                 | 100       |
| \((\text{NH}_4)_2\text{SO}_4\) ppt | 50          | 30211| 1080         | 27.97             | 3.14              | 70.57     |
| Ion exchange chromatography| 40          | 22198| 49           | 483.02            | 50.95             | 51.85     |
| Gel filtration            | 35          | 16123| 25.5         | 632.27            | 71.12             | 37.66     |
| Affinity Chromatography   | 14          | 10491| 6.5          | 1614.0            | 181.55            | 29.18     |

**Table 2:** Total Protein content and proteolytic activity at different larval instars of *Spodoptera litura*.

| Larval Instar stage | Total Protein Content (mg/ml) | Azocaseinolytic Activity (U) | Trypsin Activity (U) | Chymotrypsin Activity (U) |
|---------------------|-------------------------------|------------------------------|----------------------|---------------------------|
| First               | 1.11 ± 0.06                   | 0.58 ± 0.05                  | 0.16 ± 0.04          |
| Second              | 1.93 ± 0.07                   | 0.66 ± 0.11                  | 0.37 ± 0.03          |
| Third               | 1.47 ± 0.10                   | 0.62 ± 0.05                  | 0.28 ± 0.04          |
| Fourth              | 1.35 ± 0.17                   | 0.58 ± 0.09                  | 0.28 ± 0.09          |
| Early Fifth         | 1.22 ± 0.08                   | 0.48 ± 0.09                  | 0.15 ± 0.04          |
| Late Fifth          | 0.79 ± 0.09                   | 0.38 ± 0.03                  | 0.11 ± 0.05          |
| CV                  | 1.57                          |                              |                      |
| CD (5 %)            | 0.35                          |                              |                      |
| SE (±)              | 0.18                          |                              |                      |

*Comparative values converted into units are presented in table. Reported values are ± SEM of triplicate determinations.

**Means with the same letter are not significantly different and are at par (one way Annova followed by Duncan’s multiple range test, \( p = 0.05 \)).
by affinity chromatography using trypsin-Sepharose column (Table 1). The specific activity increased from 8.89 in crude extract to 1614.0 units per mg of total protein after affinity chromatography. In ion exchange chromatography on DEAE-Sepharose (fast flow) column (40.7 × 2.0 cm; flow rate 25 ml/h), subsequent elution with a linear NaCl (0.2M - 0.4M) gradient resulted in the elution profile which featured two peaks corresponding to 0.2 M and 0.3 M NaCl gradient. The second broad peak was chosen for further purification due to its high trypsin inhibitory activity as well as protein content corresponding to 0.3 M NaCl gradient (Fig 1A). The active fractions those possessing high trypsin inhibitory activity were collected loaded on to Superdex-75 column (22.5x1.5 cm; flow rate 0.5 ml/min) for gel filtration chromatography. The inhibitory activity emerged as one major peak and all fractions within the peak exhibited high trypsin inhibitory activity (Fig 1B).

Finally, pooled fractions from Sephadex G-75 column were allowed to flow through CNBr activated Sepharose-trypsin column (Fig 1C). The polypeptide composition of purified trypsin inhibitor protein obtained after affinity chromatography determined electrophoretically showed one single band on the gel with molecular weight of 24.0 kDa under reducing conditions (Fig 1D).

**Spodoptera litura gut protease activity:** Different synthetic substrates (azocasein, BApNA, BTpNA) were used under appropriate assay conditions to determine specific gut protease activity in different larval instars of *Spodoptera litura* (Table 2). It was observed that there were significant differences in the proteolytic activity at different larval stages (p <0.05). The gut protease activity decreased with development of larval instars. Maximum azocaseinolytic (1.93U), trypsin (0.66U) and chymotrypsin activity (0.37U) was observed in second larval instar (Fig 2).

Azocaseinolytic activity was significantly lower in late fifth larval stage as compared to other stages. Similarly, significantly higher trypsin and chymotrypsin activity was observed in second instar larva whereas there was no significant difference in the activity at third and fourth larval stage. Differences in total protein content among different larval stages were significant and were higher in second and third instar larvae.

