RESEARCH ARTICLE

Purification of trypsin inhibitor from seeds of *Cicer arietinum* (L.) and its insecticidal potential against *Helicoverpa armigera* (Hübner)

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Received: 27 February 2013; Accepted: 20 June 2013

ABSTRACT: Trypsin inhibitors (TI) have crucial functions in plant defense against pests, and recent studies reveal their diversity in stress response. Disease and herbivore infestations are harmful to plant growth, but the response of TI in Cicer arietinum (L.) in Central India has been less studied. In the present study, we partially characterized the TI from Cicer arietinum (L.) seeds and evaluated its insecticidal potential against Helicoverpa armigera (Hübner). A 20 kDa trypsin inhibitor was purified to homogeneity by ammonium sulfate precipitation and chromatographies with Sephadex G-100 and diethylaminoethyl cellulose (DEAE-cellulose-52) ion-exchange column. TI assay categorized 17 cultivars in three groups: with high (more than 70%), intermediate (16–70%) and very low (0–15%) TI activity. Moreover, results from the TI activity coincided with herbivore attack studies carried out in J.N.K.V.V. fields earlier. Together, our results suggest that the presence of TI triggers a signal that leads the dynamics of stress response and, in turn, regulates pest resistance in plants. Feeding experiments conducted with 5th instar larvae suggested dose-dependent decrease for both the larval weight and the survival of the larva.

KEYWORDS: bioassay, *Helicoverpa armigera* gut proteinase (HGP), resistant, susceptible, protein.

ABBREVIATIONS: TIA: trypsin inhibitor activity, HGP: *Helicoverpa armigera* gut proteinase; *p*NA: para-nitroaniline; R_{NI}: resistant non-infested cultivars; S_I: susceptible infested cultivars; CaTI: *Cicer arietinum* trypsin inhibitor; TCA: trichloroacetic acid; BA*p*NA: Nα-Benzoyl-D, L-arginine 4-nitroanilide hydrochloride.

INTRODUCTION

Cicer arietinum is an important self-pollinated diploid crop 2n=2x=16 with genome sized approximately 931 Mbp (Arumuganathan and Earle 1991). India is the largest producer of chickpea, contributing with about 66% of the total global production, followed by Turkey, Pakistan and Mexico (Thangwana and Ogola 2012). It is a major source of protein (12.4-31.5%), energy, fiber, vitamins, minerals and essential amino acids such as tryptophan and lysine (Maiti 2001).

Helicoverpa armigera Hübner (Lepidoptera: Noctuidae) is a polyphagous pest of 182 plant species across 47 families in the Indian subcontinent, out of which 56 are heavily damaged and 126 are rarely affected. *Helicoverpa armigera* (Hübner), also known as cotton bollworm or legume pod borer, is one of the most devastating crop pest of *Cicer arietinum* since it inflicts annual losses of over US$ 328 million. Losses caused only by this pest reported up to US$ 17 million in crops like cotton, pigeon pea, chickpea, groundnut, sorghum, pearl millet, tomato and others

of economic importance (Chaturvedi 2007). Migration by *Helicoverpa armigera* is a wide-spread phenomenon and causes major crop losses. As *H. armigera* has high reproductive and damage potentials, their suppression becomes inevitable.

The best counter action to control of *H. armigera* infestation in *Cicer arietinum* (L.) fields is the use of chemical insecticide and pesticides. But due to the high costs of insecticides, pesticides and their risk for the balance of nature and human health, proteinase inhibitor becomes a defense alternative by creating an insect-resistant plant (Ryan 1990). Plants synthesize proteinase inhibitors, alpha-amylase inhibitors, lectins and chitin binding proteins to resist herbivorous insects, pathogens and wounding (De Leo et al. 2001). Proteinase inhibitors have also been shown to act as a defensive compound against phytophagous insects by the direct assay or expression in transgenic crop plants (Koiwa et al. 1998, Vain et al. 1998). Studies have shown that a major part of *Helicoverpa armigera* gut proteinase activity can be blocked by soybean kunitz trypsin inhibitor (Johnston et al. 1991, Harsulkar et al. 1999). Numerous insect-feeding bioassays and experiments with transgenic plants have also shown the delayed growth and development of the insect (Koiwa et al. 1998, Parde et al. 2010).

Many studies have indicated the relevance of proteinase inhibitor (trypsin inhibitor) for plant defense. The overexpression of both endogenous and exogenous inhibitors is another option to augment the defense mechanism (Lawrence and Koundal 2005). In addition, certain parameters like *viz*. insect gut pH, larval developmental stage, concentration of PI and the better understanding of how insects respond and adapt to PIs influence its effectiveness (Dunse and Anderson 2011).

