RESEARCH ARTICLE

Purification and characterization of trypsin inhibitor from *Cicer arietinum* L. and its efficacy against *Helicoverpa armigera*

Rekha Kansal[1], Mukesh Kumar[1], Kalika Kuhar[2], Ram N. Gupta[1], Bhattiprolu Subrahmanyam[3], Kirpa R. Koundal[1], and Vijay K. Gupta[2*]

[1] National Research Centre on Plant Biotechnology, Indian Agricultural Research Institute (PUSA), New Delhi, India.

[2] Department of Biochemistry, Kurukshetra University, Kurukshetra, Haryana, India.

[3] Division of Entomology, Indian Agricultural Research Institute (PUSA), New Delhi, India.

* Corresponding author: Dr. Vijay Kumar Gupta, Tel.: +91-1744-239281; fax: +91-1744-238277, E-mail address: vkgupta59@rediffmail.com.
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Protease inhibitors in legumes are one of the most promising weapons that confer resistance against insects by inhibiting proteases present in the gut of insect larvae. In the present study, trypsin inhibitor activity was detected in the seed flour extracts of 10 selected varieties of chickpea. The presence of inhibitor was confirmed by dot blot analysis. All the varieties showed inhibitory activity *in vitro* against the gut protease of *Helicoverpa armigera* (HGP). Trypsin inhibitor has been purified to near homogeneity to 60.46 fold and 29.20% recovery from chickpea seeds using heat denaturation, ammonium sulphate fractionation, DEAE-Sephadex A-25 and Sephadex G-75. The purified inhibitor showed a single band on SDS-PAGE corresponding to molecular mass of 30,000 Da. The purified inhibitor was active over a wide pH range whereas it retained maximum activity between pH 6 and 10. The inhibitor protein was stable up to 80°C but retained only 40% of activity when heated at 100°C for 20 min. The inhibitor lost its complete activity at 121°C. The chickpea trypsin inhibitor exhibited inhibitory activity against *Helicoverpa armigera* both *in vitro* and *in vivo*. In insect bioassay, a progressive decline in larval weight, growth and survival as well as temporal extension of larval growth was observed after feeding *H. armigera* larvae on diet supplemented with increasing concentrations of chickpea trypsin inhibitor. The adult emergence was also adversely affected by the inhibitor. It may be concluded that chickpea trypsin inhibitor has insecticidal potential against *H. armigera*.

Key words: chickpea*,* dot blot, insect bioassay, pH stability, thermostability

INTRODUCTION

Protease inhibitors (PIs) active against trypsin have been studied as phytochemical resistance factors against herbivorous insects (Broadway, 1996). Protease inhibitors are natural products which are concentrated in seeds and tubers of plants belonging to Gramineae, Leguminosae and Solanaceae families (Connors et al., 2002). Plants contain two major families of PIs, the Kunitz and Bowman-Birk inhibitors. Kunitz inhibitors are usually 8,000-22,000 Da proteins, with two disulphide linkages and a single reactive site of trypsin, whereas Bowman-Birk inhibitors are usually 8000-10000 Da proteins, with seven disulfide linkages and two reactive sites of trypsin and chymotrypsin (Laskowski and Qasim, 2000). Protease inhibitors play important role in plant defense mechanism by preventing proteolysis in the midgut of insect larvae leading to their starvation and subsequent death (Gatehouse et al., 1999). This fact can be interpreted as a potential strategy for

increasing the level of plant defense against insects (Koiwa et al., 1997). Many reports have demonstrated retardation in the growth and development of insect pests fed on diets incorporating PIs, or on transgenic plants expressing PIs (deLeo and Gallerani, 2002; Murdock and Shade, 2002; Telang et al., 2003). Therefore, it is important to biochemically characterize the protease inhibitors from various indigenous cultivated legumes and evaluate their insecticidal potential

Chickpea, the world's third most important pulse crop (FAO Production Yearbook, 1993), suffers severe losses due to insect predation. Most of these losses are caused by the podborer *Helicoverpa armigera*, a polyphagous pest of the developing seeds of several legume species (Giri et al., 1998). For this reason, it became important to assess the levels of protease inhibitors from chickpea and their interaction with the gut protease of *H. armigera*. In view of the facts mentioned above, the present investigation is focused on purification and characterization of trypsin inhibitor from chickpea and its antimetabolic effects on the growth and development of *H. armigera.*

MATERIALS AND METHODS

Seed material: Seeds of 10 cultivated varieties viz. Pusa Pragati, P-329, P-256, P-372, P-261, Pusa-1053, P-244, P-1048, P-391 and P-209 of chickpea (*Cicer arietinum L.*) were obtained from the Division of Genetics, Indian Agricultural Research Institute, New Delhi, India.

Insects: The larvae of *Helicoverpa armigera* for isolation of gut protease and insect bioassay were obtained from the Division of Entomology, Indian Agricultural Research Institute, New Delhi, India.

Chemicals: Acrylamide, bis-acrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulphate, agarose,bovinetrypsinandNα-benzoyl-DL-arginine4-nitroanilide hydrochloride (BApNA) were purchased from Sigma Chemical Company, St. Louis, MO, USA. Diethylaminoethyl (DEAE) - Sephadex G-25 and Sephadex G-75 were procured from M/S Amersham Pharmacia, Germany. All other general chemicals used were of highest purity grade available commercially.

Extraction of trypsin inhibitor protein: Seeds of chickpea were ground in a Wiley laboratory mill and the flour obtained was defatted with acetone (1:10 w/v) 3-4 times, air-dried and used for protein extraction. The protein was extracted following the protocol of Maggo et al. (1999). Defatted flour (100 g) was shaken with 1.0 L (1:10 w/v) of 50 mM sodium phosphate buffer (pH 7.6) in a shaking water bath for 4 h at room temperature and the suspension was centrifuged at 10000 *g* for 30 min. The supernatant (crude extract) thus obtained was used for determining the activity of trypsin inhibitor.