**Inhibitory potential of rice bean protease inhibitor against *S. litura* gut protease activity:** Inhibition potential of rice bean trypsin inhibitors towards gut proteases of *Spodoptera litura* was evaluated by inhibitory enzyme assays (Table 3). The results clearly demonstrated that rice bean protease inhibitors exhibited differential inhibitory activity towards gut proteases. Bioassay revealed that rice bean trypsin inhibitors are strong inhibitor of *Spodoptera litura* and showed inhibitory action more than 80% for trypsin and around 69% for chymotrypsin activity. Maximum trypsin and chymotrypsin inhibition was observed in third larval stage (83.08 % and 69.23%) followed by fourth larval stage (80.39 % and 66.66 %) whereas minimum inhibitory activity was noticed in early fifth stage.

Inhibitory assay showed that trypsin inhibition was higher as compared to chymotrypsin inhibition. These observations indicated that *Spodoptera litura* larvae have high trypsin like proteases in its gut and rice bean trypsin protease inhibitors are highly specific to them whereas it could moderately inhibit chymotrypsin activity as well. Trypsin inhibitory activity (%) at first and second larval stage was statistically at par whereas chymotrypsin inhibition varied significantly among all the stages (Fig 3).

**Assessment of inhibitory potential of rice bean on *Spodoptera litura* survival:** Pls contribution to the plant defense mechanisms relies on the inhibition of proteases present in insect’s gut causing reduction in the availability of amino acids necessary for their growth. The effect of protease inhibitor on insect larvae is chronic rather than acute, but the effects on pest population are usually dramatic as they increase the mortality of insect population. In order to determine the effects of rice bean protease inhibitors, feeding experiments were conducted on *Spodoptera litura* larvae. Larvae were reared on diet preparation of rice bean flour along with artificial diet. The results revealed that addition of rice bean flour in the diet had a significant effect on mortality rate of *Spodoptera litura*. Larvae fed on 75 and 100% rice bean flour composition resulted in 80% larval
Fig 1: Trypsin inhibitor activity (%) and protein content (mg) in various (A) DEAE-Sepharose chromatographic fractions (B) Superdex-75 chromatographic fractions (C) Sepharose-Trypsin affinity chromatographic fractions (D) Protein molecular weight marker and lane 1 contained 20 µg purified trypsin inhibitor after affinity chromatography.
mortality just after 48 and 72 hrs of the feeding experiment, respectively. 100% mortality rate was observed after 72 hrs when rice bean flour was given as sole diet (Fig 4). In all treatments the larval mortality increased with increase in duration after feeding. After 96 hrs (4 days) of the experiment, 100 percent mortality was observed in all treatments.

Protease inhibitors are among the best-studied candidates for genetic engineering of plant resistance to insect pests. There is a growing interest in identification of novel PIs and their use in developing resistance in otherwise susceptible plants. In this study the identification of PIs from rice bean seed that are potent inhibitors of the gut proteinases of Spodoptera litura have been reported. In this study rice bean protease inhibitor has been purified to homogeneity by using phosphate buffer (0.1 M, pH 7.5) as extracting medium. Previously, different extracting media have been reported to be suitable for extraction of protease inhibitor by various workers (Maggo et al. 1999). Similarly, Klomklao et al. (2011) extracted and characterized trypsin inhibitor from mung bean (Vigna radiata (L.) R. Wilczek) seeds. The inhibitor was purified to 13.51 fold with a yield of 30.25 percent. Molecular weight distribution and inhibitory activity staining showed that the purified protease inhibitor had a molecular weight of 24.0 kDa. Using similar purification methods, protease inhibitors have been purified to homogeneity from other legumes by various workers (Haq and Khan 2003). It is likely that the inhibitor purified is of Kunitz type because it has been well documented that the Kunitz type inhibitors are proteins of a molecular weight of more than 20.0 kDa, with low cysteine content and a single reactive site, whereas the Bowman-Birk type inhibitors have a molecular weight of 8–10 kDa, as well as a high cysteine content and two reactive sites (Richardson 1991). A higher
molecular mass 24.0 kDa (> 20 kDa) of the purified inhibitor may either be due to monomer-dimer equilibrium or due to oxidation of cysteine residues as reported by Ferrason et al. (1997).

