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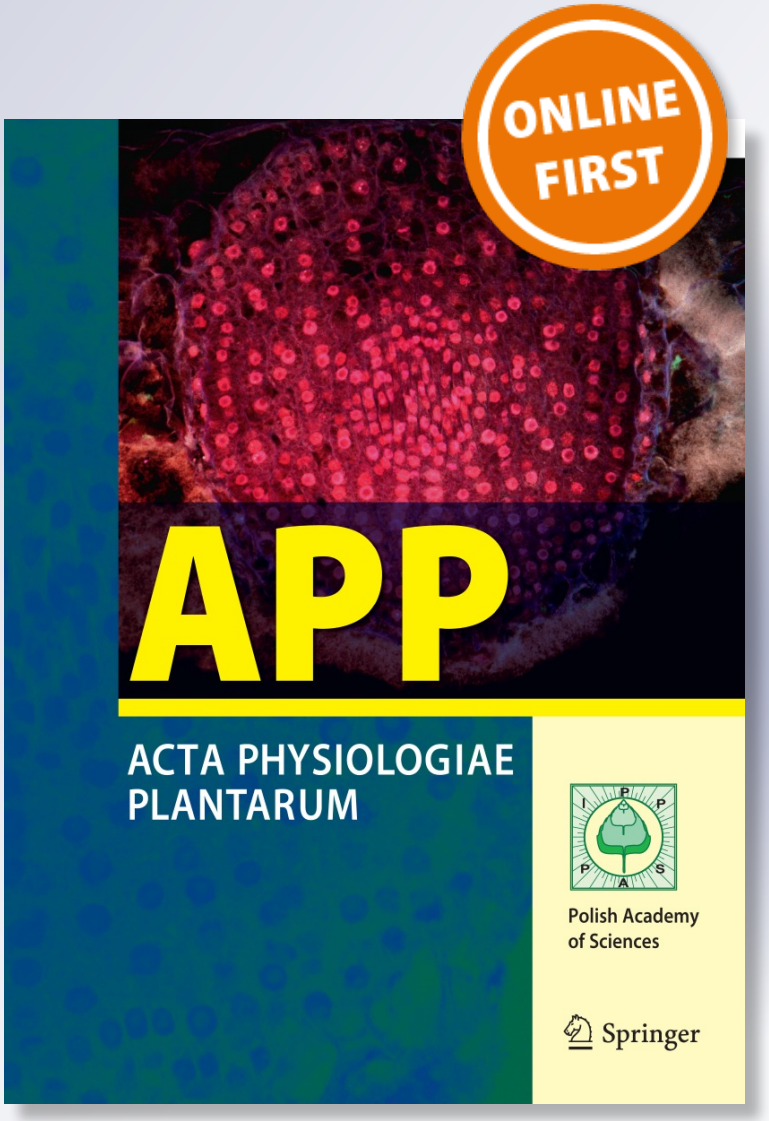

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ORIGINAL PAPER

A kidney bean trypsin inhibitor with an insecticidal potential against *Helicoverpa armigera* and *Spodoptera litura*

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Abstract In the present study, trypsin inhibitor extracts of ten kidney bean seed (*Phaseolus vulgaris*) varieties exhibiting trypsin and gut trypsin-like protease inhibitor activity were tested on *Helicoverpa armigera* and *Spodoptera litura.* Trypsin inhibitor protein was isolated and purified using multi-step strategy with a recovery of ~15 % and purification fold by ~39.4. SDS-PAGE revealed a single band corresponding to molecular mass of ~15 kDa and inhibitory activity was confirmed by reverse zymogram analyses. The inhibitor retained its inhibitory activity over a broad range of pH (3–11), temperature (40–60 °C) and thermostability was promoted by casein, $CaCl_2$, BSA and sucrose. The purified inhibitor inhibited bovine trypsin in 1:1 molar ratio. Kinetic studies showed that the protein is a competitive inhibitor with an equilibrium dissociation constant of 1.85 μM. The purified trypsin inhibitor protein was further incorporated in the artificial diet and fed to second instar larvae. A maximum of 91.7 % inhibition was obtained in *H. armigera*, while it was moderate in *S. litura* (29 %) with slight varietal differences. The insect bioassay showed 40 and 22 % decrease in larval growth followed by 3 and 2 days delay in pupation of *H. armigera* and *S. litura*, respectively. Some of the adults emerged were deformed and not fully formed. Trypsin inhibitor protein was more effective against *H. armigera* as it showed 46.7 % mortality during larval growth period compared to *S. litura* (13.3 %).

Keywords Kidney bean · Trypsin inhibitor · *Helicoverpa armigera* · *Spodoptera litura* · Artificial diet · Insect bioassay

Introduction

Plants are under constant attack by insect pests, and to produce inhibitors against the insect's gut proteases is one of the potent plant defense responses (Ryan 1973). Several studies have demonstrated that these inhibitor proteins are specifically produced in the plant upon biotic stress, protecting the plant tissue from damage (Ryan 1990; Tatyana et al. 1998). Some of these inhibitors include proteins such as protease inhibitors (PIs), amylase inhibitors, lectins and class of pathogenesis-related proteins (Ryan 1990; Tatyana et al. 1998). Plant PIs have been well established to play a potent defensive role against predators and pathogens. Developing resistance to pesticides in Lepidopteran insect pests is a significant economic, ecological and public health issue. Agricultural industries develop alternative chemical pesticides effective against these insect pests. Since the use of these chemical pesticides has a deleterious effect on human health, a recent trend is to use other safer strategies to enhance the defense mechanism of crops. Bate and Rothstein (1998) proposed a 'copy nature' strategy for insect pest

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control according to which PIs could be exploited to produce plants resistant against pests and pathogens.

PIs are present in significant amounts in storage systems of plants, especially legume seeds. There are two major types of protease inhibitors: the Kunitz type with a molecular weight of ~21 kDa consisting of 121 amino acids with 4 cysteine residues forming 2 intra-chain disulfide linkages and a single reactive site and the Bowman–Birk type with a molecular weight of 8 kDa consisting of 60–80 amino acids with 14 cysteine residues forming 7 disulfide linkages (Bhattacharyya et al. 2006). These inhibitors act on serine proteases such as trypsin, chymotrypsin and elastase (McNiven et al. 1992). Kunitz-type inhibitors primarily inhibit trypsin but also weakly inhibit chymotrypsin (Laskowski and Qasim 2000). In developing insect resistance in plants, PIs are the most exploited class of plant defense proteins (Jouanin et al. 1998). They are present in plant parts produced in enhanced levels in response to insect attack (Tamayo et al. 2000) and wounding (Zhao et al. 1996). When ingested by an insect, they result in starving of the insect for amino acids by inhibiting digestive proteases and thus retarding the growth and development (Giri et al. 2005). Plant PIs have multifold significance, for instance, signaling receptors interaction in animals and in plant defense against insect and pathogen attack (Ryan 1990), as bio-specific ligands (Graber and Condoret 1992), in digestion of proteins (Franco-Fraguas et al. 2003) and in the prevention of cancer, Dengue fever, and inflammatory and allergic disorders (Scarpi et al. 2004).

Lepidopteran insects are the destructive field pests of many important crops causing severe economic losses (Manjunath et al. 1989). Analysis of digestive proteases has revealed the presence of serine proteases, predominantly trypsin and chymotrypsin-like enzymes (Bown et al. 1998; Patankar et al. 2001). In polyphagous insects, diverse specificities and intricate changes in the expression of proteases are responsible for the inactivation of host plant and newly exposed PIs (Gruden et al. 2004). Hence, the identification of PIs having specificities towards different insect gut proteases with high binding efficiency is necessary for effective inhibition of midgut proteases. Such PIs may have direct relevance and application in the development of transgenic plants with insect tolerance trait.

Although PIs, which are effective against insect pests, have been isolated and characterized from various plant sources, yet the information on characterization and in vivo anti-feedant activity of TI from kidney bean seeds is scanty. Therefore, the present study was undertaken to purify and characterize the TI protein as well as to study the anti-feedant activity against Lepidopteran insects (*Helicoverpa armigera* and *Spodoptera litura*) under both in vitro and in vivo conditions.