Assay of trypsin inhibitor activity: The activity of trypsin inhibitor was assayed by determining the residual trypsin activity following the method of Kakade et al. (1969) with slight modifications using BApNA as the substrate and bovine trypsin as the standard enzyme. The reaction mixture contained 0.3 mL diluted trypsin inhibitor (seed extract), 0.3 mL trypsin (2 mg in 40 mL 0.001M HCl) and 2.1 mL of BApNA (30 mg dissolved in minimum volume of DMSO and adjusting its final volume to 100 mL with 0.05 M Tris-HCl, pH 8.2, containing 0.03 M $CaCl_2$) in a final volume of 2.7 mL. The final concentration of BApNA in the reaction mixture was 0.54 mM and the number of trypsin units was 180. After incubating the mixture at 37ºC for 15 min in a shaking water bath, the reaction was stopped by adding 0.3 mL of 30% (v/v) glacial acetic acid. A blank and a trypsin control were run simultaneously. The absorbance was recorded at 410 nm against the blank. Trypsin inhibitor activity (TIA) is defined as the number of trypsin units inhibited (TUI).

Extraction and assay of larval midgut protease of Helicoverpa armigera: Midguts from actively growing, lab cultured fourth instar larvae were dissected out on ice and homogenized in 50 mM phosphate buffer (pH 7.6) containing 20 mM NaCl, 5 mM $CaCl_2$ and 1 mM dithiothreitol in chilled polycarbonate tubes using a glass rod. The supernatant obtained by centrifuging the homogenate at 12,000 *g* for 15 min at 4°C was used for protease assay. An aliquot of midgut extract was incubated with BApNA (prepared as mentioned earlier) for 15 min at 37°C. The reaction was stopped by adding 0.3 mL of 30% glacial acetic acid and absorbance was recorded at 410 nm.

Study of larval gut protease inhibition by chickpea trypsin inhibitor: The inhibition of larval gut protease by trypsin inhibitor from chickpea seeds was studied in the same manner as described above in the assay of trypsin inhibitor activity except that bovine trypsin was replaced by larval midgut extract.

Protein estimation: The protein was estimated by the Lowry's method (Lowry et al., 1951).

Purification of trypsin inhibitor: The crude extract of chickpea seeds was heated at 80°C for 20 min, snap cooled to 4°C and centrifuged at 10,000 *g* for 30 min at 4°C. The supernatant was saturated to 85% by adding solid $NH_4(SO_4)_2$ and the proteins were allowed to precipitate for one hour at 4°C. After centrifugation at 10,000 *g* for 30 min at 4°C, the precipitates were dissolved in 50 mM phosphate buffer (pH 7.6) and dialyzed against the same buffer. The dialyzed sample was then subjected to ion exchange chromatography on DEAE-Sephadex A-25 (15 cm x 2 cm) at a flow rate of 35 $mL.h^{-1}$. The column was first washed with one bed volume of the same buffer to remove the unbound proteins. The bound proteins were eluted with a linear salt gradient of 0.1 – 1.0 M NaCl in 50 mM phosphate buffer (pH 7.6). Fractions (5 mL each) were collected and analyzed for protein content and trypsin inhibitor activity. Fractions containing the active inhibitor were pooled, concentrated by lyophilization and chromatographed on Sephadex G-75 column (50 cm x 2 cm, flow rate 12 $mL.h^{-1}$), pre-equilibrated with the same buffer. Fractions of 3 mL each were collected and monitored for protein (A_{280}) and trypsin inhibitor activity as done before. The active fractions were pooled and used for further studies. The purity of the fractions obtained during the course of purification was checked by native-PAGE and SDS-PAGE.

Electrophoretic analysis: The homogeneity of the purified protein was checked by native-PAGE (7.5%) as per the protocol of Davis (1964) and SDS-PAGE (15%) carried out essentially by the method of Laemmli (1970). Activity staining was done according to the protocol followed by Felicioli et al. (1997).

Determination of molecular mass: Molecular mass of the purified trypsin inhibitor was determined by gel filtration through Sephadex G-75 as well as by SDS-PAGE. Sephadex G-75 column was calibrated with standard marker proteins viz. bovine serum albumin (66,000 Da), carbonic anhydrase (29,000 Da), cytochrome C (12,400 Da) and aprotinin (6,500 Da).

pH stability: The pH stability of the chickpea trypsin inhibitor was tested by incubating an aliquot of the purified inhibitor for 24 h at room temperature using buffers of varying pH. The buffers used in the indicated pH range included glycine-HCl (pH 2.0-3.0), citrate-phosphate (pH 4.0-7.0), Tris-Cl (8.0), glycine-NaOH (pH 9.0-10.0) and phosphate-NaOH (pH 11.0-12.0), each at a concentration of 50 mM. The residual trypsin inhibitor activity was measured using assay as described above.

Thermostability: Thermostability of the chickpea trypsin inhibitor was determined by heating the purified inhibitor at 40, 60, 80 and 100ºC for 20 min in a water bath. These extracts were then immediately placed on ice and the residual trypsin inhibitor activity was determined.

Isolation of genomic DNA: The genomic DNA was isolated from 10 day old etiolated seedlings of 10 selected varieties of chickpea by the method of Dellaporta et al. (1983).

Dot blot assay: Dot blot assay of genomic DNA isolated from chickpea was done according to the protocol of Sambrook et al. (1989) using pigeon pea protease inhibitor gene as heterologous probe. Pigeon pea (*Cajanus cajan*) protease inhibitor clone was obtained from Lab No. E # 118, NRC on Plant Biotechnology, IARI, New Delhi, India. Plasmid DNA was isolated using QIAGEN Plasmid mini kit from overnight incubated culture of pigeon pea protease inhibitor clone grown in 10 ml Luria Broth containing ampicillin (1 mg/ml). The recombinant plasmid (5 μg) containing 1 kb amplicon was restricted with *Eco*RI (10 U/μl) in the presence of high salt buffer (10X). The restricted sample was electrophoresed on a low melting point agarose gel (0.8 %) and the insert band was eluted from the agarose gel by following the protocol of QIAquick Gel Extraction Kit.

