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Evaluation and characterization of trypsin inhibitor from rice bean with inhibitory activity against gut proteases of *Spodoptera litura*

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Abstract: Trypsin inhibitor (TI) in rice bean (*Vigna umbellata*) varied spatio-temporally in different parts of the plant, with the highest level (30.9 mg/g d.w.) noted in the maturing seeds of genotype BRS-2 at 160 days after planting (DAP). The TI from rice bean seeds was isolated and purified approximately 182-fold, with a final yield of 29% using ammonium sulfate precipitation, ion exchange chromatography through DEAE-Sepharose, gel permeation through Superdex-75, and finally by affinity chromatography using a trypsin-Sepharose column. The purified TI showed a single band on SDS-PAGE under reducing conditions with an apparent molecular mass of 24 kDa. The highest activity of purified inhibitor (about 90%) was recorded at pH 4.0 at 37 °C, suggesting the stability of the inhibitor under acidic conditions. The TI exhibited an inhibitory effect against *Spodoptera litura* larvae. A progressive decline in larval weight, growth, and survival rate of larval development was observed after feeding *S. litura* larvae on a diet supplemented with increasing concentrations of rice bean TI. The highest TI content in the seeds nearing maturity correlates to the role of TIs in protecting against insect pests. The study clarifies the role of rice bean protease inhibitors as a potential strategy against insectpests of economic importance.

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1 Introduction

Insect pest menace is one of the major factors destabilizing crop productivity in agricultural ecosystems, and is responsible for severe reduction in crop yields, despite the extensive use of chemical pesticides [1]. Many plants have developed a certain degree of resistance against insect pests through the production of defense compounds [2]. Leguminous plants are the second most important food source for animals and humans and are known to produce protease inhibitors (PI) that may play defense roles in these plants (Boulter et al. 2010; [3, 4]).

Protease inhibitors are the largest class of proteins to have undergone extensive investigations; consequently, their structure, properties, function, and metabolism have been well documented [5–8]. Production of proteinaceous inhibitors that may interfere with the digestive biochemistry of insect pests is one of the naturally occurring defense mechanisms in plants, which adversely affects protein digestion, causes reduction in the availability of essential amino acids, and exerts physiologic stress on the insect leading to growth retardation [9]. Protease inhibitors have been involved in the protection of plants against pests and possibly pathogenic microorganisms [10], making them one of the best means that can be used in crop protection strategies [11]. The majority of PIs studied in the plant kingdom originate from three main families: Fabaceae, Solanaceae, and Poaceae [12, 13]. Leguminous plant seeds usually contain two major types of serine PIs – Bowman-Birk type and the Kunitz type [14, 15]. While [16] have isolated Bowman-Birk type trypsin inhibitor (TI) from rice bean with a 29% recovery, the present study encompasses the isolation and characterization of Kunitz type rice bean PIs.

The pests, the majority of which belong to Lepidoptera family, have become a serious threat to many important crops and claim a major share in crop losses every

year [17]. Among them, *S. litura* has a large host range of more than 120 plant species. Several outbreaks of this pest on cotton, tobacco and chillies have been reported, resulting in 25.8% to complete losses depending on the stage of crop level of infestation [18]. The pest is resistant to many insecticides [19, 20] including new chemical insecticides such as lufenuron [21]. The persistent use of synthetic pesticides on pests and their subsequent impacts on ecologic imbalance [22] demands sustainable alternatives [23]. Several studies have demonstrated that PIs might provide adequate protection against a variety of economically important Lepidopteran insects [4]. Identification, purification, and characterization of such anti-metabolites from potential sources are imperative for their exploitation in plant breeding programmes as well as in production of transgenic plants [17].

Vigna umbellata (Thunb.), a multipurpose crop, commonly known as climbing bean, kidney bean. and mambi bean, is one of the summer legumes grown under diverse conditions ranging from marginal lands to rain-fed and drought-prone areas [24]. It is a native of South East Asia and is mainly grown in Malaysia, China, USA, Australia, Sri Lanka, Nepal, and India. Rice bean seeds have remarkable storage characteristics and remain unaffected from storage pests for a longer time compared to other legumes of the genus *Vigna* [17, 25]. Different anti-metabolic substances have been reported from rice bean, which inhibit the digestive proteases of insect-pests [26, 27]. This has invited a comprehensive investigation of anti metabolites in different parts of the rice bean plant at different stages of growth and development to assess their inhibitory potential against insect gut proteases, while assessment of natural variance for TI in different landraces will provide an insight into potential superior genotypes. In view of the above background the present investigation was undertaken to evaluatei and characterize TI from rice bean and studies on its inhibitory potential against gut proteases of *S. litura* larvae.