Materials and methods

Seed material

Seeds of ten kidney bean (*Phaseolus vulgaris*) varieties viz., IC-260299; IC-260292; IC-262840; IC-260307; IC-262837; EC-589388; EC-590329; EC-590326; EC-572720 and EC-589468 were procured from NBPGR Regional Agricultural Research Station, Phagli, Shimla (Himachal Pradesh) for experimental purposes.

Insect cultures and diet

Helicoverpa armigera and *Spodoptera litura* for the isolation of gut protease and insect bioassay were obtained from the Division of Entomology, Indian Agricultural Research Institute, New Delhi, India, and maintained at 25 ± 1 °C, 60–70 % relative humidity with a 16-h day length. Insects were routinely maintained on rearing diet as described by Nagarkatti and Prakash (1974) modified by Kalia et al. (2001). Composition of the insect diet was as follows (for 450 ml distilled water): 84.0 g Bengal gram, 11.0 g dried yeast powder, 5.0 g casein, 3.0 g L-ascorbic acid, 2.0 g methyl-*p*-hydroxybenzoate, 1.0 g sorbic acid, 0.2 g streptomycin sulfate, 0.2 g cholesterol, 1.0 ml formaldehyde, 1 ml multivitamin drops and 11.0 g agar–agar. The same diet was used for both (*H. armigera* and *S. litura*) insects.

Chemicals

Acrylamide, bis-acrylamide, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), ammonium persulfate, bovine trypsin, *N*-α-benzoyl-L-arginine-*p*-nitroanilide (BApNA) were purchased from Sigma Chemical Company, St. Louis, MO, USA. Diethylaminoethyl (DEAE)-Cellulose, Carboxymethyl (CM)-Sephadex C-50, and Sephadex G-100 were procured from M/S Amersham Pharmacia, Germany. All other general chemicals used were of highest purity grade available commercially.

Extraction of trypsin inhibitor

Trypsin inhibitor (TI) protein was extracted following the protocol of Maggo et al. (1999) with some modifications. Defatted seed flour was shaken with 0.1 % NaCl (1:40 w/v) in a shaking water bath for 52 min at 60 °C. The suspension was then centrifuged at 10,000×*g* for 30 min at 4 °C and collected the supernatant (crude extract). Protein content was estimated following the Lowry's method (Lowry et al. 1951) and TI activity was determined as per the protocol of Hajela et al. (1999) with slight modifications.

Purification of TI

The crude extract of kidney bean seeds was used for inhibitor purification. The supernatant was subjected to ammonium sulfate precipitation (20–70 % saturation) and protein was allowed to precipitate overnight at 4 °C. The precipitated protein was collected by centrifugation at 10,000×*g* for 20 min at 4 °C and the pellet was resuspended in minimum volume of 0.05 M Tris–HCl buffer (pH 8.0) and dialyzed against the same buffer. The dialyzed sample was subjected to anion-exchange chromatography on DEAE-Cellulose (20 × 2.5 cm) at a flow rate of 60 ml h^{-1}. The column was first eluted with 0.05 M Tris–HCl (pH 8.0) buffer to wash out the unbound proteins. The unbound fractions were collected till the absorbance at 280 nm approached zero. The bound protein was eluted with linear concentration gradient of 0.0–1.0 M NaCl using a gradient mixer. The fractions with TI activity were pooled and then subjected to gel filtration chromatography on Sephadex G-100 (82 × 0.8 cm) at a flow rate of 15 ml h^{-1} and eluted with 0.05 M Tris–HCl buffer (pH 8.0). The active fractions eluted were pooled and followed by cation-exchange chromatography. The pooled fractions were loaded on the CM-Sephadex C-50 (15 × 1.0 cm) at a flow rate of 60 ml h^{-1}. The column was first eluted with 0.05 M Na-acetate buffer (pH 4.6) to wash out the unbound proteins. The bound proteins were eluted with linear concentration gradient of 0.0–1.0 M NaCl. After each step of chromatography, the unbound and bound fractions were assayed for the activities of TI; fractions containing the active inhibitor were pooled and dialyzed overnight against the succeeding column buffer. The pooled active fractions were used for further studies. A multi-step strategy has been employed for the purification of PI from other legumes as well (Maggo et al. 1999; Oliveira et al. 2007; Kansal et al. 2008a).

Estimation of TI activity

The activity of TI was assayed by determining the residual trypsin activity following the slightly modified method of Hajela et al. (1999) using BApNA as the substrate and bovine trypsin as the standard enzyme. The reaction mixture containing 50 µl diluted TI (seed extract), 50 µl trypsin (1 mg in 20 ml 0.05 M Tris–HCl, pH 8.0, containing 0.03 M $CaCl_2$) and 100 µl 0.05 M Tris–HCl, pH 8.0, containing 0.03 M $CaCl_2$ was incubated at 37 °C for 10 min in a shaking water bath. The residual activity was measured by adding 1 ml of BApNA (7 mg dissolved in minimum volume of DMSO and adjusting its final volume to 20 ml with 0.05 M Tris–HCl, pH 8.0, containing 0.03 M $CaCl_2$), incubating the reaction mixture at 37 °C for 10 min in a shaking water bath followed by termination of the reaction by adding 20 µl of 30 % (v/v) glacial acetic acid. A blank and a trypsin control were run simultaneously. In blank, acetic acid was added prior to BApNA, and in trypsin control, distilled water was added in place of the inhibitor. The absorbance was recorded at 410 nm against the blank using a UV–Visible spectrophotometer. One trypsin unit (TU) was defined as an increase of 0.01 absorbance units at 410 nm per 1.2 ml of the reaction mixture. TI activity was expressed as the number of trypsin units inhibited (TUI).

Electrophoretic analysis and inhibitory activity of TI by electrophoresis

The homogeneity of the purified protein was checked by native-PAGE (12 %) as per the protocol of Davis (1964) and SDS-PAGE (15 %) according to the method of Laemmli (1970). Protein bands were visualized by staining with Coomassie brilliant blue R-250.

Trypsin inhibitory activity staining was performed using casein as substrate based on the slightly modified method of Garcia-Carreno et al. (1993) and Klomklao et al. (2010b). The gels were washed in 2.5 % Triton X-100 for 15 min to remove SDS and renature the proteins. The gel was washed with distilled water before soaking in trypsin solution (0.2 mg/ml) at 0–4 °C for 45 min. The gels were then washed again with distilled water and incubated with 1 % casein in 0.1 M glycine–NaOH, pH 9.0 for 90 min at 37 °C. The gel was washed again with distilled water, fixed and stained with Coomassie blue R-250. After destaining, the bands with inhibitory activity were compared with the control gel and molecular weight markers.

High performance liquid chromatography

High performance liquid chromatography (HPLC) was performed using HPLC system from Shimadzu, fitted with LC-10 AT VP pump and a highly sensitive SPD-10 AVP UV detector. The reverse phase column (C-18, 5 µm) of 4.6 × 250 mm size (Cat No. 1.51456.0008) purchased from E. Merck, Germany, was used to check the purity and resolve the components of the pooled active fractions eluted from cation-exchange chromatography. The chromatographic solvents were of HPLC grade. The solvents were filtered through 0.22 µm filter (Millipore) and degassed before use. The mobile phase used for separation of sample components was acetonitrile–water in a ratio of 70:30. The column was washed with acetonitrile and water sequentially. After proper washing with acetonitrile, the column was equilibrated with acetonitrile–water (70:30). A volume of 10 µl of protein sample was injected into the loop of column with the help of HPLC loading syringe and eluted with acetonitrile–water at a flow rate of 1 ml min^{-1}.

The detector was set to read the absorbance at 280 nm and HPLC chromatogram was obtained by printing the data.