The present study has also demonstrated that efficacy of this novel rice bean protease inhibitor in inhibition of gut proteinases of Spodoptera litura larvae. Proteinases are the major digestive enzymes in the insect gut. They are responsible for a continuous supply of essential amino acids and energy from the food source for development. The larval stage of Spodoptera litura is the actively feeding stage responsible for accumulation of nutrients to complete its life cycle. When this insect ingests PIs along with food proteins from plants, interaction of their gut proteinases with PIs determines the success or failure of PIs as an antidigestive factor. Earlier and current reports on PIs have dealt with the identification and characterization of PIs such as inhibitors of trypsin, chymotrypsin, or subtilisin (Ser proteinases) and papain (Cys proteinases). However, it is necessary to identify and evaluate PIs having specific inhibitory activity against Spodoptera litura gut proteinases for which first mandatory step was to evaluate the total gut proteinase (azocaseinolytic) activity, trypsin and chymotrypsin activity specifically in different larval stages (first larval stage to late fifth). The results revealed that enzymatic activities at each larval stage of Spodoptera varied which may be attributed to different feeding habits of larvae with developing instars. Protease activity in the early larval stages (second and third) was high and decreased gradually. This decline might have result from a greater degradation or a lower synthesis of digestive proteases produced by a quantitative decrease of the feed intake when a larva is near of the next molt stage. Therefore, the diminution in enzymatic activity could be related with anatomical and physiological modifications of larvae gut (Alarcon et al. 2002). The results obtained were similar to the findings of Satheesh and Murugun (2012) who studied the insecticidal potential of protease inhibitor from leaves of Coccinia grandis (L.) against Spodoptera litura and observed that maximum protease activity was in 3rd and 4th larval stage corresponding to ninth day of larval development.

The results were also in direct conformity with the findings of Mendiola-Olaya et al. (2000) who observed protease activity at highest value in the second larval instar of P. truncates. The enzyme activity in second larval stage was more than third instar and only a little activity was detected in pupal stage. Thus, high protease activity in early larval stage and especially decreasing activity with developing insects may depend on feeding behavior, as lepidopteran larvae have a long midgut and food immediately pass from mouth to midgut, the digestive enzymes works on it and after digestion absorbance also occurs simultaneously in midgut (Nation 2000). Our next strategy was to characterize the biochemical interaction of PIs with the S. litura gut proteinases so as to select candidate PIs from rice bean seeds that are capable of inhibiting the dynamic combination of gut proteinases of this insect. The inhibitory potential of the purified protease inhibitors revealed more than 80 per cent inhibition of trypsin and around 69 per cent inhibition of chymotrypsin activity. The inhibition in proteolytic activities is attributed to enzyme - inhibitor interaction (Broadway 1995). PIs reduce the quantity of proteins that can be digested, and also cause hyperproduction of the digestive enzymes which enhances the loss of sulfur amino acids as a result of which, the insects become weak with stunted growth and eventually die of starvation.

Likewise, in two other lepidopterans, Heliothis zea and S. exigua, Broadway and Dufley (1986a) hypothesized that massive overproduction of the protease inhibitor-insensitive enzyme, which meant that essential amino acids were no longer available for the production of other proteins, causes reduction in the protease activities and also reduction in the growth of larva. The inhibitory potential of rice bean protease inhibitor was higher than that observed in Dimorphandra mollis seed trypsin inhibitor (DMTI) which showed 67% inhibition among bruchid (Callosobruchus maculatus) (Macedo et al. 2002). Since rice bean protease inhibitor is effective against both trypsin and chymotrypsin protease, it could generate a better approach to combat the overall growth and developmental physiology of insect larvae. Previous studies on insect protease-protease inhibitors interaction have reiterated in the fact that the best inhibitory effects are obtained with an inhibitor with multiple inhibitory activities (Babu et al. 2012). Similar results were also reported by Sadaati and Badani (2011) that a significant decrease in tryptic activity in digestive tract extracts of Helicoverpa armigera fed with a diet containing soybean trypsin inhibitor paved the beginning of the strategy adopted by insects to counter the deleterious effects of protease inhibitors. An emerging trend emphasizes that the leaf chewing lepidopterans induce not only over production of PI insensitive protease of same class as that of the inhibitor but may also switch over to alternate pathways involving enzymes with the same mechanistic class but with a swapped substrate specificity class. Therefore, it may be possible that the down regulation of tryptic activity by one type of serine protease inhibitor is compensated by an upregulation of another class of serine proteases.

The feeding experiments, revealed the potency of rice bean protease inhibitor in response to larval digestive enzymes after incorporating the rice bean flour to the artificial diet as the adverse effects were significant at higher concentrations of PIs. The results revealed that addition of rice bean flour in the diet had a significant effect on mortality rate of Spodoptera litura. Qin et al. (2004) also observed total larval mortality of S. litura larvae after eight days of
feeding on cowpea (Vigna radiata) flour, which was four days longer than observed in the present study. Similarly, Elbadry et al. (2009) observed total larval mortality of Spodoptera after nine days of feeding on sweet potato (Ipomoea batatas) and cotton (Cocos nucifera). PIs of the kind reported in this paper would serve the objective of inhibiting larval growth, thereby reducing the crop damage. Thus, tandem use of rice bean trypsin inhibitor to develop transgenic crop plants could lead to sustainable resistance against Spodoptera litura.