Insect bioassay - Larval diet: The larvae were fed on artificial diet as per the composition given by Veera Reddy and Bhattacharya (1990) with some modifications: Bengal gram (12.44 g), yeast (1.63 g), casein (0.74 g), ascorbic acid (0.44 g), methyl hydroxybenzoate (0.30 g), sorbic acid (0.15 g), streptomycin sulphate (0.03 g), cholesterol (0.03 g), multivitamins (0.74 g), tocopherol (0.015 mL), formaldehyde (0.15 mL), agar (1.63 g) and distilled water to make the final volume 100 mL. Two types of diets were prepared: control diet (without the inhibitor) and the diet containing the purified trypsin inhibitor from chickpea. The inhibitor was added to the diet in three different concentrations i.e. 10,000 TUI; 20,000 TUI and 30,000 TUI. These treatments were referred to as T1, T2 and T3, respectively. All the ingredients, except the agar, were mixed well with 34 mL of distilled water. The inhibitor

was first dissolved in 12 mL of water and then mixed with the above ingredients. The agar was dissolved by heating in 54 mL of the distilled water and allowed to cool to 60°C. It was then quickly added to and mixed with the above ingredients to produce a homogenous solution. The diet was then poured into petri dishes, allowed to set and cubes of equal size were cut.

Rearing of H. armigera larvae on artificial diet: Four days old larvae (mean weight 8-9 mg) of *H. armigera* were reared individually at 26°C with 40% relative humidity in culture bottles (3 cm dia x 4 cm high) and fed on both types of diets. Fresh cubes of food (approx. 10 mm^3 size) were provided to the larvae. Fifteen larvae per diet were used. The food was changed every second day to reduce microbial contamination. The larval weight was monitored at 2 day intervals up to 18 days and thereafter, at 4 day intervals up to 26 days. Larval growth, extended larval growth, survival (%) and moulting of larvae and pupal weight were also recorded. The observations on the date of pupation and adult emergence were also taken.

RESULTS AND DISCUSSION

Extraction: The defatted flour of chickpea was extracted using different media like water, 0.2% NaCl, 50 mM sodium phosphate buffer (pH 7.6) and 100 mM sodium phosphate buffer (pH 7.6) containing 100 mM NaCl in 1:10 (w/v) ratio. The TIA varied from 37.43 to 39.47 TUI indicating that all the five media could extract equally well. However, the maximum activity was observed when the seed flour was extracted with 50mM phosphate buffer (pH 7.6). Therefore, in all the subsequent experiments, 50 mM phosphate buffer (pH 7.6) was used for extraction. Previously, different media have been reported to be suitable for extraction of trypsin inhibitor by various workers (Sessa and Nelsen, 1991; Domoney et al., 1993; Marconi et al., 1993; Hajela et al., 1999; Maggo et al., 1999).

Screening of chickpea varieties for trypsin inhibitor: Seed flour extracts of ten selected cultivars of chickpea were screened for the presence of trypsin inhibitor and the results are shown in Table 1. TIA was present in all the cultivars but it showed inter-varietal variations. The highest TIA was observed in the variety P-256 whereas it was lowest in Pusa Pragati. Presence of trypsin inhibitor was further confirmed by dot blot analysis which showed positive signal in all the varieties (Fig. 1).

Table 1. Trypsin inhibitor activity in seeds of chickpea varieties and its effect on larval gut protease of *Helicoverpa armigera*. Values represent the mean ± S.D. of three samples.

Variety	Trypsin inhibitor activity (TUI.mg^{-1} protein)	Inhibition of HGP (%)
P-209	8.71± 1.21	28.56± 1.32
P-391	7.67± 0.9	27.14± 2.65
P-1048	16.72± 2.3	46.16± 0.68
P-244	9.32± 2.12	33.64± 1.16
P-1053	11.36± 3.62	35.71± 4.09
P-261	8.02 ± 1.54	28.56± 0.15
P-372	18.88± 4.78	53.00± 3.73
P-256	39.47± 1.91	66.34± 5.66
P-329	8.98± 2.67	30.07± 4.32
Pusa Pragati	6.19± 0.56	22.36± 1.22

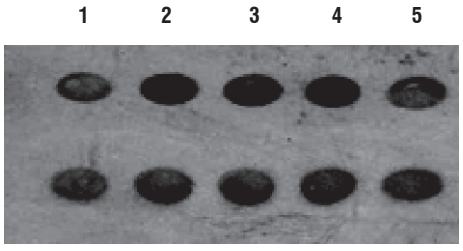

Figure 1. Dot blot assay of different cultivated varieties of chickpea for the presence of trypsin inhibitor gene **1**. P-209, **2.** P-391, **3.** P-1048, **4.** P-244, **5.** P-1053, **6.** P-261, **7.** P-372, **8.** P-256, **9.** P-329, **10.** Pusa Pragati.

The seed extracts of different chickpea varieties were screened *in vitro* for their inhibitory activity against the gut protease of *Helicoverpa armigera* (HGP). Trypsin inhibitor from all the varieties under study showed inhibition against HGP indicating its insecticidal potential. Compared to other varieties, P-256 exhibited maximum inhibition (66%) of HGP (Table 1). The inhibition of HGP by trypsin inhibitor is in agreement with the earlier report that primary digestive enzymes of *H. armigera* larvae are predominantly serine proteinases of trypsin and chymotrypsin type, which are extra cellular and active at alkaline pH (Johnston et al., 1991; Purcell et al., 1992). Patankar et al. (1999) showed a significant

variation in the trypsin inhibitor (TI) and the *Helicoverpa armigera* gut proteinase inhibitor (HGPI) content in the seeds of eight chickpea cultivars. They observed highest TI (198 units/g) and HGPI (23 units/g) activities by mature seeds of cv ICCV-2, whereas the lowest inhibitor activities were exhibited by cv PG8505–7 (96.1 TI units/g) and cv Vijay (5 HGPI units/g). Srinivasan et al. (2005) observed 60%inhibition of HGP activity by PI from seeds of chickpea. Gomes et al. (2005) have reported 73% inhibition of *Anthonomus grandis* proteinase larvae by a chickpea trypsin inhibitor. Harsulkar et al. (1999) showed 0-55% inhibition of HGP activity *in vitro* by PIs from host plants (chickpea, pigeonpea, cotton etc.) whereas complete inhibition was reported by PIs from non-host plants (groundnut, potato and winged bean). Kansal et al. (2008) found the inhibition of HGP in the range of 37-59% by PI isolated from eight mungbean varieties. Giri et al. (2003) isolated seven major trypsin inhibitors from dry mature seeds of winged bean (WBTIs) with different binding potentials towards HGP. WBTI-1 (28 kD) was identified as the most potent inhibitor of HGP relative to trypsin and among the other WBTIs; it inhibited 94% of HGP activity while at the same concentration it inhibited only 22% of trypsin activity.