Biochemical characterization of purified protein

Determination of molecular weight

Molecular weight (MW) of the purified TI was estimated both by SDS-PAGE under reducing condition (with β-mercaptoethanol) and gel filtration chromatography through Sephadex G-100. In SDS-PAGE, the molecular weight marker proteins were co-electrophoresed with the purified protein to determine the molecular weight of the enzyme. The mobility of each protein was calculated as follows:

$$\text{Relative mobility } (R_{\text{m}}) = \frac{\text{Distance moved by a protein band}}{\text{Distance moved by the tracking dye}}$$

A logarithmic plot between molecular weight of marker proteins versus corresponding relative mobility resulted in a straight-line standard graph. After calculating the relative mobility of inhibitor in the gel, its molecular weight was determined from the standard graph prepared for the following standard analyzed by SDS-PAGE (molecular weight in parentheses): albumin bovine (66,000), albumin egg (45,000), glyceraldehyde-3-phosphate dehydrogenase rabbit muscle (36,000), carbonic anhydrase bovine (29,000), trypsinogen bovine pancreas (24,000) trypsin inhibitor soybean (20,000) and α-lactalbumin bovine milk (14,200).

The void volume of the Sephadex G-100 column was determined by loading 2.0 ml of 0.2 % blue dextran-2000 (MW $\sim 2 \times 10^6$) on the column which was eluted at a flow rate of 15 ml h^{-1} by passing the buffer. The standard protein markers used for calibration of the gel filtration column were albumin (66,000), carbonic anhydrase (29,000), cytochrome C (12,400), and aprotinin (6,500). The proteins were eluted with the elution buffer at a flow rate of 15 ml h^{-1}. Fractions of 3.0 ml each were collected and their absorbance was recorded at 280 nm. The elution volume (V_e) of each protein was determined. The purified TI was eluted separately from the same column under identical conditions and the elution volume of each inhibitor protein was determined. A graph was plotted between V_e/V_o vs. Log MW which was used for calculation of molecular weight.

Effect of temperature on activity and stability of TI

The optimum temperature of the purified TI was determined by measuring its activity at different temperatures (30–80 °C) according to the standard assay procedure. Appropriate control for trypsin was run simultaneously at each temperature. The temperature showing the maximum activity was designated as the optimum temperature. Relative inhibitory activity (%) at different temperatures was calculated relative to its activity at the optimum temperature which was taken as 100 %.

Thermostability of the purified inhibitor was studied by pre-incubating an aliquot of the purified TI at different temperatures ranging from 40 to 100 °C separately for different time intervals: 15, 30, 45 and 60 min. These samples were then cooled immediately to 0 °C and the activity was measured using standard assay procedure at 37 °C. Residual inhibitory activity (%) at different pre-incubation temperatures was calculated with reference to its maximum activity which was taken as 100 %.

Effect of pH on activity and stability of TI

The pH optimum was determined using different buffers over the pH range 3.0–12.0 in the standard assay mixture. The buffers used in the indicated pH range included Tris–Phosphate (pH 3–7), Tris–HCl (pH 7–9), Glycine–NaOH (pH 9–11) and phosphate–NaOH (pH 11–12) each at 0.05 M. Appropriate control for trypsin was run simultaneously at each pH separately. The substrates were dissolved in respective buffers so as to observe the exact change in the activities. The optimum pH was designated as the pH which displayed the highest inhibitory activity of inhibitor proteins. Relative inhibitory activity (%) at different assay pH values was calculated relative to its activity at the optimum pH which was taken as 100 %.

The pH stability was studied by pre-incubating an aliquot of the purified inhibitor with buffers of different pH ranging from 3.0 to 11.0 for 24 h at room temperature followed by measurement of activity by the usual assay procedure at the optimum pH. The buffers (50 mM each) used in the indicated pH range included Tris–Phosphate (pH 3–7), Tris–HCl (pH 7–9) and Glycine–NaOH (pH 9–11). Residual inhibitory activity (%) at different pre-incubation pH values was then calculated relative to its maximum activity which was taken as 100 %.

Effect of stabilizers on thermal stability

The enhancement of thermal stability of protease inhibitor at 40, 50 and 60 °C was evaluated by the addition of thermal stabilizers like glycine (1 M), PEG 8000 (10 mM), glycerol (10 %), casein (1 %), $CaCl_2$ (10 mM), sucrose, BSA, and starch (at 1 % level) (arbitrarily selected based on the available literature on protease). Appropriate controls for trypsin and inhibitor were run simultaneously. Samples were drawn after 30 min and assayed for trypsin inhibitor activity.

Effect of various metal ions

Effect of various metal ions like K^{+}, Hg^{2+}, Fe^{3+}, Mn^{2+}, Co^{2+}, Cu^{3+}, Na^{1+}, Zn^{2+}, Ni^{1+}, Cd^{2+}, Ca^{2+} and Mn^{2+} each at 1 mM final concentrations in the reaction mixture was studied for inhibitor activity. The counter ion of all the metals used was chloride. The purified inhibitor was pre-incubated with each metal ion solution separately for 30 min at 37 °C, and assayed for TI activity. The percent inhibition was calculated by taking the activity in control (where no metal ion was added) as 100 %.

Inhibitory properties and determination of K_i value

The inhibitory activity of TI against trypsin was determined by measuring the hydrolytic activity toward BApNA. Kinetic studies were also conducted using trypsin. In this experiment, trypsin activity was determined after pre-incubating 50 µl of trypsin with 50 µl of different concentrations of TI (0, 6.67 and 13.33 µM) for 10 min at 37 °C. Later the pre-incubated mixtures were added separately to 0–1.2 mM BApNA for another 10 min at the same temperature. After incubation, the reaction was terminated by adding 20 µl of 30 % (v/v) acetic acid. The liberated *p*-nitroaniline was measured at 410 nm in a UV–Visible spectrophotometer. Using the inhibition data a double-reciprocal plot ($1/v$ versus $1/[S]$) was plotted to study the pattern of inhibition (competitive, uncompetitive or non-competitive).

K_i against trypsin was determined from the Dixon's plot ($1/v$ versus [I]). In this experiment, the activity of trypsin was determined by the standard assay after incubation with various concentrations of TI (0.00, 0.33, 0.67, 1.34, 2.68, 4.00 and 5.36 µM) at two different concentrations of BApNA (1 and 3 mM). The Dixon's plot was then drawn to calculate K_i.

Extraction of insect gut proteases

Midguts from actively growing, lab cultured fourth instar larvae were dissected out on ice in 0.1 M NaCl (to maintain the osmolarity) and homogenized in 0.2 M glycine–NaOH (pH 10.0) containing 2 mM DTT (dithiothreitol) and 10 % PVP (polyvinylpyrrolidone) in chilled pestle and mortar. The supernatant obtained by centrifuging the homogenate at $10{,}000 \times g$ for 15 min at 4 °C was used for estimation of the activity of trypsin. An aliquot of the midgut extract was incubated with BApNA (7 mg dissolved in minimum volume of DMSO and adjusting its final volume to 20 ml with 0.05 M Tris–HCl, pH 8.0, containing 0.03 M $CaCl_2$) for trypsin activity at 37 °C for 15 min. The reaction was stopped by adding 20 µl of 30 % (v/v) glacial acetic acid and absorbance of the resulting color was recorded at 410 nm using a double beam spectrophotometer (Model 2202, Systronics, India).

Effect of kidney bean TI on larval gut protease (in vitro assay)

The inhibition of fourth instar larval gut trypsin-like protease by the extract (containing TI) was studied in the same manner as described for the assay of insect gut trypsin-like protease except that the seed extract was also added to the reaction mixture. All the experiments were carried out in triplicate.

Dose response bioassay

The chronic growth bioassay was carried out by incorporating a series of four different concentrations (1,250, 2,500, 5,000 and 10,000 TUI) of purified TI (from the variety showing maximum inhibitory activity during in vitro assay) into the artificial diet. The control diets were treated only with the buffer in which TI was dissolved. Bioassays were performed using freshly hatched neonate larvae of *H. armigera* and *S. litura*. Each experiment was carried out for 1 week. The EC_{50} (effective concentration to inhibit growth by 50 % relative to control) was calculated by regression equation using probit analysis. As the response is always binomial (death or no death), the relationship between response and various concentration is sigmoid, so probit analysis was used for transformation from sigmoid to linear plotting.