There is a continuous search for new inhibitors that are able to oppose such pest adaptation *via* antimetabolic activity. Moreover, with genetic engineering replacing one or more PI domains in the multidomain precursor, with PIs tailored w.r.t., gut proteinases of a target insect are also being considered (Ellis and Jones 1998). Thus, the present investigation was focused on the purification and characterization of trypsin inhibitor from the chickpea (CaTIs), the concentration of purified trypsin inhibitor, and the effectiveness under insect gut pH with its antimetabolite effect on the growth and development of *H. armigera*.

MATERIALS AND METHODS

Plant material : *Cicer arietinum* cultivar seeds (17) were obtained from the Department of Plants Genetics & Breeding, Jawaharlal Nehru Krishi Vishvavidyalaya (J.N.K.V.V.), Jabalpur, India. Each plant was grown from a single seed, planted in a rectangular and maintained under greenhouse conditions. Prior to *H. armigera* infestation, studies on all 17 cultivars had been carried out (M. Nair, unpublished results).

Extraction of trypsin inhibitor from seeds of *Cicer arietinum* (L.) cultivars: Dekernalised *Cicer arietinum* seeds (100 g) were crushed, depigmented and defatted with chilled acetone (300 mL) four to five times, and with n-hexane (200 mL) twice, respectively. These solvents were filtered and seed powder was recovered after air drying. The resulting flour was suspended in 10 mM phosphate buffer, pH 7.2, at 5°C for 6 h with continuous stirring. After centrifugation at 12,000 x g for 30 min at 4°C, crude fraction was used to determine trypsin inhibitor activity via subsequent dot blot assay and proteinase inhibitor activity.

Analysis of trypsin inhibitor by the dot blot method : Crude extracts of all 17 cultivars were tested for trypsin inhibitor activity using two natural substrates (casein and gelatin) with trypsin (EC 3.4.21.4) being the enzyme. Gelatin plates were prepared by adding 1 g of agar, 0.1% (w/v) gelatin to autoclaved 100 mL of 100 mM Tris-HCl buffer (pH 8.0) *via* continuous stirring in a water bath at 80°C. The mixture was then poured into sterile petri dishes under sterile conditions and solidified. Similarly, casein plates were prepared by adding 1 g of hammerstein casein to 50 mL of autoclaved Tris-HCl buffer (100 mM, pH 8.0). The solution was dissolved in a water bath at 80°C with precautionary measures to avoid the coagulation of the casein due to overheating. The solution was then added to 50 mL of 1% (w/v) premelted autoclaved agar in 100 mM Tris-HCl buffer (pH 8.0). The resulting mixture was later poured into sterile petri plates and left undisturbed until becoming solid.

Crude extracts of different concentrations (10 to 1000 µg) were pipetted into respective wells with trypsin enzyme as control. The reaction was terminated by the addition of 5 mL of 5% TCA, in case of casein, and 2% $HgCl_2$, in case of gelatin. Observations were recorded for control (where no crude extract was added) and experimental sets. Casein plates were stained with Coomassie Brilliant Blue for 30 min. Protein concentration was estimated by the Lowry method using bovine serum albumin as the standard (Lowry et al. 1951).

Trypsin inhibitor assay : The trypsin inhibitor (TI) activity was assessed by incubating 50 µl of crude extract with 20 µl of commercial bovine trypsin (1 mg mL^{-1}) and incubating at 37°C for 15 min, by adopting the method given by Kakade et al. (1974) and Nagashima et al. (2004). Then, 40 µl (from the stock solution of 10 mg mL^{-1} in Dimethyl Sulfoxide) BA*p*NA was added to the assay solution and the mixture was further incubated at 37°C for 30 min. Reaction was terminated by adding 500 µl of 10% glacial acetic acid and the absorbance of the reaction mixture was

measured at 410 nm against a reference blank without trypsin and a blank containing crude extract without BA*p*NA in order to subtract the absorbance from the yellow pigment of the crude extract. Trypsin inhibitory activity was measured by obtaining the difference between the enzyme activity in the absence and in the presence of inhibitors.

Purification of trypsin inhibitor : The crude extract was filtered and subsequently saturated with ammonium sulfate in three stages [0 to 40% (105.27 g), 40 to 60% (58.50 g) and 60 to 90% (100.74 g)] to precipitate the protein at 4°C. After the centrifugation at 12,000 x *g* for 30 min at 4°C, the resulting precipitate was dissolved in the minimum volume of distilled water (pH 7.5) and dialyzed against 3 × 500 mL of 10 mM Tris/HCl buffer, pH 7.5, and loaded on Sephadex G-100 (Pharmacia, Sweden), which was earlier equilibrated with chilled 10 mM Tris/HCl buffer, pH 7.5. When the sample had thoroughly percolated into the column, it was then washed with 10 mM Tris-HCl buffer (pH 7.8) (five times the column length). Fractions (2 mL each) were collected and tested for protein (using the Lowry method) and trypsin inhibitory activity (using BA*p*NA as substrate).