2 Materials and methods

2.1 Materials

Rice bean germplasm: For the present study, seeds of 12 different rice bean genotypes *viz*., JCR-54, JCR-163, IC-137194, IC-140795, IC-140808, IC-140798, IC-137200, LRB-141, LRB-176, EC-48223B, Baroi, and BRS-2 were procured from the Biochemistry Laboratory, Department of Crop Improvement, CSK HPKV, Palampur, India. The crop was raised in a green house at 27 °C temperature and 12–14 h of light per day. For the assessment of TI, the leaves, tendrils, and stem were sampled 105 days after planting; sampling of intact pods and seeds was carried out at intervals of 105, 120, 135, 145, and 160 days after planting. All the experiments were carried out in triplicates and the values appearing in the tables are the mean of three experiments. Rice bean seeds were sown in 8.0 cm dia. earthen pots. The pots were filled to about 0.75 cm from the top with Soilrite supplied by Kelperlite, Banglore, India, which consisted of peat moss, perlite and vermiculite in 1:1:1 proportion. Seven seeds were sown in each pot, which were kept in a glasshouse under controlled conditions. No fertilizer was added and plants were irrigated with tap water throughout the experiment.

2.2 Chemicals

All chemicals such as glacial acetic acid, trypsin, benzoyl-DL-arginine-*p*-nitroanilide (BApNA), N-benzoyl-DL-tyrosine-*p*-nitroanilide (BTpNA), DEAE-Sepharose, Sephadex G-75 and Tris-HCl were procured from Sigma-Aldrich (St Louis, MO, USA). Acrylamide, bisacrylamide and SDS were procured from Merck (Bangalore, India).

2.3 Estimation of trypsin inhibitor content

Trypsin inhibitor content was estimated in different parts (stem, leaf, tendril, pod, seeds) of the rice bean plant. The samples were defatted and depigmented with several washes of hexane and acetone. For each sample, 0.2 g powder was extracted with 25 mL 0.1 M potassium phosphate buffer pH 7.5 by grinding it in a prechilled mortar and pestle and placed at 4 °C. The homogenate was centrifuged at 13,000 *g* for 20 min at 4 °C. The supernatant obtained was used for determining TI activity.

The TI assay was carried out with a slight modification in the method described by [28] by using BApNA as substrate and bovine trypsin as standard enzyme. Duplicate sets of 0–1 mL of the purified fraction were transferred to test tubes – one to serve as control and the other as the test. Volume was raised to 2 mL with 0.1 M Tris HCl buffer (pH 8.2) in the blank and 1 mL in the test set. One mL of trypsin enzyme solution (20 μg) was added to each tube in the test set. All the tubes were incubated in a water bath at 37 °C and after a few minutes, 2.5 mL of substrate (1 mg BApNA) was added to each tube. The solutions were again incubated for 10 min at 37 °C and the reaction was stopped by adding 30% glacial acetic acid. For each sample, a separate standard and blank were run simultaneously. Absorbance was recorded at 410 nm. One unit of activity corresponds to that amount of TI in μg protein that provides 50% inhibition of enzyme activity under experimental conditions. The TI activity is expressed in trypsin inhibitory units (TIU) per milligram protein using the following equation:

$$\text{TIU mg/g of samples} = \frac{\text{differential absorption} \times \text{dilution factor}}{0.019 \times 1000}$$

2.4 Purification of rice bean protease inhibitor

Rice bean seeds were thoroughly cleaned and ground using a Willey mill to pass through a 2 mm mesh and stored in air tight bags. The seed powder (50 g) was homogenized in 200 mL 0.1 M phosphate buffer pH 7.5 at room temperature (25 °C) for 4 h, filtered, and then centrifuged at