Insect feeding bioassays

Insect feeding bioassays were conducted on second instar larvae (mean weight 8 ± 1 mg) of *H. armigera* and *S. litura.* The required amount of purified kidney bean TI giving EC_{50} was incorporated into the diet of these insects to study its effect on larval growth and development. Newly molted first instar larvae were taken from the culture and divided into control and experimental groups in triplicates. Thirty larvae were placed on the control diet (lacking TI) and the same numbers of larvae were placed on the TI-containing diets individually in culture bottles (3 cm diameter × 4 cm high), ensuring minimal variation between populations. The larvae were kept in a controlled temperature cabinet at 25 ± 1 °C and 60–70 % relative humidity. The diet was replaced if it became dehydrated or if more than 75 % of it was consumed. The weight of each larva was measured at alternate days after treatment, till larvae start undergoing pupation and the mean weight ± SE was calculated. The duration of larval and pupation period was also recorded. The number of adults that emerged was counted to determine the percentage

Table 1 Inhibition of trypsin and insect gut protease by kidney bean trypsin inhibitor in vitro studies

Variety	TUI/mg protein	% inhibition of HGP	% inhibition of SGP
IC-260299	2954.84 ± 28.15	91.7 ± 4.3	29.0 ± 2.5
IC-260292	1562.29 ± 28.10	59.5 ± 4.9	6.7 ± 1.3
IC-262840	2351.15 ± 35.01	73.9 ± 3.6	15.1 ± 1.8
IC-260307	1711.79 ± 16.54	68.8 ± 2.9	29.2 ± 2.6
IC-262837	2228.97 ± 4.03	72.5 ± 0.7	17.6 ± 2.0
EC-589388	1808.33 ± 8.60	74.4 ± 1.5	15.6 ± 1.9
EC-590329	1926.55 ± 10.39	77.5 ± 1.6	7.4 ± 1.5
EC-590326	1905.20 ± 8.00	71.8 ± 1.4	16.5 ± 2.1
EC-572720	2121.52 ± 10.01	78.4 ± 1.7	4.7 ± 1.0
EC-589468	2138.10 ± 7.01	77.1 ± 1.2	20.9 ± 2.3

Values are the mean ± SD

HGP Helicoverpa gut trypsin-like Protease, *SGP Spodoptera* gut trypsin-like Protease

Table 2 Purification of trypsin inhibitor from kidney bean (*P. vulgaris*) seeds

Purification step	Total activity (TUI)	Total protein (mg)	Specific activity (TUI/mg)	Recovery (%)	Purification fold
Crude extract	17,35,000	1336.67	1,298.00	100.00	1.00
Ammonium sulfate	15,08,000	608.33	2,478.92	86.92	1.91
DEAE-Cellulose	8,52,000	35.83	23,778.96	49.11	18.32
Sephadex G-100	3,60,500	7.30	49,383.56	20.78	38.04
CM-Sephadex C-50	2,60,800	5.10	51,137.25	15.03	39.40

survival to adult emergence (*S*). The time at which the adults emerged was also recorded to allow estimation of the mean time of development (*T*). Howe's index was calculated according to Howe (1971) as given below:

$$\text{Howe's index} = \frac{\text{Log of percent survival to adult emergence (\%S)}}{\text{Mean time of development (T)}}$$

Statistical analysis

All data were examined using one-way analysis of variance (ANOVA) (General Linear Models on GLM procedure). p value <0.05 was considered to be significant. EC_{50} values were calculated by probit analysis using Finney table (Finney 1952).

Results and discussion

Defatted seed flour extracts of ten varieties of kidney bean were screened for the presence of TI. The activity of TI was present in all the varieties but showed slight inter-varietal variation (Table 1). The highest activity was exhibited by mature seeds of IC-260299, whereas the lowest were observed in IC-260292. Screening of varieties for PI activity has earlier been reported in different legumes including chickpea (Kansal et al. 2008a) and mungbean (Kansal et al. 2008b) but with varying magnitude.

Purification of TI

Trypsin inhibitor was purified to apparent homogeneity from seeds of kidney bean by a multi-step strategy. The overall purification was 39.40-fold (Table 2) with a recovery of 15.03 %. After ammonium sulfate fractionation, the overall fold purification was 1.19 with a recovery of 86.92 %. The ammonium sulfate fraction containing the inhibitor was dialyzed and subjected to anion-exchange chromatography on DEAE-Cellulose column. The elution profile of the inhibitor through DEAE-Cellulose column is shown in Fig. 1. TI eluted through the anion-exchange column with 49.11 % recovery, which was chromatographed on Sephadex G-100 at pH 8.1, resulting in the recovery of 20.78 %. The partially purified fraction obtained from gel filtration column was dialyzed and chromatographed on CM-Sephadex C-50 at pH 4.6 which resulted in a single peak of activity for the inhibitor. The fractions containing inhibitor activity were pooled and analyzed for purity by native and SDS-PAGE.

Checking of purity and reverse zymography

The purity of the TI obtained after cation-exchange column chromatography was checked by native and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The purified TI appeared as a single band on native-PAGE (Fig. 2a) as well as on SDS-PAGE under reducing conditions (Fig. 2b) which indicated that the

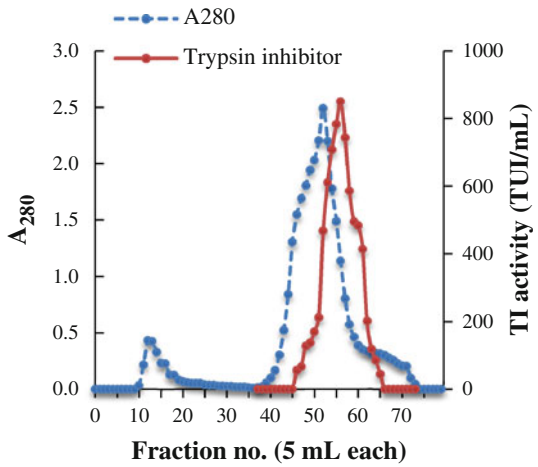

Fig. 1 Elution profile of purification of trypsin inhibitor from kidney bean seeds by DEAE-Cellulose column chromatography

purified inhibitor was apparently homogeneous. The activity staining for the purified TI showed a single blue colored activity band corresponding to the single protein band on SDS (Fig. 2b).

The purity of the pooled active fractions obtained was further checked by HPLC. Reverse phase HPLC was performed isocratically with the solvent system acetonitrile:water (70:30). HPLC profile showed a single peak with a retention time of 2.569 min for the TI purified by multi-step strategy (Online Resource 1).

Determination of molecular weight

The purified TI showed a single protein band on SDS-PAGE under reducing condition (with β-mercaptoethanol) corresponding to a molecular weight of ~15 kDa (Fig. 2b). On analysis by gel filtration through Sephadex G-100 column, the purified enzyme eluted as a single peak with a molecular weight of ~15 kDa as calculated from the standard graph. An identical MW obtained by SDS-PAGE and by analytical gel filtration chromatography indicated that the inhibitor consisted of a single polypeptide chain. Klomklao et al. (2010a) reported that the apparent molecular weight of the TI was estimated to be 14 kDa based on SDS-PAGE. The molecular weight of the trypsin inhibitor from bambara groundnuts was 13 kDa (Benjakul et al. 2000). Two trypsin inhibitory activity bands were observed for cowpea (10 and 18 kDa) and pigeon pea (15 and 25 kDa) (Benjakul et al. 2000).

Temperature profile

The optimum temperature for TI was determined by measuring the inhibitor activity at various temperatures ranging from 30 to 80 °C. The optimum temperature was found to be 37 °C (Fig. 3a). The activity of TI increased gradually up to 37 °C but decreased sharply thereafter and was completely inactive at 80 °C. Bodhe (1991) showed maximum activity of PI from horse gram at 37 °C. Novillo et al. (1997) also reported an optimum temperature of 37 °C. Thermostability of the TI was studied by pre-incubating the purified inhibitor at different temperatures ranging from 40 to 100 °C for different time intervals (15, 30, 45 and 60 min) followed by assay at 37 °C. The inhibitor retained 80 % of activity after 30 min and 50 % of the activity was left after 60 min of incubation at 40–60 °C (Fig. 3b). Thus, the inhibitor was thermo-tolerant and likely to be useful in biotechnological application.