Active fractions with trypsin inhibitor activity (Fraction A) were further purified by anion exchange chromatography. Active fractions were precipitated with the addition of chilled acetone (in ice cold condition) and centrifuged at 10,000 x *g* at 4°C for 15 min. Pellets were redissolved in minimum volume of distilled water (pH 7.5) and loaded to Diethylaminoethyl cellulose (DEAE-cellulose-52) ion-exchange column, which was pre-equilibrated with 100 mL of chilled 50 mM Tris/HCl buffer, pH 7.5. Bound proteins were eluted with a linear salt gradient of 0–2 M NaCl. Fractions of 2 mL were collected and monitored for protein (A_{280}) and trypsin inhibitor activity, as aforementioned.

Electrophoretic analysis of trypsin inhibitor : Electrophoretic analysis was performed in two stages: one undergoing 12% (w/v) SDS polyacrylamide gel and another gel containing 0.1% gelatin (w/v) to determine the trypsin inhibitor. Ge1 was run at a constant voltage of 50 V at 4°C in Tris Glycine-SDS (TGS) buffer (25 mM Tris/HCl pH 8.8, 192 mM glycine and 0.1% (w/v) SDS). Activity staining was carried out according to the protocol followed by Mulimani et al. (2002). Molecular mass of the protein was determined by comparing them with soybean trypsin inhibitor (Calbiochem®, USA) as standards, and the protein molecular weight marker.

Extraction of proteinase from larvae of *Helicoverpa armigera* (Hüber) : For extraction of proteinase, fifth instar *Helicoverpa armigera* larvae were collected from chickpea fields of J.N.K.V.V., Jabalpur. Mid-guts of *H. armigera* were isolated by dissecting the larvae and mixing the gut tissue with three volumes of chilled 0.1 M glycine-NaOH buffer, pH 10.0. Gut luminal contents were allowed to stand for 15 min centrifuged at 10,000 x *g* for 10 min at 4°C analyzed for protein content and proteinase activity.

Determination of proteinase activity in *Helicoverpa armigera* gut proteinase (HGP) : Total gut proteinase activity was measured by performing the dot blot assay and proteinase activity using casein and BA*p*NA without trypsin inhibitor, according to the protocol by Erlanger et al. (1964) with some modifications.

***In vitro* activity of CaTI in HGP :** The experiment was carried out in three slots. Firstly, the activity of trypsin inhibitor towards the midgut of *Helicoverpa armigera* larvae (HGP) at pH 7.8 and 10 was determined. Secondly, the trypsin inhibitory activity using bovine trypsin (BT) and HGP was compared to determine their proteolytic activity, and thirdly, the trypsin inhibitor activity of a crude extract, fraction A (gel filtrate fraction) and anion exchange fractions were determined with bovine trypsin and HGP as an enzyme. BA*p*NA was used as a substrate at the standard conditions described earlier, in order to test the *in vitro* activity of CaTI on HGP.

***In vivo* activity of trypsin inhibitor (TI) against *H. armigera* :** Bioassays were conducted by feeding *H. armigera* larvae on chickpea TIs incorporated to an artificial diet at 28°C and 55% stable humidity (Giri and Kachole 1998). Bioassays were conducted in a 40 mL artificial diet [140 g of chickpea seed meal, 14 g of yeast extract, 0.4 g of Bavistin (BASF, Mumbai), 0.2 mL of formalin, 4.3 g of ascorbic acid, 1.3 g of sorbic acid, 2.6 g of methyl benzoate, 0.5 g of tetracycline, one tablet of vitamin-B complex and two drops of vitamin E], which was added to 450 mL of distilled water. To this mixture, 17 g of agar dissolved in 500 mL of water (50–60°C) was added, mixed and poured into trays which were cut into cubes (2 g) and used in feeding experiments. Trypsin inhibitor was incorporated to the diet at concentrations of 10, 100 and 500 µM, and weight was monitored in intervals of 2 d until pupation. In negative controls, distilled water was added with no chickpea TIs added to the artificial diet.

RESULTS

Cicer arietinum (L.) trypsin inhibitor was purified to homogeneity in four steps: by the preliminary identification *via* the dot blot assay, trypsin inhibitor assay, ammonium

sulfate precipitation, Sephadex G-100, and anion exchange chromatography (DEAE-cellulose-52 column). In the present study, trypsin inhibitor activity was detected in all 17 *Cicer arietinum* (L.) cultivars' crude extract by both the dot blot and the proteinase inhibitor assays.