10,000 *g* for 15 min. The supernatant obtained was precipitated overnight with 80% saturated ammonium sulfate. 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 frozen to solidify, followed by lyophilization for concentration and purification. The concentrated extract was loaded onto a DEAE-Sepharose column (40.7 × 2.0 cm), equilibrated with the 0.1 M phosphate buffer pH 7.5. Stepwise elution was carried out with a gradient of 0.1–0.5 M NaCl in 0.1 M phosphate buffer (pH 7.5). Fractions (3 mL) were collected with a flow rate of 0.5 mL/min. The eluted fractions were analyzed for protein content and TI activity. The active fractions with high trypsin inhibitory activity were pooled and were used for electrophoresis. A freeze-dried sample collected after passing through the DEAE-Sepharose column was dissolved in 0.1 M phosphate buffer pH 7.5 and applied to a Sephadex G-75 column (22.5 × 1.5 cm; flow rate 0.5 mL/min) for gel permeation chromatography. The active fractions were further purified by trypsin-Sepharose affinity chromatography for which the column was prepared with a slight modification in the method described by [29] and [30]. The pass-through fractions collected from the Sephadex G-75 column were applied to a column of CNBr activated Sepharose (0.9 cm × 14 cm) for further purification. Fractions 1.5 mL each were eluted at a flow rate of 0.5 mL/min with 0.05 M Tris-HCl buffer pH 7.8. The active fractions (465 mg/mL protein) with trypsin inhibitory activity were pooled and stored for further analysis.

2.5 Electrophoretic analysis

The purity of purified TI was checked by polyacrylamide gel electrophoresis using 12.5% acrylamide gels as described by [31].

2.6 Estimation of protein content

The protein content was determined per the method of [32] using bovine serum albumin (BSA) as the standard protein.

2.7 Insect rearing

Culture stocks of *S. litura* (second instar) larvae were procured from Biological Control Lab, CSK HPKV (Palampur, India). Larvae of *S. litura* were reared on an artificial diet at standard conditions – of 27 ± 5 °C, 75 ± 5% relative humidity as described earlier by [33]. The artificial diet comprised soaked mungbean (15 g), baking yeast (1 g), sorbic acid (0.15 g), ascorbic acid (0.3 g), casein (0.5 g), agar (1.4 g), vitamin stock (3 g/1000 mL) (mixture of niacin, calcium pantothenate, thiamine, riboflavin, pyridoxin monohydrochloride, folic acid, biotin, vitamin B_{12}), 40% formalin, and distilled water. The final composition of the test diet was made 25, 50, 75 and 100% (v/w) with purified rice bean inhibitor having 465 mg/mL protein. The control was an artificial diet only.

2.8 Mid-gut luminal enzyme preparation from *S. litura*

Spodoptera larvae were anesthetized by exposing them to diethyl ether for a few seconds and the digestive tracts were dissected using ice cold iso-osmotic saline (0.15 M NaCl) [26]. They were cleaned of unwanted adhering tissues and the gut luminal contents were collected into a prechilled microfuge tube. A total of 8–10 clean midguts/larval instar stage were extracted by homogenizing the tissue with its contents in an equal volume (5 mL) of ice cold distilled water. The gut luminal contents were removed by centrifugation at 11,000 *g* for 20 min at 4 °C. The resultant supernatant was then filtered and was designated as midgut luminal enzyme preparation. Frozen supernatant was divided into aliquots. The samples were stored at –20 °C and were used to study the inhibitory potential of rice bean PI against *S. litura* gut proteases.

2.9 Gut protease activity and inhibition assays

Protease activity was measured by slight modification in the method published by [34]. Total gut protease activity was determined using the chromogenic substrate azocasein at a final concentration of 2.0%. For the assay, 60 µL of enzyme extract was added to 200 µL of 1% azocasein (in distilled water) and incubated at 37 °C for 30 min. The reaction was terminated by the addition of 300 mL of 5% trichloroacetic acid. After incubation at room temperature for 30 min, tubes were centrifuged at 10,000 *g* for 10 min. An equal volume of 1N NaOH was added to the supernatant and activity was estimated by measuring the optical density (OD) at 450 nm. Specific gut protease trypsin and chymotrypsin activity were measured with BApNA and BTpNA as synthetic substrate, respectively. 1.35 mL of 0.1 M Tris-HCl buffer (pH 10.0) was incubated with 30 µL of gut extract for 10 min at 37 °C. 200 µL of substrate BApNA (8 mM) and BTpNA (1 mM) was added to the reaction mixture for trypsin and chymotrypsin activity, respectively. Samples were incubated at 37 °C for 10 min. Assays were run in triplicates with appropriate blanks. The reaction was terminated by addition of 750 µL of 30% acetic acid and OD was measured at 410 nm.