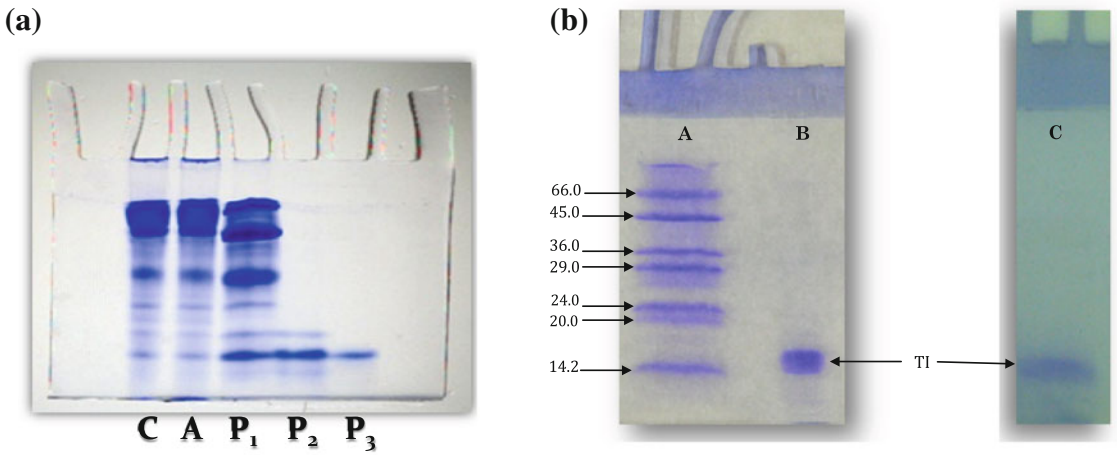

Fig. 2 **a** Native-PAGE of the purified trypsin inhibitor on a 12 % Davis gel (*C* crude, *A* ammonium sulfate fraction, *P1* Anion fraction, *P2* Gel filtration fraction and *P3* cation purified fraction); **b** SDS-PAGE (15 %) and inhibitory activity staining for trypsin (*A* molecular weight standard, *B* purified fraction, *C* reverse zymography)

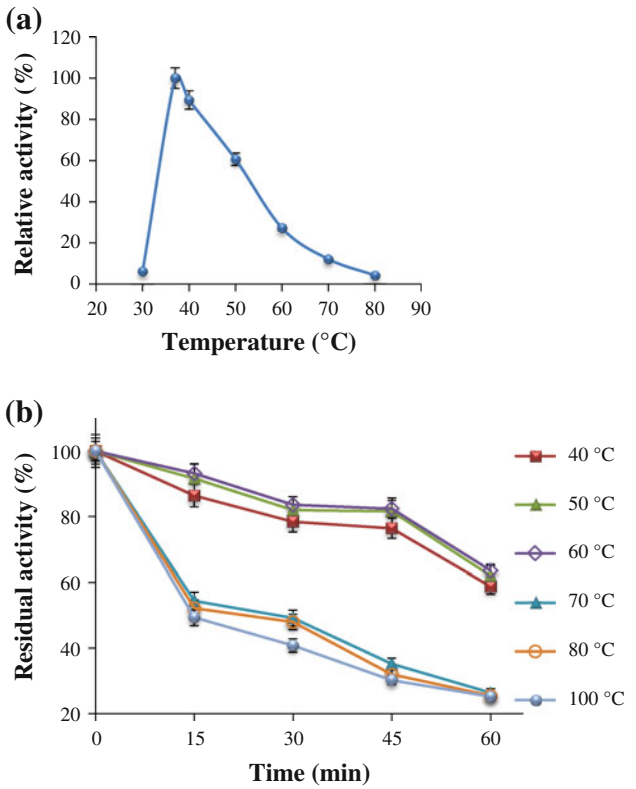

Fig. 3 Effect of temperature on the purified trypsin inhibitor: **a** temperature optima; **b** thermostability (*bars* indicate standard deviation from triplicate determination)

TI is heat sensitive in field beans, peanuts and cereals (Liener and Kakade 1969). Vacconcelos et al. (1997) reported that TIs from Brazilian soybeans were destroyed completely by heating at 92 °C for 5 min. The difference in the thermal stability of the TIs may be due to differences in the nature of the proteins, e.g. conformation and bonding involved (Cheftel et al. 1985).

pH profile

The pH optimum for the catalytic action of TI was determined using the chromogenic synthetic substrate BApNA. The enzyme activity was measured over the pH range from 3 to 12. The maximum inhibitory activity was observed at pH 8.0 (Fig. 4a). Similar pH optima for PI were observed by other researchers (Kuhar et al. 2013). The pH stability of the purified inhibitors was studied with buffers of different pH ranging from 3 to 11. The TI was found to be stable over a wide range, i.e. from pH 3 to 11 (Fig. 4b). Such wide pH stability might imply for their efficacy against Lepidopteran and Coleopteran insects. Godbole et al. (1994) found that the inhibitors from pigeon pea retained their activity between pH 7 and 10; however, Benjakul et al. (2000) reported that the inhibitors from pigeon pea and cowpea retained their activities between pH 4 and 10. The differences in the pH stability were likely the reason for the different molecular properties including bonding and stabilizing of the structure, as well as the different trypsin inhibitor conformations among the various species and anatomical locations.

Effect of stabilizers on thermal stability of TI

Since the trypsin inhibitor showed decreased activity at 40, 50 and 60 °C after a prolonged incubation, the effect of additives as thermal stabilizers was studied at the same temperatures using glycine, PEG 8000, glycerol, casein, $CaCl_2$, sucrose, BSA and starch. Data presented in Fig. 5a indicated that, in general, almost all the stabilizers promoted thermal stability and inhibitory activities compared to that of control (in the absence of any stabilizers). At 40 °C, maximal stability was promoted by casein (77 % inhibition) followed by glycerol and $CaCl_2$ (74 % inhibition). At 50 °C, maximal stability was promoted also by casein (73 % inhibition) followed by glycerol (72 % inhibition). At 60 °C, almost all stabilizers supported slight stability compared to that of control (in the absence of any stabilizers). Glycine, which supported stability at 40 and 50 °C, did not promote stability at 60 °C. Enhancement of thermal stability is desirable and in turn increases the efficiency of proteins, which is essential feature for their commercial exploitation.

Effect of metal ions

In the present study, the activity of the purified PIs was measured in the presence of various metal ions like Ka^{+}, Hg^{2+}, Fe^{3+}, Mn^{2+}, Co^{2+}, Cu^{3+}, Na^{1+}, Zn^{2+}, Ni^{1+}, Cd^{2+}, Ca^{2+} and Mn^{2+} each at 10 mM final concentrations in the reaction mixture. Among the various metal ions studied except Fe^{3+}, Mn^{2+} and Co^{2+} were found to increase the TI activity (Fig. 5b). The maximum decrease in activity of TI was observed due to Mn^{2+}; only 1.26 % activity was left as compared to control. Kuhar et al. (2013) reported that Co^{2+}, Ni^{2+} and Cu^{2+} at a concentration of 1.0 mM were found to slightly inhibit the inhibitory activities of TI. Ohtsubo (1989) reported that monovalent cations at 10 mM, such as Na^{+}, K^{+}, and NH_4^{+}, had little influence on the TI from bran of Job's tears (*Coix lacrymajobi* L.) seeds. Ogiso et al. (1975) observed no inhibition of the TI from barley by heavy metal ions at 10 mM.