Purification of trypsin inhibitor: Trypsin inhibitor from chickpea was purified to apparent homogeneity with 60.46 fold purification and 29.2% recovery using heat denaturation, $NH_4(SO_4)_2$ fractionation, ion-exchange chromatography on DEAE-Sephadex A-25 and gel filtration through Sephadex G-75 (Table 2). In ion-exchange chromatography, the activity of trypsin inhibitor was recovered in bound fractions and eluted as two peaks on applying linear 0.1-1 M NaCl salt gradient (Fig. 2*A*). The active fractions of major peak were pooled, concentrated and loaded on Sephadex G-75 column which resulted in a single peak of enzyme activity (Fig. 2*B*). Electrophoretic analysis of the purified trypsin inhibitor on SDS-PAGE revealed a single band which indicated that it was apparently homogeneous (Fig. 2*C*). The presence of an activity band at the position corresponding to the protein band in SDS-PAGE confirmed that it was a trypsin inhibitor (Fig. 2*D*). Gomes et al. (2005) purified a proteinase inhibitor from chickpea using salt fractionation followed by affinity Red-Sepharose C1-6B and reversed phase HPLC which was a monomeric protein. Using similar techniques, protease inhibitors were purified to homogeneity from other legumes (Hajela et al., 1999; Maggo et al., 1999; Lawrence and Nielsen, 2001).

Determination of molecular mass: The molecular mass of trypsin inhibitor as determined by gel filtration was 28,000 Da (Fig. 3). SDS-PAGE also showed a single sharp protein band with a molecular mass of 30,000 Da. It is likely that the inhibitor is of Kunitz type. A higher molecular mass (> 20,000 Da) may either be due to monomer-dimer equilibrium (Whitley and Bowman 1975) or due to oxidation of cysteine residues as it has been reported by Ferrasson et al. (1997). Kunitz-type protease inhibitors having different molecular masses than that of the chickpea trypsin inhibitor have been reported in soybean (19,000 Da), mustard seeds (20,000 Da) and *Cajanus cajan* (14,000 Da) (Mandal et al., 2002; Haq and Khan, 2003)

Table 2. Purification of a trypsin inhibitor from chickpea seeds

Step	Total TIA (TUI)	Protein (mg)	Specific activity ($TUI.mg^{-1}$)	Recovery (%)	Fold purification
Crude extract	30391.90	13040.00	2.32	100.00	1.00
Heat denaturation (80 °C, 20 min)	26735.40	11626.00	2.29	88.00	1.01
$(NH_4)_2SO_4$ ppt. (0-85 %)	23664.00	6246.46	3.78	77.90	1.62
DEAE-Sephadex A-25	15787.00	217.63	72.54	51.94	31.26
Sephadex G-75	8876.00	63.00	140.88	29.20	60.46

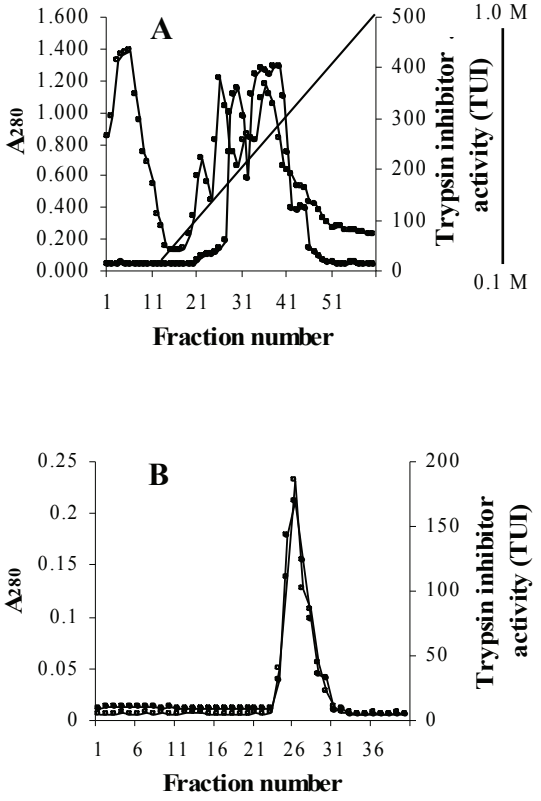

Figure 2. Purification of trypsin inhibitor from chickpea seeds. (A) DEAE-Sephadex A-25 column chromatography. (B) Sephadex G-75 gel chromatography. In (A) and (B), (○) A_{280}, (●) Trypsin inhibitor activity (C) SDS-PAGE (15%). In (C), lane 1 contained 20 μg of purified trypsin inhibitor. "M" indicated protein markers for SDS-PAGE. (D) Activity staining