Proteinase activity was calculated as µmol *p*-nitroaniline produced per minute. The molar extinction coefficient of 8800 (M^{-1} cm^{-1}) for *p*-nitroanilinide (pNA) at 410 nm [35] was taken into account to calculate trypsin and chymotrypsin-like activity (BApNA/BTpNA units/mg protein) using the formula:

$$\text{Activity units} = \frac{\text{Abs}_{410}/\text{min} \times 1000 \times \text{mL of reaction mixture}}{\text{Extinction coefficient of chromogen} - \text{mg protein in reaction mixture}}$$

For inhibitory activity, 1.35 mL of the purified rice bean protease inhibitor was added to 30 µL of gut extract for 10 min at 37 °C. 200 µL of substrate BApNA (8 mM) and BTpNA (1 mM) was added to the reaction mixture for trypsin and chymotrypsin inhibitory activity, respectively. Tubes were incubated at 37 °C for 10 min. The reaction was terminated by addition of 750 µL of 30% acetic acid and OD was measured at 410 nm. The residual proteinase inhibitory activity was estimated as described above. In all the assays, blanks without sample were run simultaneously. One TI unit was defined as the amount of inhibitor that causes inhibition of one unit of proteinase activity under given assay conditions.

2.10 pH and thermal stability of rice bean TI

The pH stability of the rice bean TI was estimated by incubating aliquot of the purified inhibitor for 45 min at room temperature using

buffers of varying pH. The buffers used in the indicated pH range included glycine-HCl buffer (pH 2.2), acetate buffer (pH 4.0), phosphate buffer (pH 7.5), Tris-buffer (9.0) and phosphate-NaOH (pH 11.0–12.0), each at a concentration of 50 mM. The residual TI activity was measured using assay as described above. For the thermostability the inhibitor in 0.1 M phosphate buffer (pH 7.5) was first incubated at 20, 40, 80, 90 or 100 °C for 45 min. The inhibitor was then added to the assay mixture (described above) and the incubation was continued. The OD was measured at 410 nm and used to calculate TI activity.

3 Results and discussion

3.1 Variation in TI activity in different parts of the rice bean plant

Twelve different rice bean genotypes were investigated for TI activity. Trypsin inhibitor activity was estimated in the leaves, tendrils, and stem 105 days after planting (DAP). The trypsin inhibitor content varied from 13.9 to 15.3 mg/g in rice bean leaves. Highest TI activity in the leaves was observed in genotype IC-140795 followed by genotype JCR-163. Minimum TI content was observed in genotype Baroi. In tendrils, the TI content varied from 17.1 to 18.1 mg/g. Highest TI content in tendrils was recorded in LRB-141, whereas genotype EC-48223B had the lowest TI content. As compared to tendrils and leaves, TI content was higher in the stem, ranging in value from 18.4 to 19.7 mg/g. Highest TI in stems was observed for genotype IC-137194 followed by IC-137200; minimum TI content was observed in genotype IC-140808 (Supplementary Table 1).

3.2 TI content in intact pods, developing and mature seeds of different rice bean genotypes

In legumes, the seed is the economically most important structure generally targeted by an insect pest. We assessed the TI content in intact pods as well as in seeds during different growth stages. An increasing trend in inhibitor content from 105 to 160 DAP was observed, particularly in the seeds. In the intact pods, the highest TI content was observed 145 DAP, ranging from 15.9 to 16.9 mg/g followed by 135 DAP (14.5–15.7 mg/g) and 105 DAP (6.1–7.9 mg/g). In intact pods, the highest TI content was observed in genotype IC-140808 followed by EC-48223B (16.9 mg/g), whereas, the lowest TI content was observed in IC-140798 at 145 DAP. The highest TI content in seeds was observed 160 DAP ranging from 27.4 to 30.6 mg/g (Supplementary Table 2) followed by 145 DAP (16.6–18.6 mg/g), 135 DAP (9.9–10.6 mg/g), and 105 DAP (8.7–10.0 mg/g). Maximum TI content was observed in seeds close to maturity in genotype BRS-2 (30.6 mg/g) at 160 DAP.