Kinetic studies

The inhibitory activity of purified TI against trypsin was determined by measuring the hydrolytic activity toward BApNA. The protein completely inhibited trypsin at a molar ratio of 1:1 (Fig. 6a). The purified inhibitor was

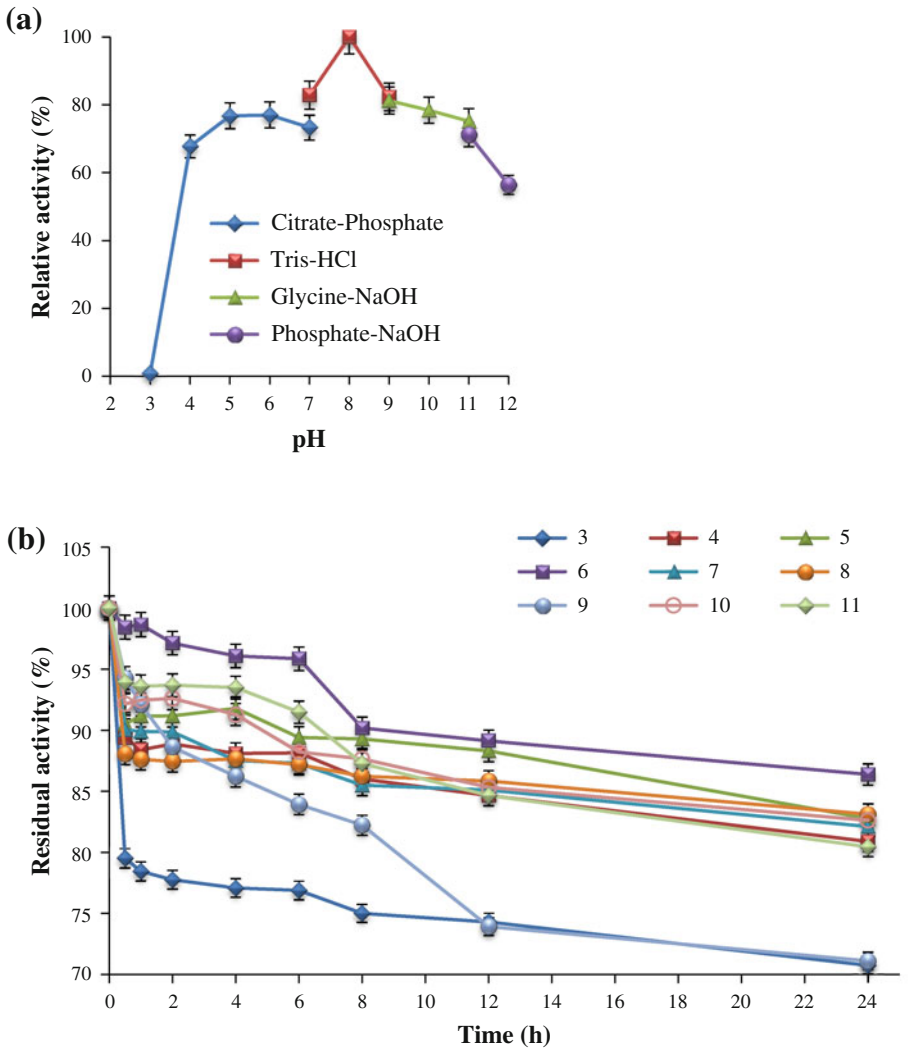

Fig. 4 Effect of pH on the purified trypsin inhibitor: **a** pH optima; **b** pH stability (*bars* indicate standard deviation from triplicate determination)

tested for type of inhibition and K_i determination against bovine trypsin using specific substrate BApNA. The double-reciprocal plot for the inhibition of trypsin by TI showed competitive inhibition (Fig. 6b). The K_i value of TI against trypsin was found to be 1.85 μM as calculated from the Dixon's plot (Fig. 6c). The Dixon plot analysis also showed that TI is a competitive inhibitor where two lines corresponding to each substrate interact above the *x*-axis, a characteristic of competitive inhibition. A low K_i value indicated high potency of the inhibitor towards enzyme. There are variable reports with regard to type of inhibition and the K_i values. The analysis of Lineweaver–Burk plot showed the inhibitor to be of non-competitive type like trypsin inhibitors from *Adenanthera pavonina* (Macedo et al. 2004) and *Vicia faba* (Gupta et al. 2000). Oliveira et al. (2007) and Bhattacharyya et al. (2006), however, found the inhibitors to be of competitive type. PIs isolated from different leguminous plants possessed K_i values for trypsin in the range between 0.1 and 52,000 nM (Tetenbaum and Miller 2001). Earlier high K_i value has been reported for trypsin inhibitor from faba bean (Gupta et al. 2000). Other researchers have also reported higher affinity against trypsin (Macedo et al. 2000).

In vitro inhibition of insect gut protease

Inhibition of trypsin and insect gut trypsin-like protease by kidney bean TI was studied using in vitro assay. The seed extracts of different kidney bean varieties were screened for their inhibitory activity against the gut trypsin-like protease of *H. armigera* and *S. litura*. TI from all the varieties under study showed inhibition against insect gut protease, indicating its insecticidal potential. Amongst the varieties (Table 1), IC-260299 exhibited maximum inhibition of *Helicoverpa* (91.7 %) and *Spodoptera* (29.0 %). So, efficacy of the kidney bean TI was higher against *Helicoverpa* as compared to *Spodoptera.* The inhibition differed with variety, the % inhibition of *Helicoverpa* gut trypsin-like protease varied from 59.5 to 91.7 % while in *Spodoptera* varied from 4.7 to 29 %. Kansal et al. (2008a)

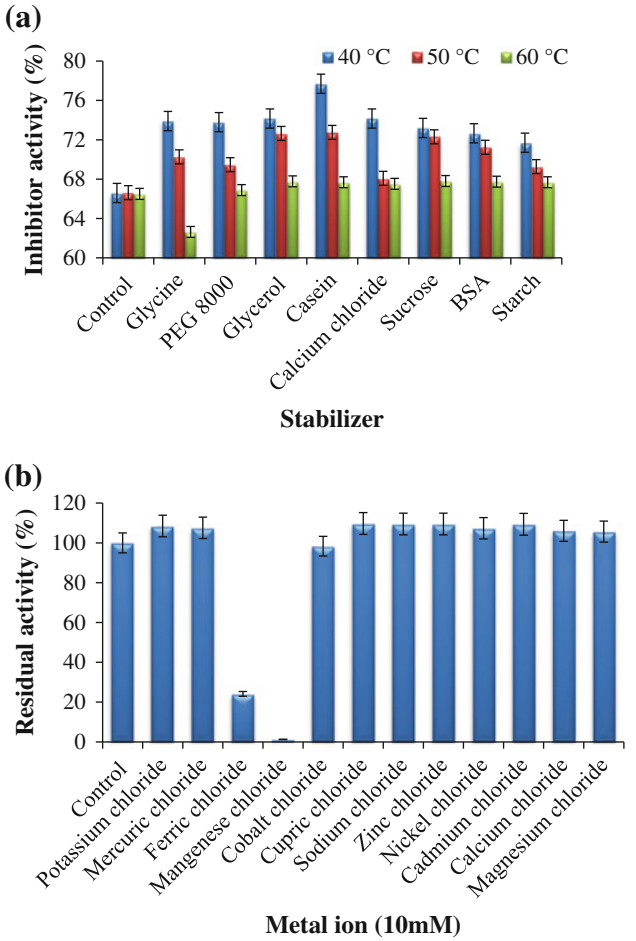

Fig. 5 **a** Effect of stabilizers on the activity of trypsin inhibitor at 40, 50 and 60 °C: trypsin inhibitor was incubated with different thermostabilizers and inhibitor activity was measured after 30 min of incubation. **b** Effect of metal ions on trypsin inhibitor activity, measured after incubating with 1 mM of metal ion final concentration for 30 min