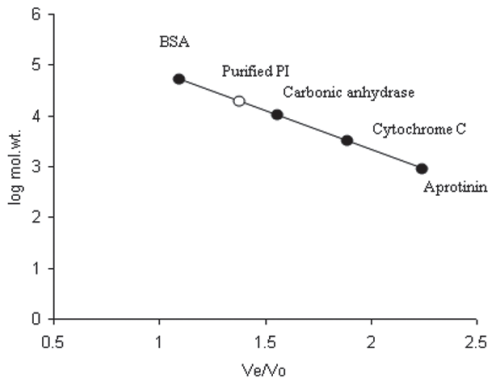

Figure 3. Standard curve of molecular weight determination by gel filtration

pH stability: More than 90% activity from chickpea was observed between pH 6 and 10 while at pH ranging from 3 to 5, it showed 71 to 85% activity (Fig.4*A*). Singhal (2004) also reported that the trypsin inhibitor from mungbean seeds was active between pH 4.0 and 10.0. Annapurna et al. (1991) reported that the trypsin inhibitor from jack fruit seeds was stable over still a wider range of pH (3.0 to 12.0). In contrast, the trypsin inhibitor from pigeon pea seeds retained its full activity between pH 7.0 and 10.0, but when exposed to acidic pH from 3.0 to 5.0, 20% of the activity was lost (Godbole et al., 1994). Johnston et al. (1991) observed that the midgut of lepidopteran larvae is highly alkaline and the digestive proteases have optimal activity between pH 10.0 and 11.0, while the larvae of phytophagous coleopteran have acidic conditions in their midgut region, with pH optima for digestive enzymes typically in the range 4.0-5.0. The stability of chickpea trypsin inhibitor over a wide range of pH might suggest its efficacy in controlling a variety of phytophagous insects.

Thermal stability: Varying temperature from ambient to 121°C, it was observed that trypsin inhibitor was stable up to 80°C but thereafter the activity decreased gradually retaining about 40% of activity at 100°C when heated for 20 min. At 121°C, the inhibitor lost its activity completely (Fig. 4*B*). In contrast, Marquez and Alonso (1999) observed completed inactivation of chickpea protease inhibitor on boiling for 300s. In general, protease inhibitors from legumes are quite stable up to 80°C but lose activity above this temperature (Hajela et al., 1999; Maggo et al., 1999; Gupta et al., 2000). The stability of the inhibitor at high temperatures may be attributed to its rigid and compact protein structure stabilized by a number of disulfide linkages as suggested for PI from pea seeds (Sierra et al., 1999).

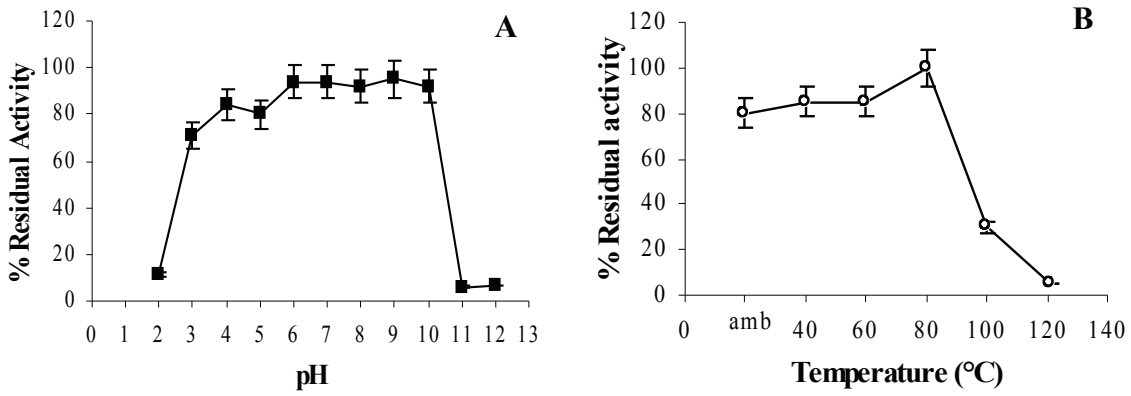

Figure 4. pH and thermal stability of purified chickpea trypsin inhibitor. Each point represents the average of three experimental observations. The vertical bars represent standard deviations.

Insect bioassay: The feeding of chickpea trypsin inhibitor reduced the larval weight significantly as compared to control diet (without inhibitor) indicating a possible response of ecdysis to semi starvation. When fed with 30,000 TUI of the inhibitor, the larvae showed about 50% reduction in weight (Table 3). The increasing concentration of the inhibitor in the diet resulted in progressive reduction of larval growth (Fig. 5*A*). Johnston et al. (1993) also reported a reduction of total biomass by 50% in larvae fed on diet containing soybean protease inhibitor. Sudheendra and Mulimani (2002) observed significant reduction in larval growth of *H. armigera* fed with chickpea and mungbean inhibitors. After feeding on diet containing the inhibitor, some larvae showed abnormality in moulting and subsequently died (Fig. 5*B*). Out of 15 larvae, six did not pupate in the presence of the highest concentration of the inhibitor. The trypsin inhibitor also caused a marked reduction in pupal growth (Fig. 5*C*). Adult emergence from the larvae feeding on diet containing chickpea trypsin inhibitor was lower. Further, some of these adults were abnormal (Fig. 5*D*) and could not survive.

Table 3. Effect of chickpea trypsin inhibitor on larval weight of *Helicoverpa armigera*. Values represent the mean ± S.D. of three samples. Values in parentheses represent the number of larvae. T1, T2 and T3 refer to different concentrations (viz. 10,000 TUI; 20,000 TUI and 30,000 TUI) of the inhibitor fed to *H. armigera* larvae.

Age of larvae (Days)	Weight of larvae (mg)			
	Control	T1	T2	T3
6	9.32±2.34 (15)	9.64±1.32 (15)	8.74±3.99 (15)	8.98±4.34 (15)
8	51.21±2.01 (15)	41.32±1.51 (15)	21.42±2.02 (15)	22.41±5.07 (15)
10	126.86±3.15 (15)	88.78±3.73 (13)	76.72±1.72 (12)	70.72±1.88 (12)
12	350.61±1.42 (15)	136.62±1.77 (13)	120.20±1.01 (11)	98.10±5.46 (10)
14	395.70±3.30 (15)	268.87±1.59 (13)	224.60±1.29 (10)	130.20±1.84 (9)
16	-	361.05±4.32 (13)	280.21±4.45 (10)	157.10±2.52 (9)
18	-	-	299.94±2.03 (10)	211.20±1.62 (9)
22	-	-	320.21±1.56 (10)	219.50±3.87 (9)
26	-	-	-	194.40±3.53 (9)