The inhibitor content increased with seed development and accumulated to its highest level in seeds nearing maturity at 160 DAP. The stem, tendrils, and intact pods revealed decreasing TI contents as compared to the seeds, with lowest levels recorded in the leaves. Inhibitor content was recorded in all the tissues. In the seeds, the TI content was comparatively high even at early

Table 2: Activity of rice bean trypsin protease inhibitor against *S. litura* larval gut proteases.

Set no.	Larval instar	Trypsin inhibition (%)*	Chymotrypsin inhibition (%)*
1	First	$78.46^{d} \pm 0.9$	$71.23^{e} \pm 1.2$
2	Second	$79.51^{d} \pm 1.6$	$73.25^{d} \pm 1.0$
3	Third	$97.08^{a} \pm 1.5$	$80.56^{a} \pm 1.4$
4	Fourth	$89.39^{b} \pm 1.7$	$79.23^{b} \pm 0.6$
5	Early fifth	$82.27^{d} \pm 2.1$	$78.12^{e} \pm 0.7$
6	Late fifth	$79.45^{c} \pm 1.5$	$75.63^{c} \pm 0.6$

*Data are reported as mean ± standard error of the mean of three independent determinations and are expressed as% inhibitory activity relative to control. Means with the same letter are not significantly different and are at par (one way Annova followed by post hoc testing using Duncan's multiple range tests. A significant level of 0.05 was used for all tests; p=0.05).

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

Step	Volume (mL)	Total activity (TIU)	Protein (mg)	Specific activity	Fold purification	Yield (%)
Crude extract	100	42,806	4811	8.9	1	100
$(NH_4)_2SO_4$ ppt	50	30,211	1080	27.9	3.14	70.6
Ion exchange chromatography	40	22,198	49	483.0	50.95	51.8
Gel filtration	35	16,123	25.5	632.3	71.12	37.6
Affinity chromatography	14	10,491	6.5	1614.0	181.55	24.5

stage of seed development. The changes in TI content are consistent with the overall metabolic changes during seed development that favor net accumulation of seed reserves, including storage proteins, from early stages till seed maturation.

Among the different genotypes, the highest inhibitor content was observed 160 DAP in mature seeds of genotype BRS-2 (30.9 mg/g). The high TI content is the contributing factor for resistance against insect pests in rice bean seeds. The relatively low amount of TI in the leaves and tendrils of rice bean indicates a trivial role for TI of in the regulation of plant proteases during plant growth. However, the high TI content in the seeds of most rice bean genotypes demonstrate a regulatory and defensive role for the trypsin inhibitor in rice bean [36].

Ryan et al. [37] observed that the concentration of TI trypsin inhibitor content in plant tissues during different developmental stages is influenced by meristematic growth and was highest at a time just preceding new growth. This indicates that these inhibitors might be closely associated with internal changes in the plant, which are necessary for the initiation of new growth or may be a major defensive effort by plant systems to protect their newly developing tissues from pests. The high levels of TI in the seeds as compared to other tissues ensures protection of seeds from storage insect pests. The quantitative differences in the inhibitor proteins among genotypes are likely due to inherent genotypic differences.

3.3 Isolation and purification of protease inhibitor from rice bean

The results from the previous section revealed that genotype BRS-2 had the highest TI content. Therefore, the same genotype was selected for further isolation and purification of TI. Rice bean seed powder was extracted with different extraction media *viz.*, 0.001 N NaOH, distilled water and 0.1 M phosphate buffer (pH 7.5). Because the highest TI activity was observed when the extraction was carried out in 0.1 M phosphate buffer, further extractions were carried out in this buffer.

Trypsin inhibitors from the mature seeds of genotype BRS-2 of rice bean were purified to homogeneity with 181.5-fold purification (Table 1) and 29% yield using a series of different chromatography columns (Figure 1). The specific activity increased from 8.9 in crude extract to 1614 units per mg of total protein after affinity chromatography. In ion exchange chromatography on a DEAE-Sepharose column, elution with a NaCl gradient (0.1 M–0.5 M) resulted in an elution profile that featured two peaks corresponding to 0.2 M and 0.4 M NaCl, respectively. The second broad peak was selected for further purification because of its high TI activity and its protein content, which was eluted by 0.3 M NaCl (Figure 1A). The active fractions of those possessing high TI activity were collected, concentrated in a freeze dryer, and loaded on to a Superdex-75 column for gel filtration chromatography. The inhibitory activity emerged as one major peak and all fractions within the peak exhibited high TI activity (Figure 1B).