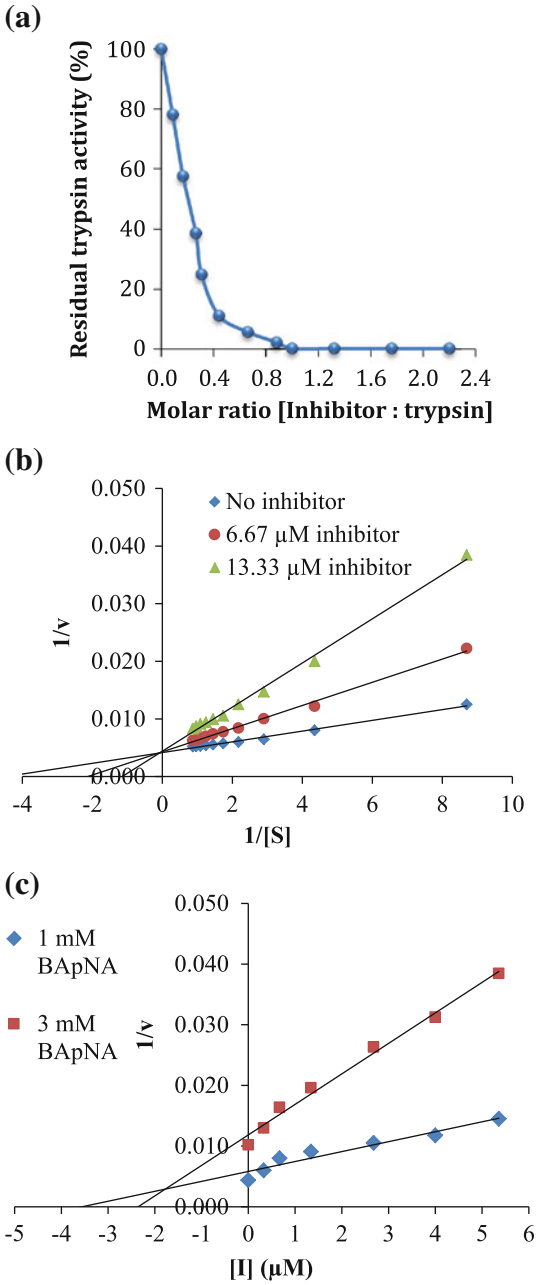

Fig. 6 Kinetic analysis of trypsin inhibitory activity of TI. **a** Trypsin inhibitory activity showing residual trypsin activity in percent as function of the inhibitor concentration using BApNA as substrate; **b** Lineweaver–Burk plot for inhibition of trypsin by trypsin inhibitor (0, 6.67 and 13.33 µM) at various concentrations of BApNA (0–1.2 mM); **c** Dixon's plot for determination of dissociation constant (K_i) value of trypsin inhibitor against trypsin at two different concentrations of BApNA (1 and 3 mM)

reported 95 % inhibition of larval gut proteinase of *H. armigera* by TI present in seed extract of soybean (P-20). Kumar et al. (2007) also found inhibitory proteins in seed extracts of various legumes specific against *H. armigera* gut proteinase (HGP). Patankar et al. (1999) and Srinivasan et al. (2005) reported an inhibition of HGP activity by PIs from seeds of chickpea by 2–33 and 60 %, respectively. Harsulkar et al. (1999) showed an in vitro inhibition of HGP activity by 0–55 % in host plants, whereas total inhibition of HGP activity in non-host plants. It is evident that the extent of inhibition of HGP by the kidney bean TI was greater than several other reports.

TI dose response on larval mortality

The insect larval mortality varied significantly at different TI concentrations (1,250, 2,500, 5,000 and 10,000 TUI). The above inhibitor concentrations were used to calculate the EC_{50} values calculated by probit analysis (Fig. 7). At low concentration, the kidney bean TI did not cause a significant mortality but at high concentration it was more toxic (higher mortality) to *H. armigera* (EC_{50} ~5,400

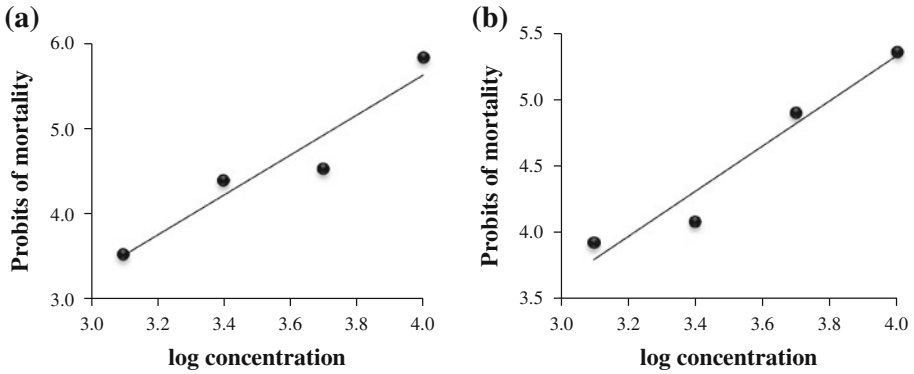

Fig. 7 EC_{50} estimates analyzed by regression of probit analysis graph plotted between log of the concentration and probits of mortality. The inverse log of concentration corresponding to probit of 5 is EC_{50} value: **a** *Helicoverpa armigera*; **b** *Spodoptera litura*

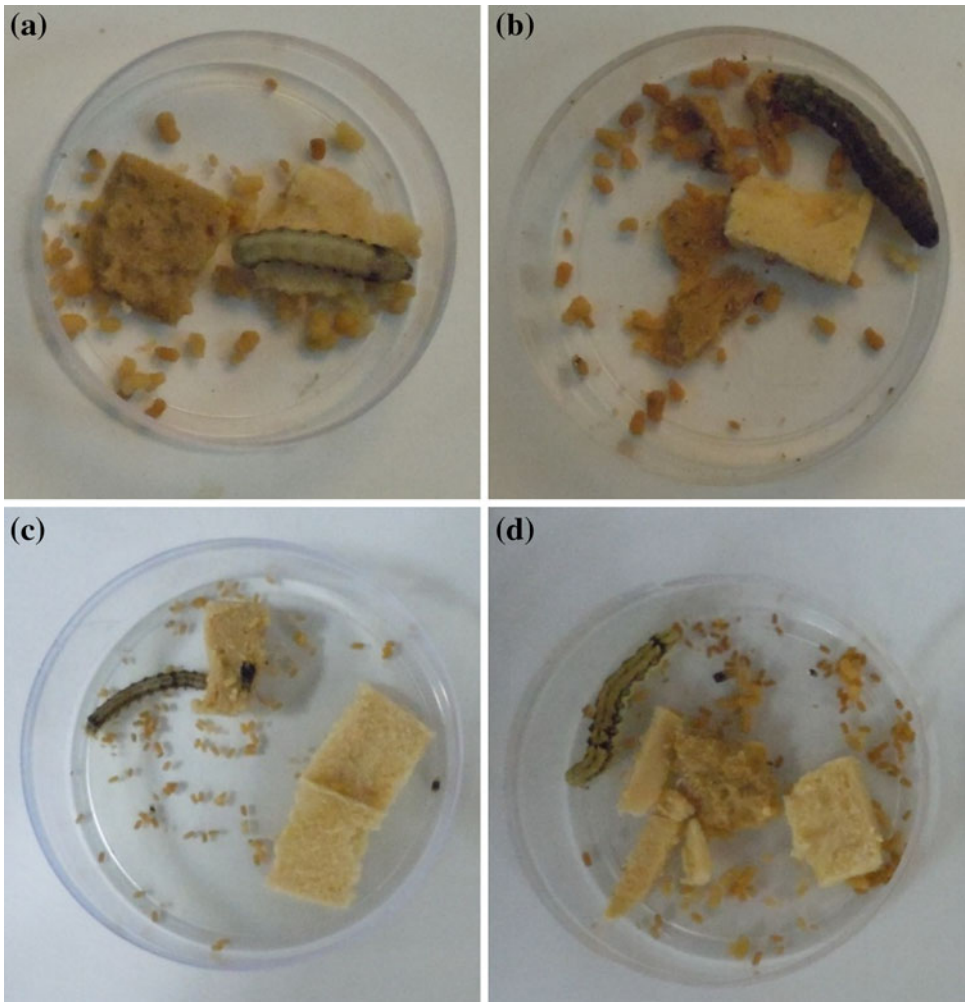

Fig. 8 The morphological difference observed during the larval growth when fed on diet impregnated with purified trypsin inhibitor in **a** *S. litura* and **c** *H. armigera* as compared to the control diet **b** and **d**, respectively

units; $R^2 = 0.89$) than to *S. litura* (EC_{50} ~6,400 units; $R^2 = 0.94$).

Insect bioassay

To test the potency as an insect-control agent, effect of TI on insect growth and development was investigated by incorporating purified TI in the artificial diet of second instar larvae having a known weight (8 ± 1 mg). Development of pupae and adults was also monitored to evaluate the effect on larval weight, pupation, adult emergence and mortality.