CONCLUSION

The salient findings of the study emerged out to be decisive and have some practical utility for crop improvement by conferring resistance to plants against insect pests attack. The analysis of the purified inhibitor on SDS-PAGE under reducing conditions showed a single band protein (24 Da). A comprehensive investigation on the stability of trypsin inhibitory activity showed that maximum trypsin inhibitory activity was at 37°C (65.50%) and lowest at 100°C (35.30%). The inhibitor was more stable over a wide range of acidic pH than in alkaline solutions. The stability of rice bean protease inhibitor in acidic pH suggest its efficacy in controlling a variety of phytophagous insects as larvae of lepidopterans have acidic conditions in their midgut region, with pH optima for digestive enzymes typically in the range of 4.0–5.0. The metabolic interaction results showed that maximum azocaseinolytic (1.93U), trypsin (0.66 U) and chymotrypsin activity (0.37 U) was observed in second larval instar which gradually decreased up to late fifth larval instar stage. This turn down may be a consequence of degradation or a lower synthesis of digestive proteases produced by a quantitative decrease of the feed intake when a larva is near to the next molt stage. Bioassay revealed that rice bean trypsin inhibitors are strong inhibitor of Spodoptera litura. Feeding experiments on Spodoptera litura larvae and Callosobruchus maculatus showed PIs adversely affects the protein intake at the larval stage, which caused developmental abnormalities and increased mortality of insect. The disruption of amino acid metabolism by the inhibition of protein digestion by the PIs is the basis of PI-based defense in plants; however, in nature, it might be coupled with other factors. The adverse effects on insect gut proteases were significant at higher concentrations of PIs. Significant mortality of larvae was also apparent. From the foregoing account, it is evident that rice bean protease inhibitors have expediency and potential which can be exploited, to develop genetically modified plants with resistance to this insect.

REFERENCES

Ahmad, M., Arif, M.I. and Ahmad, M. (2007). Occurrence of insecticide resistance in field populations of Spodoptera litura (Lepidoptera: Noctuidae) in Pakistan. Crop Prot. 26: 809-817.

Alarcon, F. J., Martinez, T.F., Barranco, P., Cabello, T., Daz, M. and Moyano, F.J. (2002). Digestive proteases during development of larvae of red palm weevil, Rhynchophorus ferrugineus (Olivier, 1790) (Coleoptera: Curculionidae). Insect Biochem. and Mol. Biol. 32:265–274.

Babu, S.R., Subrahmanyam, B., Srinivasan and Santha, I.M. (2012). In vivo and in vitro effect of Acacia nilotica seed protease inhibitors on Helicoverpa armigera (Hübner) larvae. J. of Biosci. 37(2): 269-276.

Broadway, R.M. and Dufley, S.S. (1986a). Plant protease inhibitors: mechanisms of action and effect on the growth and physiology of larval Heliothis zea and Spodoptera exigua, J. of Insect Physiol. 32:827-834.

Broadway, R.M. (1995). Are Insects resistant to plant protease inhibitors? J. of Insect Physiol. 41: 107-116.

Chang, C.R. and Tsen, C.C. (1981). Isolation of trypsin inhibitors from rye, triticale and wheat samples Cereal Chem. 58:207-210.

Charity, J.A., Anderson, M.A., Bittisnich, D.J., Whitecross, M. and Higgins, T.J.V. (1999). Transgenic tobacco and peas expressing a transgene for mammalian trypsin inhibitor have increased insect resistance, Mol. Breeding. 5(4):357-365.

Chitra, L. and Sadasivam, S. (1986). Studies on trypsin inhibitor of black gram [Vigna mungo (L.) Hepper] Food Chem. 21(4): 315-320.

Elbadry, E.A., Abdel-Salam, F.A., Abo Elghar, M.R., Hassan, S.M. and Asal, M.A. (2009). The Effect of Four Different Host Plants on the Larval Development of the Cotton leafworm Spodoptera littoralis (Bois.) J of Applied Entomol. 68: 138-142.

Erlanger, B., Kokowsky, N. and Cohen, W. (1961). The preparation and properties of two new chromogenic substrates of trypsin. Arch. of Biochem. and Biophys. 95: 271–278.