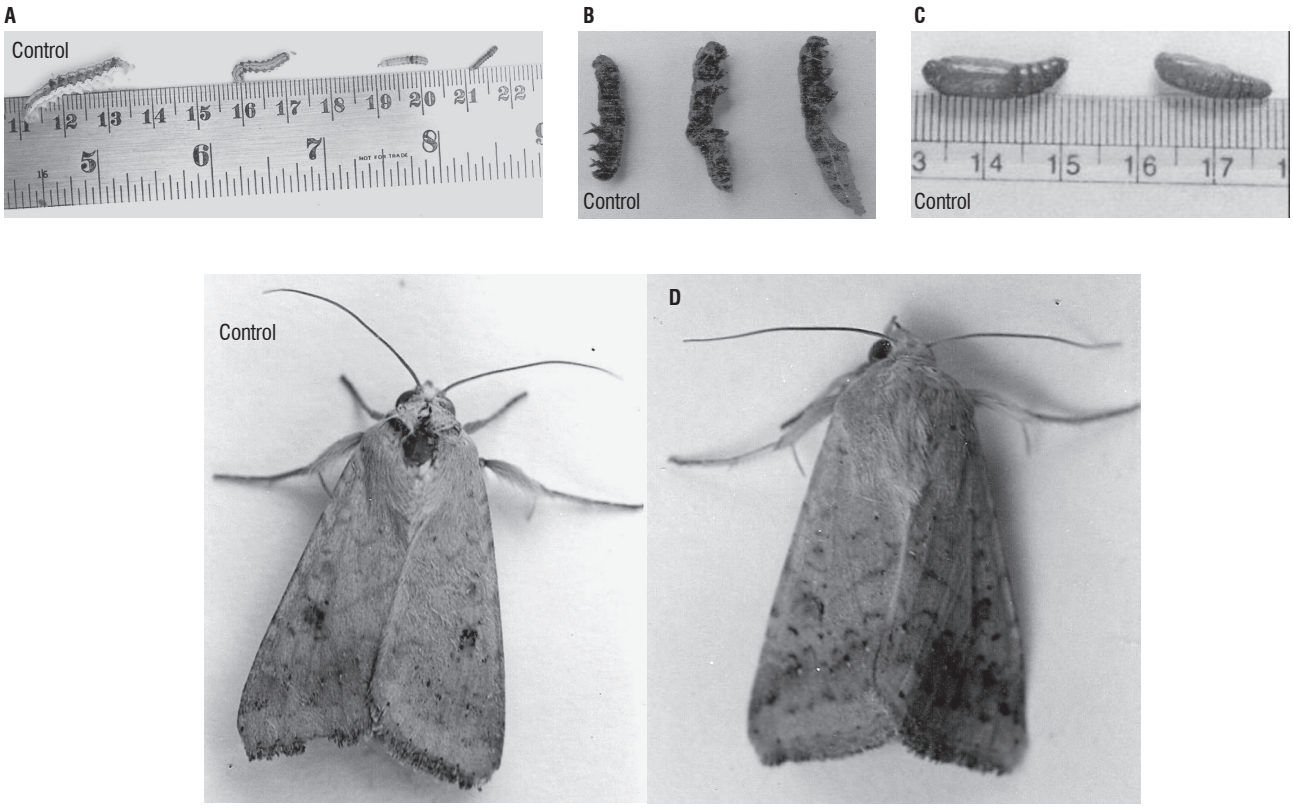

Figure 5. Insect bioassay: Effect of purified chickpea trypsin inhibitor on **(A)** larval growth **(B)** moulting **(C)** pupal growth **(D)** adult emergence.

Some of the larvae survived even in the presence of chickpea trypsin inhibitor but the percentage of survival decreased with increase in the concentration of trypsin inhibitor in the diet (Fig. 6*A*). Broadway (1996) observed that herbivorous insects could overcome the activity of these proteinase inhibitors by secreting inhibitor resistant enzymes. The insect mid-gut contains a number of different proteins with trypsin-like activity and this allows the insects to digest dietary protein in the presence of protease inhibitor. The mortality of *H. armigera* larvae fed with chickpea PI was 13, 33 and 40% at T1, T2 and T3 respectively (Fig. 6*B*). Gomes et al. (2005) observed 45% mortality when 1.5 % (w/w) of chickpea trypsin inhibitor was fed to *Anthonomus grandis* larvae. A 33% mortality of H. armigera larvae was observed on feeding mungbean TI (10,000 TUI) impregnated diet (Kansal et al., 2008). Srinivasan et al. (2005) have also reported a Kunitz trypsin inhibitor from chickpea effective against *H. armigera* larvae. The chickpea PI fed larvae showed extended larval growth in all the three concentrations of the inhibitor. The temporal extension of larval growth was 10 days when fed with diet containing 30,000 TUI of the inhibitor as compared to the control (Fig. 6*C*). The trypsin inhibitor also caused a marked reduction in pupal weight (Fig. 6*D)*. These results revealed the efficacy of chickpea trypsin inhibitor against *H. armigera* larvae*.* Several reports suggested that trypsin inhibitors could affect pest development when expressed in a heterologous system (Broadway and Duffey, 1986; Gatehouse et al., 1999)