Effect on larval weight

The larvae feeding on the kidney bean inhibitor showed a difference in weight as compared to ones fed on

control diet. The average weight of the inhibitor fed larvae was 25.6 ± 1.55 and 26.9 ± 1.29 mg, which was 22.0 and 36.4 % lower than the average control weight of 32.9 ± 2.04 and 42.4 ± 2.32 mg in *S. litura* and *H. armigera*, respectively, after 2 days of treatment. Similarly, on the fourth day, average weight of the inhibitor fed larvae was 98.0 ± 7.95 and 91.0 ± 12.4 mg, which was 18.6 and 48.3 % lower than the control (120.4 ± 7.73 and 176.1 ± 10.30 mg), while by the 6th day, the inhibitor fed larvae weighed 277.9 ± 13.79 and 250.1 ± 46.39 mg, 11.8 and 40.2 % lower than the control at 314.9 ± 12.54 and 418.4 ± 109.68 mg in *S. litura* and *H. armigera*, respectively. The one-way analysis of variance showed that the kidney bean extract had a very significant effect on weight in *H. armigera* ($F = 28.18$; $df = 1$; $p < 0.0001$) as compared to *S. litura* ($F = 7.98$; $df = 1$; $p < 0.01$) at maximum inhibition. These results implied that larval growth was adversely affected in the presence of inhibitor (Fig. 8). A significant reduction in weight of *H. armigera* larvae when reared on artificial diet containing PI was reported by Sudheendra and Mulimani (2002) and Shukla et al. (2005). Srinivasan et al. (2005) also observed that *H. armigera* gut proteinase inhibitors inflicted maximum adverse effect on third instar larvae which progressively decreased until the fifth instar.

Effect on larval mortality

In larvae fed with control diet, no mortality occurred. Treatment provoked a highly significant effect causing 46.7 % larval mortality of *H. armigera* during the treatment ($F = 420.25$; $df = 1$; $p < 0.0001$). At the same time, mortality of *Spodoptera* was 13.3 % during larval growth ($F = 48$; $df = 1$; $p < 0.01$). The larval mortality was higher than those reported in the literature. Shukla et al. (2005) reported larval mortality of 20 % when reared on diet impregnated with soybean TI as compared to 10 % mortality in control. On feeding mungbean TI-impregnated diet to *H. armigera* larvae, 33 % mortality was observed (Kansal et al. 2008b). Larval mortality ranging from 10 to 20 % of the total larval population was reported by Telang et al. (2003) in *S. litura* by Bitter gourd proteinase inhibitors.

Effect on larval and pupation period

In control, larval and pupal duration was 14 and 12 days, respectively, in *H. armigera*, while in *S. litura* it was 18 and 9 days, respectively. The *H. armigera* and *S. litura* showed a significantly longer larval period (17 and 20 days, respectively) compared to the control. The one-way analysis of variance showed that the TI had a very significant effect on larval development period of both insects studied. The pupal life duration in both species was increased by 1 day so that it was 13 and 10 days after treatment in *H. armigera* and *S. litura*, respectively. These observations revealed the efficacy of kidney bean TI against the insect. Telang et al. (2003) reported that larval and pupal periods were marginally reduced in *H. armigera*, while larval period was not affected but pupal period was delayed by up to 3 days in *S. litura*.

Effect on emergence rate and adult mortality

The emergence rate in control was 100 %. The number of adults emerging from the pupae was significantly reduced in TI-treated cultures. The mortality in *H. armigera* and *S. litura* was 60 and 13.3 %, respectively, when fed on treated diet. Further, morphology of the survived adults fed on TI was found to be deformed in *H. armigera* (37.5 %) and *S. litura* (30 %) as compared to control adults (Fig. 9). Thus, kidney bean TI shows fatal effect on insect growth and development. The developmental abnormalities in the adults might be due to the fact that TI interferes with normal proteolysis and ingestion thereby adversely affecting the protein intake at the larval stage. Kansal et al. (2008b) reported that the adult emergence from larvae

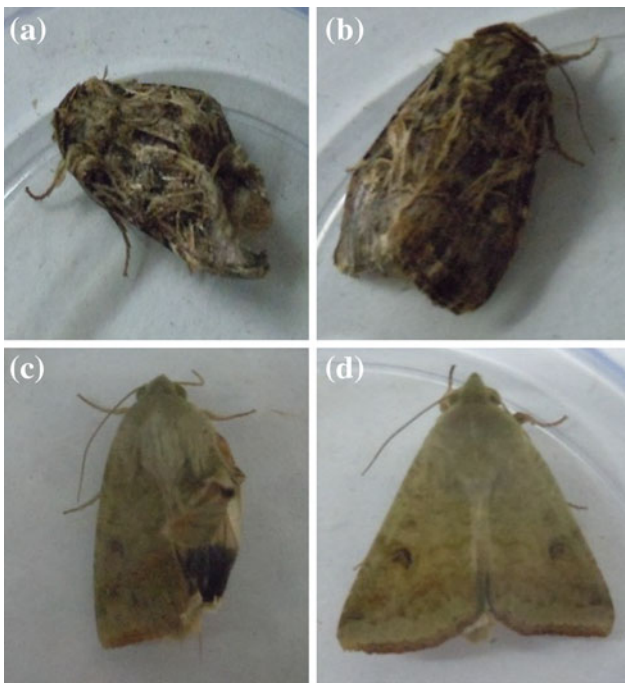

Fig. 9 The morphological difference observed by the adults developed from the larvae fed on diet impregnated with trypsin inhibitor in **a** *S. litura* and **c** *H. armigera* as compared to the control diet **b** and **d**, respectively

Table 3 Effect of purified trypsin inhibitor from kidney bean on the development of the *H. armigera* and *S. litura*

Insect	Diet	%S[a]	T[b] (days)	Log S^c/T
H. armigera	Control	90.00	26	0.075
	Inhibitor	36.67	30	0.052
S. litura	Control	100.00	27	0.074
	Inhibitor	86.67	30	0.062

[a] Percentage survival to adult emergence

[b] Mean time of development

[c] Howe's index

feeding on diet containing chickpea TI was lower, with some of the adults being abnormal and could not survive.

The mean time of development (in days) was slightly higher in the diet containing TI (Table 3) with a difference of 4 and 3 days in *H. armigera* and *S. litura*, respectively, as compared to control diet fed larvae. Howe's index of 0.052 and 0.065 was obtained in *H. armigera* and *S. litura*, respectively, for the artificial diet containing kidney bean TI as compared to the value of 0.075 and 0.074 for the control artificial diet, respectively. This suggested that the inhibitor diet was not as suitable as the control diet for Lepidopteran development. Macedo et al. (2003) reported Howe's index of 0.048 for the artificial diet containing *Peltophorum dubium* TI compared to the value of 0.041 for the control diet for *A. kuehniella* development. Thus, the efficiency showed by purified TI from kidney bean seeds against insect growth and development indicates its insecticidal potential.

Conclusion

Considering the high complexity of protease inhibitor interaction in host–pest system and the diversity of proteolytic enzymes used by pests and pathogens to hydrolyze dietary proteins or to cleave peptide bonds in more specific processes (Graham and Ryan 1997), it is important to choose an appropriate protein inhibitor or set of protein inhibitors representing a primary determinant in success or failure of any pest control strategy relying on protease inhibition. In this study, the TI from kidney bean seeds has been shown to exert deadly effects on growth and development of *H. armigera* and *S. litura*, being much more effective against the former. The significant inhibition during the in vitro and in vivo assay suggested that kidney bean seeds TI can be used as biocontrol and deployed in developing transgenic plants for enhancing the resistance to lepidopteran insects. Therefore, the trypsin inhibitor gene of *P. vulgaris* can be used to transform agronomically important crop plants to develop resistance against insect pests of Lepidoptera order.

Author contribution A. Mittal, R. Kansal and V.K. Gupta designed and performed the experiments, made data analysis, and prepared the manuscript. V. Kalia and M. Tripathi helped in performing the insect bioassay studies.

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