Dot blot Assay : In the dot blot assay, ten cultivars (JG-63, JG 99-115, ICCV-2, KAK-2, JG 2004-944, JG 2003-108, JG 2001-12, JGK-334, JGK-333, JGK-8) produced no halo zone, whereas seven cultivars (C-134, JG-16, ICC-11550, JGK-1, JGK-7, JGK-313, JGK-3) showed complete halo zone as shown in Figure 1 and Table 1. These results were compared to infestation trials held up earlier in J.N.K.V.V. fields (M. Nair, unpublished results), which completely showed the direct variation between *H. armigera* infested and non-infested cultivars, thereby creating two groups: (1.) Non-infested cultivars as being resistant (R_{NI}), and (2.) Infested cultivars as being susceptible (S_I).

Trypsin Inhibitor Assay : Results of trypsin inhibitor activity (TIA) were similar to those in the dot blot assay, in which trypsin inhibitor activity was found to be the lowest in seven cultivars (JGK-313, JGK-1, JGK-3, JGK-7, JG-16, C-134, ICC-11550 and JGK-334) and the highest in ten cultivars (JG-63, JG 99-115, ICCV-2, KAK-2, JGK-333, JG 2004-944, JG 2003-108, JGK-8 and JG 2001-12), which have been earlier categorized as being susceptible infested (S_I) and resistant non-infested (R_{NI}) cultivars in infestation studies as described in Table 3.

Trypsin proteinase activity using both natural (casein) and synthetic substrate (BA*p*NA), without inhibitor, was 227.66±0.5 nmol tyr min^{-1} mg^{-1} protein and 172.01±0.5 µmol *p*NA min^{-1} mg^{-1} protein, respectively. The result of TIA indicated the inhibition in trypsin activity as the release of the *p*NA [*p*-nitroaniline] product. In case of R_{NI} cultivars, the release of *p*NA was very low, being substantially higher in S_I cultivars in relation to control, in which trypsin enzyme activity was observed to be 100%.

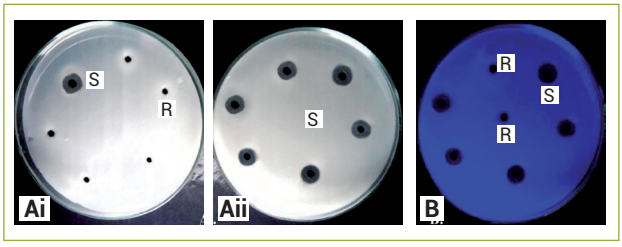

Figure 1. Dot Blot assay using crude extract of *Cicer aritienum* (L.) cultivars. (Ai,ii) No halo zone was found in R_{NI} (JG-63, JG 99-115, JG 2003-108, JG 2001-12, JGK-8) with respect to SI (C-134, JG-16, ICC-11550, JGK-1, JGK-7, JGK 313, JGK-3) cultivars which showed clear halo zone in gelatin amended plates. (B) Casein amended plates were stained with Coomassie Brilliant Blue stain for 30 min.

In resistant non-infested plants (R_{NI}), the trypsin inhibitory activity was higher with increased inhibitor concentration, or it was in intermediate concentrations, whereas no inhibition was seen in susceptible infested cultivars (S_I). These data are shown in Table 2

Trypsin inhibition percentage varied from 0–99% and 10–99.4% using casein and BA*p*NA, respectively. The low percentage of inhibition (0-15%) was due to the absence of trypsin inhibitor in crude extract, whereas in R_{NI} plants the hydrolysis of casein and BA*p*NA was maximally inhibited, thereby showing a 99.9% inhibition. These data are shown in Table 2. Cultivars were therefore ranked into three classes according to trypsin inhibition percentage using BA*p*NA results:

1. Low TIA group of cultivars, from 0–15% of inhibition
2. Intermediate group of cultivars, from 16–70%
3. A high TIA group of cultivars, higher than 70%

Cultivars (i.e. JGK-1, JGK-7, JGK-313) with intermediate values were also grouped into resistant (R_{NI}) ones, as shown in Table 3.

Purification of *Cicer arietinum* Trypsin Inhibitor (CaTI) : JG 2001-12 indicated a 99.9% trypsin inhibitory activity, as shown in Table 2, hence it was further used for purification, *in vitro* and *in vivo* studies against *H. armigera.* Trypsin inhibitor was purified to homogeneity with 65.4 fold purification using ammonium fractionation and ion exchange

Table 1. Dot Blot Assay

Cultivars	Halo zone	Trypsin Inhibitor	Infestation studies
C-134	-	Absent	S_I
JG-16	-	Absent	S_I
JG-63	+	Present	R_{NI}
JG 99-115	+	Present	R_{NI}
JG 2004-944	+	Present	R_{NI}
JG 2003-108	+	Present	R_{NI}
JG 2001-12	+	Present	R_{NI}
ICC-11550	-	Absent	S_I
JGK-1	-	Absent	S_I
JGK-3	-	Absent	S_I
JGK-8	+	Present	R_{NI}
JGK-7	-	Absent	S_I
JGK-334	+	Present	R_{NI}
JGK-333	+	Present	R_{NI}
JGK-313	-	Absent	S_I
KAK-2	+	Present	R_{NI}
ICCV-2	+	Present	R_{NI}

R_{NI}: Cultivar Resistant to *Helicoverpa armigera* infestation; S_I: Cultivar susceptible to *Helicoverpa armigera* infestation, per infestation studies.

chromatography where trypsin inhibitor was eluted, using a NaCl gradient (0 to 2 M) as shown in Figure 2B.