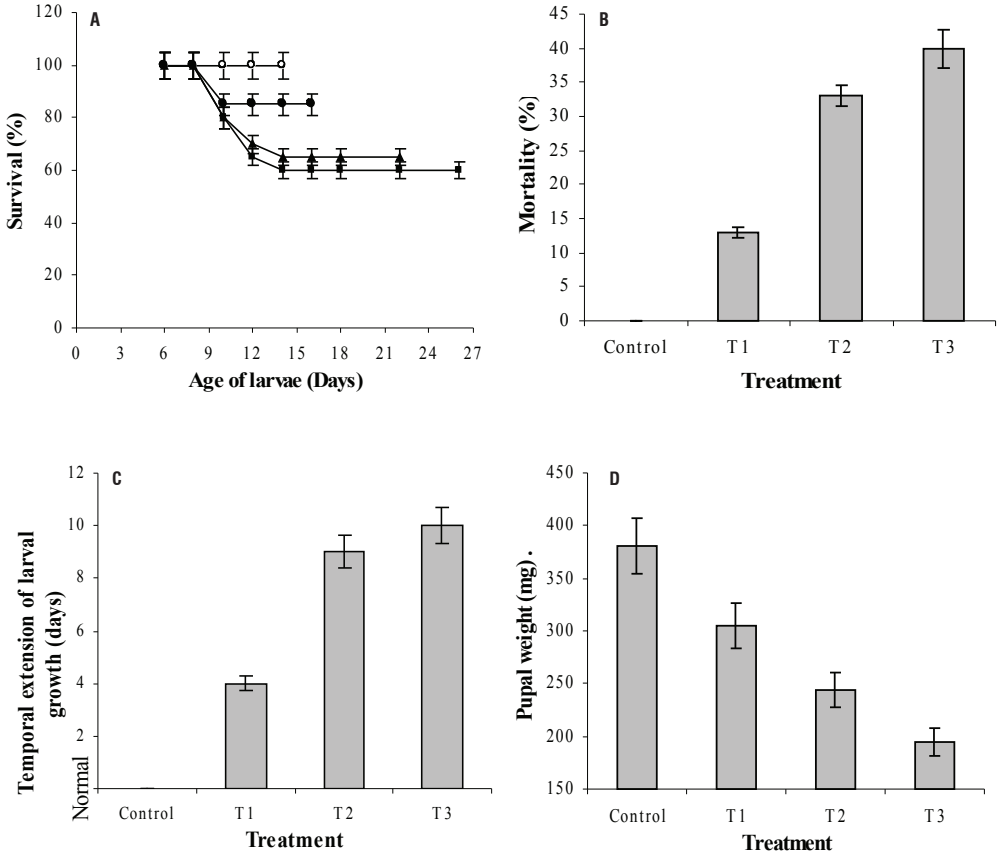

Figure 6. Effect of chickpea trypsin inhibitor on **(A)** survival, **(B)** mortality, **(C)** temporal extension of larval growth and **(D)** pupal weight of *Helicoverpa armigera.* In (A), the diet was supplemented with 10000 TUI (●), 20000 TUI (▲) and 30000 TUI (■). Control (○) was without inhibitor.

CONCLUSION

The trypsin inhibitor from different cultivars of chickpea was found to inhibit *H. armigera* gut protease. The inhibitor was purified to homogeneity and was found to be stable over a wide pH range. The results of the insect bioassay studies point out the insecticidal potential of chickpea trypsin inhibitor against *H. armigera*. Furthermore, a combination of different plant protease inhibitors might produce a greater insecticidal effect. It is likely that PIs expressed and produced in higher amounts in the agronomically important crops would lead to development of resistance against a variety of polyphagous insects. In addition, the trypsin inhibitor proteins often contain high levels of nutritionally essential amino acids which after denaturation during cooking will add to the nutritional quality of the crop.

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REFERENCES

Annapurna SS, Ramadoss CS, Prasad DS (1991) Characterization of a trypsin/chymotrypsin inhibitor from jack fruit (*Artocarpus integrifolia*) seeds. J. Sci. Food Agric. 54:605-618.

Broadway MM (1996) Resistance of plants to herbivorous insects: Can this resistance fail? Canadian J. Plant Pathol. 18:476-481.

Broadway RM, Duffey SA (1986) Plant proteinase inhibitors: mechanism of action and effect on the growth and digestive physiology of the larvae *Heliothis zea* and *Spodoptera exigua*. J. Insect Physiol. 34:1111-1117.

Connors BJ, Laun NP, Maynard CA, Powell WA (2002) Molecular characterization of gene encoding a cystatin expressed in the stems of American Chestnut (*Castanea dentate*). Planta 215:510-514.

Davis BJ (1964) Disc electrophoresis. II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. 121:404-427.

deLeo F, Gallerani R (2002) The mustard trypsin inhibitor 2 affects the fertility of *Spodoptera littorallis* larvae fed on transgenic plants. Insect Biochem. Mol. Biol. 32:489-496.

Dellaporta SL, Wood J, Hecks JB (1983) A plant DNA minipreparation:Version II. Plant Mol. Bio. Rep. 1:19.

Domoney C, Welham T, Sidebottom C (1993) Purification and characterization of *Pisum* seed trypsin inhibitors. J. Exp. Bot. 44(261):701-709.

Felicioli R, Garzelli B, Vaccari L, Melfi D, Balestreri E (1997) Activity staining of Protein inhibitors of Proteases on Gelatin-containing Polyacrylamide gel electrophoresis. Anal. Biochem. 244:176-179.

Ferrasson E, Quillien L, Gueguen J (1997) Proteinase inhibitors from pea seeds: Purification and characterization. J. Agric. Food Chem. 45:127-131.

Food and Agriculture Organization of the United Nations, (1993) FAO Production Yearbook, vol 46. Food and Agriculture Organization of the United Nations, Rome, Italy, pp. 105-115.

Gatehouse AMR, Norton E, Davison GM, Babbe SM, Newell CA, Gatehouse JA (1999) Digestive proteolytic activity in larvae of tomato moth, *Lacanobia oleracea*; effects of plant proteinase inhibitor in vitro and in vivo. J. Insect Physiol. 45:545-558.

Giri AP, Harsulkar AM, Deshpanade VV, Sainani MN, Gupta VS, Ranjekar PK (1998) Chickpea defensive proteinase inhibitors can be inactivated by podborer gut proteinases. Plant Physiol. 116:398-401.

Giri, AP, Harsulkar, AM, Ku, MS, Gupta, VS, Deshpande, VV, Ranjekar, PK, Franceschi, VR (2003) Identification of potent inhibitors of *Helicoverpa armigera* gut proteinases from winged bean seeds. Phytochemistry 63:523-532.