Ferrasson, E., Quillien, L. and Gueguen, J. (1997). Protease inhibitors from pea seeds: purification and characterization. J. of Aigr. and Food Chem. 45 (1): 127-131.

Ferry, N., Edwards, M. G., Gatehouse, J.A. and Gatehouse, A.M.R. (2004). Plant-insect interactions: molecular approaches to insect resistance. Current Opinion in Biotech. 15:1–7

Haq, S.K. and Khan, R.H. (2003). Characterization of a proteinase inhibitor from Cajanus cajan (L.). J. of Protein Chem. 22: 543-554.

Klomklao, S., Benjakul, S., Kishimura, H. and Chaijan, M. (2011). Extraction, purification and properties of trypsin inhibitor from Thai mungbean [Vigna radiata (L.) R. Wilczek] Food Chem. 129: 1348–1354.

Kowalska, J., Pszczola, K., Wilmowska-Pelc, A., Lorenc-Kubis, I., Zuziak, E., Lugowski, M., Legowska, A., Kwiatkowska, A., Sleszynska, M., Lesner, A., Walewska, A., Zablotna, E., Rolka, K. and Wilusz, T. (2007). Trypsin inhibitors from the garden four o’clock (Mirabilis jalapa) and spinach (Spinacia oleracea) seeds: Isolation, characterization and chemical synthesis. Phytochemistry. 68: 1487-1496.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage. Nature. 277: 680–685.
Lowry, O.H., Rosebrough, N.J., Fan, A.L. and Randall, R.J. (1951). Protein measurement with Folin phenol reagent. J. of Biol. Chem. 193: 256–275.

Macedo, M.L.R., Mello, G.C., Md, G.M., Freire, J.C., Novello, S., Marangon, I S. and de Matos, D.G.G. (2002). Effect of a trypsin inhibitor from Dimorphandra mollis seeds on the development of Callosobruchus maculatus. Plant Physiol and Biochem. 40: 891-898.

Maggo, S., Malhotra, S.P., Dhawan, K. and Singh, R. (1999). Purification and characterization of protease inhibitor from rice bean (Vigna umbellata). J of Plant Biochem. Biotech. 8: 61-64

Mendiola-Olaya, E., Valencia-Jimenez, A., Valdes-Rodriguez, S., Delano-Frier, J. and Blanco- Labra A. (2000). Digestive amylase from the larger grain borer, Prostephanus truncates Horn, Compar. Biochem. and Physiol. Part B, 126: 425-433.

Nation, J.L. (2000). Insect Physiol. and Biochem. CRC Press p: 485.

Paiva, P.M.G and Coelho, L.C.B.B. (1992). Purification and partial characterization of two lectins isoforms from Cratylia mollis Mart. (Camaratu Bean). Applied Biochem. and Biotech. 36 (2): 113-118

Qin, H.G., Ye, Z.X., Huang, S.J., Ding, S.J. and Luo, J. (2004). The correlations of the different host plants with preference level, life duration and survival rate of Spodoptera litura Fabricius. Chinese J. of Eco-Agric. 12(2):40-42.

Richardson, M.J. (1991). Seed storage proteins: The enzyme inhibitors. In: Richardson, M.J. ed. Methods in Plant Biochem., New York, Academic Press, 259.

Saadati, F. and Bandani, A.R. (2011). Effects of serine protease inhibitors on growth and development and digestive serine proteinases of the Sunn pest. Eurygaster integriceps. J of Insect Sci. 11(72): 1-12.

Satheesh, S.L. and Murugan, K. (2012). Protease inhibitors from Coccinia grandis (L.)Voigt. Leaves: purification, characterization and kinetic properties. Int J of Pharmacy and Pharmac Sci. 4(1):565-573

Shorey, H.H. and Hale, R.L. (1965). Mass-Rearing of the larvae of Nine Noctuid species on a simple artificial medium J of Econ. Entomol. 58:522–524.

Srinivasan, A., Giri, A.P. and Gupta, V.S. (2006). Structural and functional diversities in lepidopteran serine proteases. Cellular and Mol. Biol Letters. 11(1): 132-154.

Telang, M.A., Giri, A.P., Sainani, M.N. and Gupta, V.S. (2005). Elastase like proteinase of Helicoverpa armigera is responsible for inactivation of a proteinase inhibitor from chickpea J of Insect Physiol. 51:513–522.