The active fractions of anion exchange peak were pooled, concentrated and analyzed later for TIA. This protocol yielded a purified trypsin inhibitor, with 140 µM *p*NA min^{-1} mg^{-1} specific activity, and low protein content, of 0.1 mg. The data in Table 4 showed an overall 65.4 fold increase in purificarion fold specific activity with 10% activity yield. The purity of the protein was analyzed by SDS-PAGE, which showed a single thick polypeptide band with molecular mass of approximately 20 kDa. The molecular mass was apparently homogenous to the standard molecular weight marker and soybean trypsin inhibitor, thus indicating its purity. The presence of an activity band in reverse zymography gel at the position corresponding to the protein band in SDS-PAGE confirmed the protein band as a trypsin inhibitor; these data are shown in Figure 3A and 3B.

Proteolytic activity of HGP extract : Proteolytic activity of HGP was 148.3 nmol min^{-1} mg^{-1} protein using casein as the natural substrate and 390±0.5 nmol min^{-1} mg^{-1} protein using BA*p*NA as substrate. HGP extracts equivalent to 40 µg showed halo zones in the dot blot assay, indicating its proteinase activity as shown in Figure 4 and Table 5.

Effect of CaTI on HGP extract : Inhibition assays using HGP extract and purified TI from *Cicer arietinum* (CaTI) demonstrated inhibition of HGP at both pH 7.8 and 10. A 45–50% inhibition of the HGP activity at pH 7.8 and

Table 2. Trypsin activity and Trypsin Inhibition (%) of crude extracts in different cultivars of *Cicer arietinum* (L.)

Addition	Trypsin Activity		Trypsin Inhibition (%)		
	Casein	BA*p*NA	Casein	BA*p*NA	
Cultivar	nmol tyr min^{-1} mg^{-1} protein	µmol *p*NA min^{-1} mg^{-1} protein	Trypsin Inhibition (%)	Trypsin Inhibition (%)	TIA Group*
None (control)	227.6±0.5	172.01±0.5	0	0	-
		Crude extract			
C-134	209±5.7	154.2±0.3	9.0	10.0	Low
JG-16	200±0.5	145.2±0.3	12.0	15.5	Low
JG-63	4.2±0.2	2.2±0.3	98.0	98.0	High
JG 99-115	3.6±0.1	1.4±0.3	98.0	99.0	High
JG 2004-944	7.0±0.2	2.2±0.3	96.0	98.7	High
JG 2003-108	5.2±0.2	3.2±0.3	97.7	98.1	High
JG 2001-12	0.7±0.4	1.0±0.3	99.0	99.4	High
ICC-11550	197±0.2	150.2±0.3	13.0	12.8	Low
JGK-1	199±0.2	144.2±0.3	12.0	16.3	Intermediate
JGK-3	197±0.2	153.2±0.3	13.0	11.1	Low
JGK-8	4.7±0.2	3.2±0.3	97.9	98.1	High
JGK-7	200±0.2	134.2±0.3	12.9	21.9	Intermediate
JGK-334	2.4±0.2	2.2±0.3	98.0	98.7	High
JGK-333	3.7±0.2	3.2±0.3	98.0	98.1	High
JGK-313	201±0.2	124.2±0.3	11.7	27.7	Intermediate
KAK-2	2.3±0.2	3.2±0.3	98.0	98.1	High
ICCV-2	4.2±0.2	5.2±0.3	98.0	96.0	High

TIA group = Classes of trypsin inhibitory activity.

Table 3. Seventeen cultivars of *Cicer arietinum* (L.) grouped into two TIA groups

Name of Cultivars	TIA units	Result of Infestation studies
JG-63, JG 99-115, ICCV-2, KAK-2, JG 2004-944, JG 2003-108, JG 2001-12	higher than 70%.	RESISTANT (R_{NI})
JGK-334, JGK-333, JGK-8	16–70%	
C-134, JG-16, ICC-11550, JGK-1, JGK-7, JGK-313, JGK-3	0–15%	SUSCEPTIBLE (S_I)

R_{NI}: Cultivar Resistant to *Helicoverpa armigera* infestation; S_I: Cultivar susceptible to *Helicoverpa armigera* infestation