Finally, pooled fractions from the Sephadex G-75 column were allowed to flow through CNBr-activated Sepharose-trypsin column and the active fractions with TI activity were collected (Figure 1C).

3.4 Electrophoretic analysis of rice bean TI

Electrophoretic analysis of the purified TI in the presence of a reducing agent (β-mercaptoethanol) revealed one sharp protein band with a molecular mass of approximately 24 kDa (Figure 2). This molecular mass is consistent with those of proteinase inhibitors from other species Proteinase inhibitors have molecular masses in the range from 21 to 26 kDa [38]. Trypsin PIs have been purified to homogeneity from the seeds of other legumes. The 13.5 fold with 30% yield purified trypsin inhibitor from mung bean [*Vigna radiata* (L.) R. Wilczek] seedshad a molecular mass of 14 kDa [39]. The inhibitor content and type in legume seeds may vary with many factors, including cultivar and stage of maturity [40]. Legume seeds normally contain trypsin and chymotrypsin inhibitors of either the Bowman-Birk (10–12 kDa) or the Kunitz family (21–26 kDa) [41]. The trypsin PI isolated from seeds of rice bean genotype BRS-2 is of the Kunitz type, as this category of inhibitors contains proteins of a molecular masses of more than 20.0 kDa [42]. [16] have also isolated a PI from rice bean with a molecular mass of 16.8 kDa, classifying it as a Bowman-Birk type inhibitorwith 29% recovery, as compared to nearly 71% of the Kunitz type PI in the present study (Table 1).

3.5 Thermal and pH stabilities of rice beantrypsin inhibitor

Incubation of the inhibitor in the pH range of 2.2–9.0 revealed maximal inhibitory activity at pH 4.0, followed by a decline at higher pH values (Figure 3A). According to [39], the inhibitor from mung bean seeds was stable in a wide pH range. [43] also reported that the trypsin