Godbole SA, Krishna TG, Bhatia CR (1994) Purification and characterization of protease inhibitors from pigeon pea (*Cajanus cajan*) seeds. J. Sci. Food Agric. 64:87-93.

Gomes APG, Dias SC, Bloch Jr. C, Melo FR, Furtado Jr. JR, Monnerat RG, Grossi-de-Sa´MF, Franco OL (2005) Toxicity to cotton boll weevil *Anthonomus grandis* of a trypsin inhibitor from chickpea seeds. Comp. Biochem. Physiol. Part B 140:313–319.

Gupta P, Dhawan K, Malhotra SP, Singh R (2000) Purification and characterization of trypsin inhibitor from seeds of faba bean (*Vicia faba* L.). Acta Physiol. Plant. 22:433-438.

Hajela N, Sharma AH, Sharma S, Rao DN, Hajela K (1999) Studies on a double headed protease inhibitor from *Phaseolus mungo.* J. Plant Biochem. Biotech. 8:57-60.

Haq SK, Khan RH (2003) Characterization of a proteinase inhibitor from *Cajanus cajan* (L.). J. Protein Chem. 22:543-554.

Harsulkar AM, Giri AP, Patankar AG, Gupta VS, Sainani MN, Ranjekar PK, Despande VV (1999) Successive use of non-host plant proteinase inhibitors required for effective inhibition of *Helicoverpa armigera* gut proteinases and larval growth. Plant Physiol. 121:497-506.

Johnston KA, Gatehouse JA, Anstee JH (1993) Effect of soybean protease inhibitors on the growth and development of larval *Helicoverpa armigera.* Insect Physiol. 39:657-664.

Johnston KA, Gatehouse JA, Anstee JH (1991) In vitro and In vivo studies of the effects of plant proteinases inhibitors on *Helicoverpa armigera* larvae. J. Exp. Bot. 42:238.

Kakade ML, Simons N, Liener IE (1969) The evaluation of natural vs synthetic substrates for measuring the antitrypsin activities of soybean samples, Cereal Chem. 46:518-526.

Kansal, R, Gupta, RN, Koundal, KR, Kuhar, K, Gupta, VK (2008) Purification, characterization and evaluation of insecticidal potential of trypsin inhibitor from mungbean (*Vigna radiata* L. Wilczek) seeds. Acta Physiol. Plant. 30:761-768.

Koiwa H, Bressan RA, Hasegawa PM (1997) Regulation of protease inhibitors and plant defense. Trends Plant Sci. 2:379-384.

Laemmli EK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227:680-685.

Laskowski Jr. M, Qasim MA (2000) What can the structures of enzyme-inhibitor complexes tell us about the structures of enzyme substrate complexes? Biochem. Biophys. Acta 1477:324-337.

Lawrence JC, Nielsen SS (2001) Partial characterization of a cysteine proteinase inhibitor from Lima bean (*Phaseolus lunatus*). J. Agric. Food Chem. 49:1020-1025.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:1265-275.

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

Mandal S, Kundu P, Roy B, Mandal RK (2002) Precursor of the inactive 2S seed storage protein from the Indian mustard *Brassica juncea* is a novel trypsin inhibitor. Characterization, post-translational processing studies, and transgenic expression to develop insect-resistant plants. J. Biol. Chem. 277:37161-37168.

Marconi E, Ng NW, Carnovale E (1993) Protease inhibitors and lectins in cowpea. Food Chem. 47:37-40.

Marquez, MC, Alonso, R (1999) Inactivation of trypsin inhibitor in chickpea. J. Food Comp. Anal. 12:211-217.

Murdock LL, Shade RE (2002) Lectins and protease inhibitors as plant defenses against insects. J. Agric. Food Chem. 50:6605-6611.

Patankar, AG, Harsulkar, AM, Giri, AP, Gupta, VS, Sainani, MN, Ranjekar, PK, Deshpande, VV (1999) Diversity in inhibitors of trypsin and *Helicoverpa armigera* gut proteinases in chickpea (*Cicer arietinum* L.) and its wild relatives. Theor. Appl. Genet. 99:719-726.

Purcell JP, Greenplate, Sammons RD (1992) Examination of midgut luminal proteinase activities in six economically important insects. Insect Biochem. Mol. Biol. 22:41–47.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, second edition, Cold Spring Harbor Laboratory Press, USA.

Sessa DJ, Nelsen TC (1991) Chemical inactivation of soybean proteinase inhibitors. JAOCS. 68(7):463-470.

Singhal R (2004) Isolation and Characterization of protease inhibitors from various indigenous legumes, Department of Biochemistry, Kurukshetra University, Kurukshetra, India. PhD Thesis.

Sierra, IL, Quillien, L, Flecker, P, Gueguen, J, Brunie, S (1999) Dimeric crystal structure of a Bowman-Birk protease inhibitor from pea seeds. J. Mol. Biol. 285(3):1195-1207.

Srinivasan A, Giri AP, Harsulkar AM, Gatehouse JA, Gopta VS (2005) A Kunitz trypsin inhibitor from chickpea (*Cicer arietinum* L.) that exerts anti-metabolic effect on podborer (*Helicoverpa armigera*) larvae. Plant Mol. Biol. 57:359-374.

Sudheendra K, Mulimani VH (2002) Effect of feeding legume proteinase inhibitors on *Helicoverpa armigera* gut proteinase activity. ICPN 9:51-53.

Telang M, Srinivasan A, Patankar A, Harsulkar A, Joshi V, Damle A, Deshapande V, Sainani M, Ranjekar P, Gupta G, Birah A, Rani S, Kachole M, Giri A, Gupta V (2003) Bitter gourd proteinase inhibitors:potential growth inhibitors of *Helicoverpa armigera* and *Spodoptera litura*. Phytochemistry 63:643-652.

Veera Reddy GG, Bhattacharya AK (1990) Development of semi-synthetic diets for the rearing of *Heliothis armigera* (Hub.). J. Insect Sci. 3:23-33.

Whitley EJ, Bowman DE (1975) Isolation and properties of navy beans proteinase inhibitor component I. Arch. Biochem. Biophys. 169:42-50.