30–35% inhibition at pH 10.0, respectively, were observed. This repression in the proteolytic activity of HGP was due to the presence of CaTI as compared to control (where no CaTI was added), in which the activity was found to be 100%, as demonstrated in Table 6. Figure 5 shows the decreased proteolytic activity of bovine trypsin (BT) and HGP in the presence of CaTI. A close examination revealed that JG 2001-12 cultivar fractions at different purification stages showed an increased inhibitory activity against HGP, as shown in Table 7. It was also observed that, with further purification steps, the inhibitory activity increased as anion exchange fraction exhibited 66.34±5.66 µmol *p*NA $min^{-1}mg^{-1}$ protein inhibition towards HGP w.r.t. crude extracts and Fraction A (gel filtrate pooled fraction) of gel filtration.

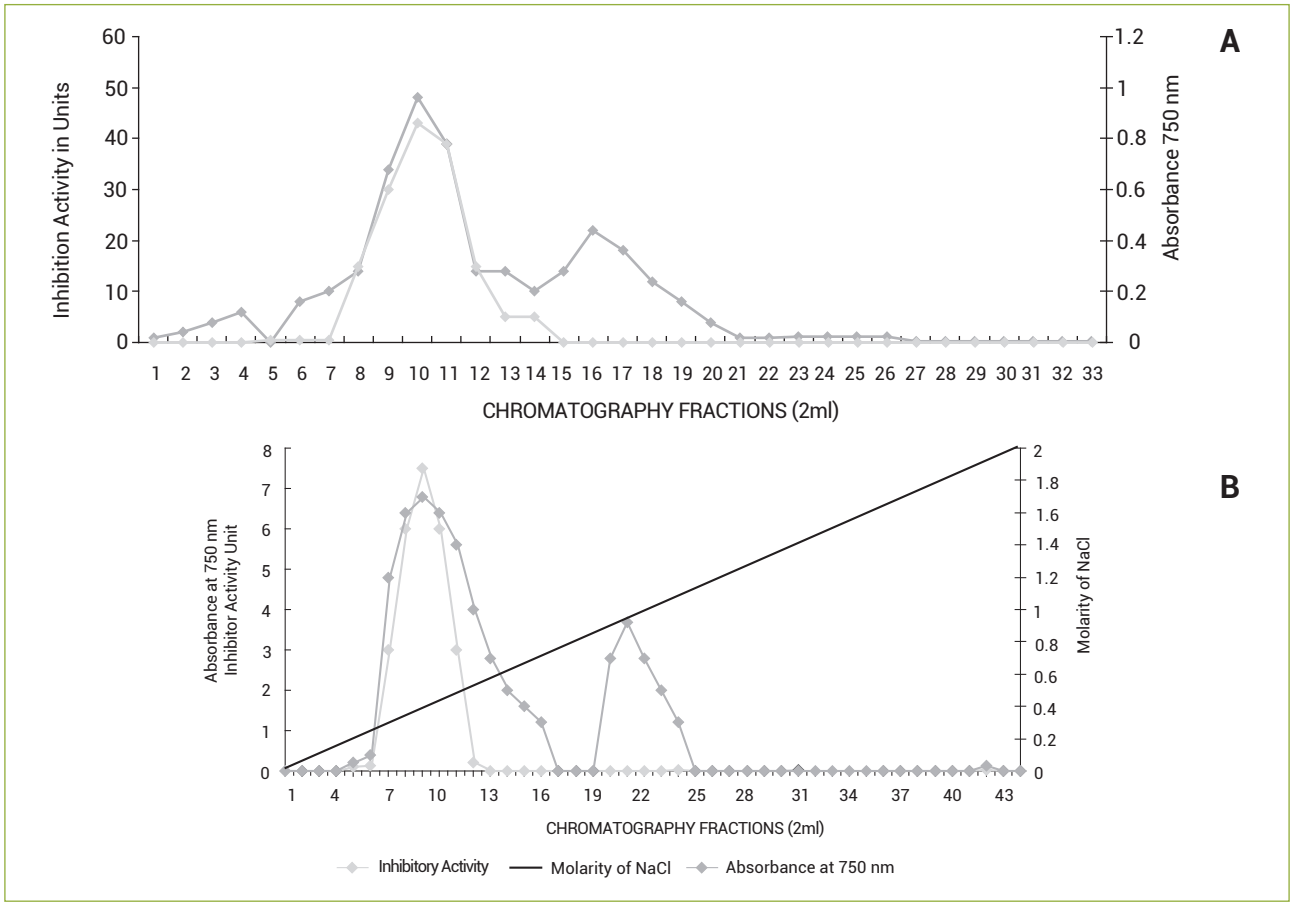

Figure 2. Elution profile of 40-60% ammonium sulfate-precipitated protein loaded onto a gel filtration column (Sephadex G-100). The protein was eluted out with 10 mM Tris-HCl buffer (pH 7.8). 2 mL fractions were collected and analyzed for their inhibitory activity using proteinase inhibitor assay and synthetic substrates (A). Elution profile of the trypsin inhibitor during DEAE-cellulose anion exchange chromatography (B).