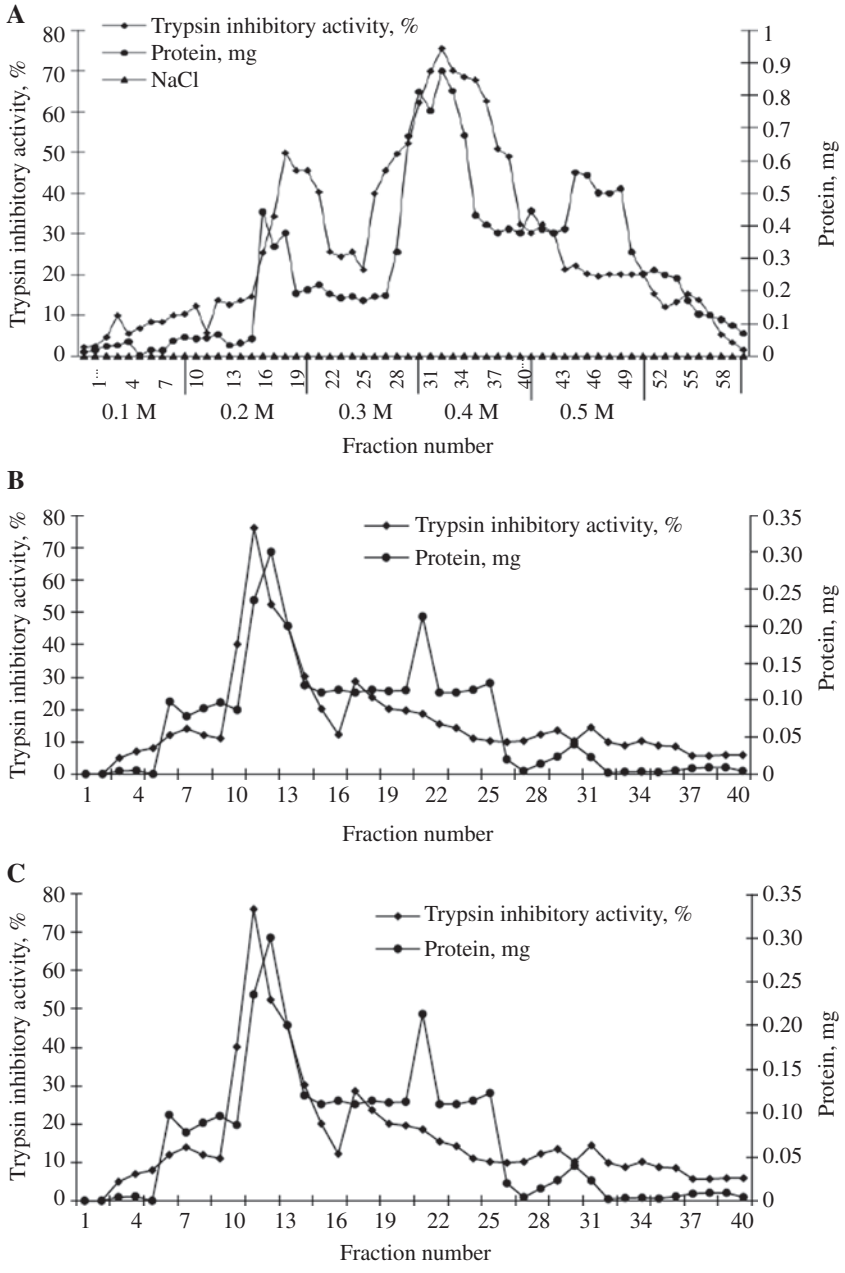

Figure 1: (A) Trypsin inhibitory activity (%) and protein content (mg) in DEAE- Sepharose chromatographic fractions eluted with a 0.1–0.5 M step NaCl gradient, (B) trypsin inhibitory activity (%) and protein content (mg) in Superdex-75 chromatographic fractions, (C) trypsin inhibitory activity (%) and protein content (mg) in Sepharose-Trypsin affinity chromatographic fractions.

inhibitor from mungbean seeds was active between pH 4.0 and 7.5. [44] also reported that inhibitors from pigeon pea and cowpearetained their activities over a wide range of pH. However, a decreased activity was observed at alkaline pH in the Bambara groundnut, while the TI purified from adzuki bean seeds was stable over the pH range of 4–10 [45].

Incubation of the rice bean TI in 0.1 M phosphate buffer (pH 7.5) at temperatures ranging from ambient to 100 °C for 45 min revealed that the inhibitor was stable up to 70 °C, but incubation above 70 °C resulted in decreased TI activity. Rice bean TI was maximally active at 37 °C (65%) but retained activity even at 100 °C (35%) (Figure 3B). [46] reported that PIs from legumes are quite stable up to 80 °C lose their activity at 100 °C. Similar results have been reported for the pigeon pea TIs [44]. The purified inhibitor from mung bean seeds was heat stable for up to 50 min at 90 °C [39].

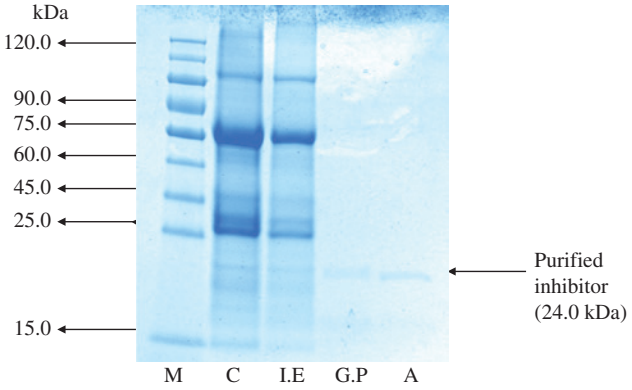

Figure 2: SDS-PAGE profile of different fractions during purification of protease inhibitor from rice bean.
M, Marker; C, crude extract; I.E, ion exchange chromatographic fractions; G.P, gel permeation chromatographic fractions; A, affinity chromatographic fractions.

The stability of the inhibitor at high temperatures may be attributed to its rigid and compact structure, which is stabilized by a number of disulfide linkages and extensive hydrogen bond networks [47]. Intramolecular disulfide bridges are presumably responsible for the functional stability of Kunitz-type inhibitors in the presence of physical and chemical denaturants such as temperature, pH and reducing agents. [48] also reported that TI from Brazilian soybean was denatured completely by heat treatment at 92 °C for 5 min.

3.6 Inhibitory activity of purified trypsin protease inhibitor from rice bean against *S. litura*

The defensive role of PIs is based on their inhibition of the digestive proteases of insects, resulting either in a critical shortage of essential amino acids or interfering with important biochemical or physiological processes of insects. The rice bean TI was evaluated for inhibitory activities against the larval gut proteases of *S. litura* (Table 2).