Table 4. Extraction Profile for Trypsin Inhibitor from the JG 2001-12 cultivar

Sample	Protein (mg ml^{-1})	Total protein (mg)	Total activity (µM *p*NA min^{-1})	Specific Activity (µM *p*NA $min^{-1}mg^{-1}$)	Trypsin Inhibition*	Yield	Purification Fold
Crude Extract	8.6	258.0	20	2.32	44.5	100	1.0
40–60% ammonium sulfate	7.8	19.6	19	2.42	47.3	95	12.4
Dialysis	5.0	14.0	17	3.40	52.7	85	55.1
Sephadex G-100	3.6	9.0	5	72.54	86.7	25	57.1
DEAE-cellulose-52 column	0.2	0.1	2	140.00	94.4	10	65.4

*The obtained values were compared with control, in which trypsin inhibitor was replaced with 0.1 M Tris/HCl buffer, pH 7.5. Trypsin inhibition was carried out after each step of extraction.

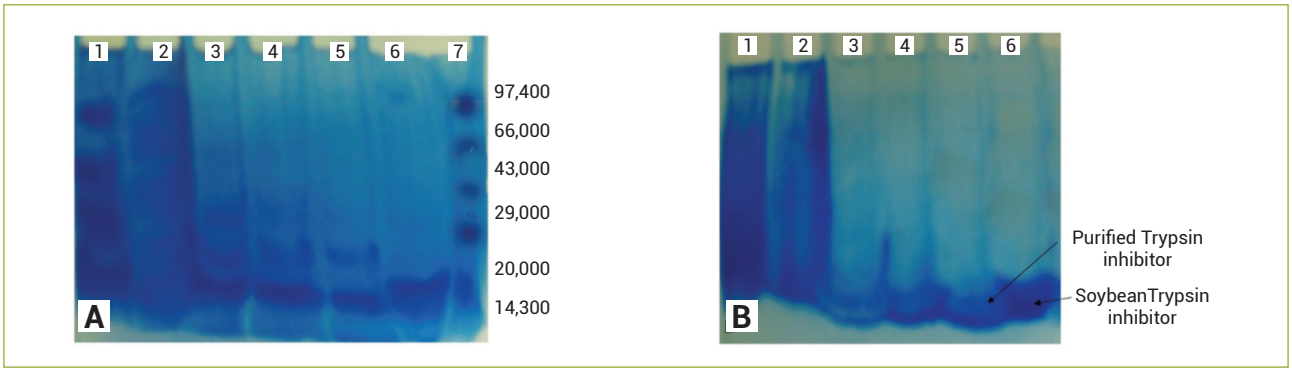

Figure 3. SDS-PAGE of trypsin inhibitor. The samples were mixed with the tracking dye containing bromophenol blue and SDS. Lane 1 Crude Extract Lane 2 40–60% ammonium sulfate cut off, Lane 3 Fraction 9, Lane 4 Fraction 10, Lane 5 Fraction 11, Lane 6 purified trypsin inhibitor after anion exchange column chromatography, Lane 7 Molecular weight marker (A). Reverse zymography of trypsin inhibitor. Lane 1, Crude Extract, Lane 2 40–60% ammonium sulfate cut off, Lane 3 Fraction 9, Lane 4 gel filtration Fraction, Lane 5 purified trypsin inhibitor after anion exchange column chromatography, Lane 6 Standard soybean trypsin inhibitor (Sigma) (B).

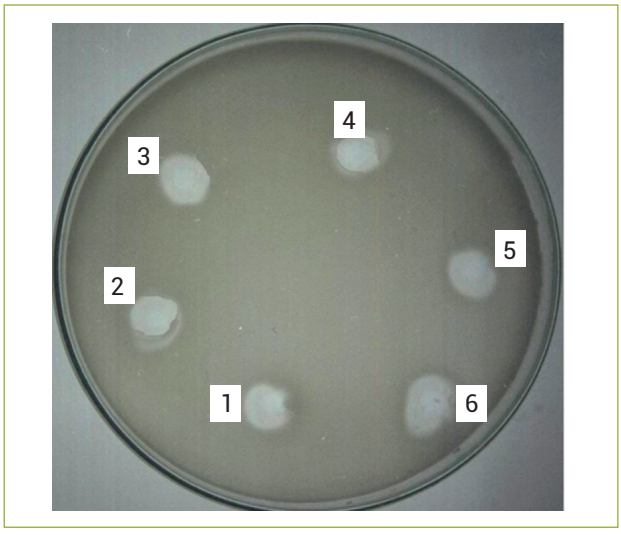

Figure 4. Dot Blot assay showing proteolytic activity of *Helicoverpa armigera* gut proteinases (HGP). Halo zone was achieved in gelatin amended plates subjected to HGP extract (1–40 µg, 2–50 µg, 3–60 µg, 4–70 µg, 5–80 µg, 6–90 µg).