The rice bean TI strongly inhibited the gut proteases of *S. litura*. As TIs are also potent chymotrypsin inhibitors, the potential of rice bean TI was also checked against chymotrypsin [49]. Maximum trypsin and chymotrypsin inhibition was observed at the third larval stage (97% and 81%). followed by the fourth larval stage (89 and 79%). The inhibitory activity is in synchronization with the feeding activity and larval stage [50]. [51] reported that the protein concentration affects the protease activity. As larval developmental stages progress, the larvae become voracious feeders. This presumably accounts for the enhanced protein concentration in the gut and hence, the noted increase in TI activity. The protein concentration declines as the larvae gradually become pupae and give up food consumption. Such changes in gut proteolytic activity during development have also been demonstrated in larvae of *Bombyx mori*. [52] suggested that the efficacy of PIs depends on the affinity or specificity of an

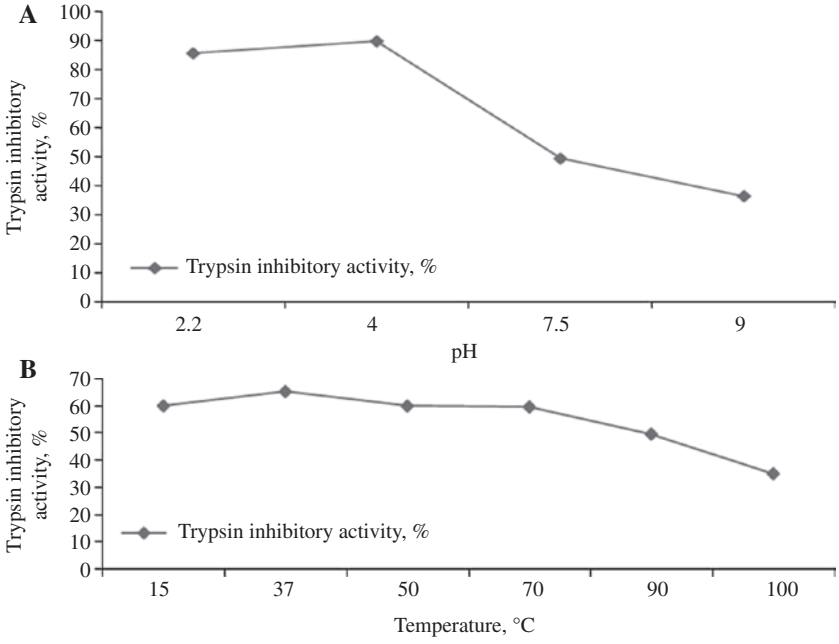

Figure 3: Effect of pH (A) and of temperature (B) on the activity of purified rice bean trypsin inhibitor. TI activity is defined as complete inhibition of trypsin activity.

inhibitor for the gut proteinases of insect. The inhibitory potential of rice bean TI was higher than that observed for the *Dimorphandra mollis* seed trypsin inhibitor (DMTI), which produced 67% inhibition in the bruchid *Callosobruchus maculates* [53]. Since rice bean trypsin PI s effective against both trypsin and chymotrypsin, it might better combat the overall growth and developmental physiology of insect larvae. Previous studies on insect protease-protease inhibitor interaction have corroborated the observation that the best inhibition of insect growth is achieved by inhibitors with multiple inhibitory activities [54].

4 Conclusions

PI activity in different tissues of rice bean has been evaluated. The highest TI content was observed in the seeds nearing maturity as compared to the other plant parts for genotype BRS-2 at 160 DAP. For intact pods, genotype IC-140808 had the highest TI content at 145 DAP, while for developing seeds it was highest in genotype JCR-54. The purified inhibitor is active in a wide range of temperature and pH. The TI inhibits both trypsin and chymotrypsin in the gut of *S. litura* larval instars. The study suggests that the TIs from rice bean could be useful in designing insect pest resistance in crop plants. It is likely that PI expressed and produced in higher amounts in agronomically important crops would lead to the development of resistance against a variety of polyphagous insects. In addition, the TI proteins often contain high levels of nutritionally essential amino acids that will add to the nutritional quality of the crop once it has become denatured during cooking.

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