Effects of ingestion of CaTI on larval digestive proteases : A reduction in weight gain among the fifth instar larvae fed on CaTI supplemented diet was observed, thus indicating an *in vivo* effect on endogenous protease activity of *H. armigera*. At low concentration (10 µM), no drastic weight loss was observed, whereas at 100 µM and 500 µM, a significant reduction in weight was observed in comparison to control. The presence of 500 µM of CaTI in the diet caused several deleterious effects, thus instigating death. As the concentration of trypsin inhibitor increased, the weight of larvae decreased, pointing to a trivial effect of trypsin inhibitor on the growth and development of *H. armigera* insect, as shown in Figure 6. The increased

Table 5. Gut proteinase activity of *Helicoverpa armigera* larvae using natural and synthetic substrates

Addition	Protease activity [nmol min^{-1} (mg protein)$^{-1}$]	
	Casein	BApNA
HGP extract	148.3±0.4	390±0.5

Table 6. Proteolytic assays of midgut extract from *Helicoverpa armigera* larvae with CaTI at two different pHs (7.8 and 10) and at a constant temperature of 25°C. Activity is presented as the percentage of the control activity

Treatments	Inhibition Percentage (%)			
	Casein		BApNA	
	pH – 7.8	pH – 10	pH – 7.8	pH – 10
Control	100	100	100	100
CaTI + HGP	45	30	50	35

CaTI: *Cicer arietinum* Trypsin inhibitor; HGP: *Helicoverpa armigera* gut proteinases

Table 7. Trypsin Inhibitor activity of JG 2001-12 cultivar in the midgut protease of *Helicoverpa armigera* (HGP)

Treatments	Trypsin Inhibitory Activity TIA mg $^{-1}$ protein	Inhibition of *Helicoverpa* Gut Proteinase
	µmol *p*NA $min^{-1}mg^{-1}$ protein	
Crude extract of JG 2001–12	8.71±1.21	28.56±1.32
Fraction A	11.36±3.62	35.71±4.09
Anion Exchange Fraction	39.47±1.91	66.34±5.66

concentration also showed abnormality and subsequent death of the larvae. Tryptic activities in the midgut from 5th instar larvae reared on artificial diets containing CaTI altered food accumulation wich led to decrease in weight, leading to growth retardation. It indicated a possible response of starvation in these instars, compared with control.

DISCUSSION

In this communication, we have isolated CaTI from resistant cultivar, JG 2001–12, which is significantly at par with that obtained from *Vigna mungo* and *Derris trifoliate* (Bhattacharyya and Babu 2009, Prasad et al. 2010).

Purification and biochemical activity of CaTI :

C. arietinum (chickpea) seeds are known to contain inhibitors of proteases, and their properties have been studied (Belew and Eaker 1976, Smirnoff et al. 1979, Jibson et al. 1981, Sastry and Murray 1987, Saini et al. 1992, Harsulkar et al. 1997). The screening of 17 cultivars was firstly carried out using the dot blot technique, which provided a direct method for screening trypsin inhibitor protein in crude extracts of various cultivars, whereas the proteinase inhibition assay resulted in a quantitative amount of trypsin inhibitor in these cultivars. *H. armigera* infestation studies on the fields provided a connection between pest resistance and the presence of trypsin inhibitor, as studies confirmed the idea that inhibitors of serine proteinase interfere with the digestive processes of insects (Rai et al. 2008, Valueva et al. 2012).

The purification of CaTI was carried out using ammonium sulfate precipitation and subsequent dialysis. Preliminary and subsequent assays to determine CaTI confirmed the presence of trypsin inhibitor in JG 2001–12. BApNA and casein substrates provided quantitative data regarding the presence and absence of CaTI in cultivars. Gel filtration chromatography and anion exchange chromatography purified trypsin inhibitor from JG 2001–12 cultivar was found to be 0.45 g of trypsin inhibitor per kilogram. Further purification steps, using reverse zymography confirmed the presence of trypsin inhibitor in R_{NI} cultivar *viz*. JG 2001–12, where an inhibitor of 20 kDa was determined by SDS-PAGE and reverse zymography. Sharma and Suresh (2011) purified an 18 kDa Kunitz-type trypsin inhibitor protein (CPTI) from chickpea seeds. On the contrary, Srinivasan et al. (2005) identified and purified a low expressing proteinase inhibitor (PI), different from the Bowman-Birk inhibitors from chickpea seeds.

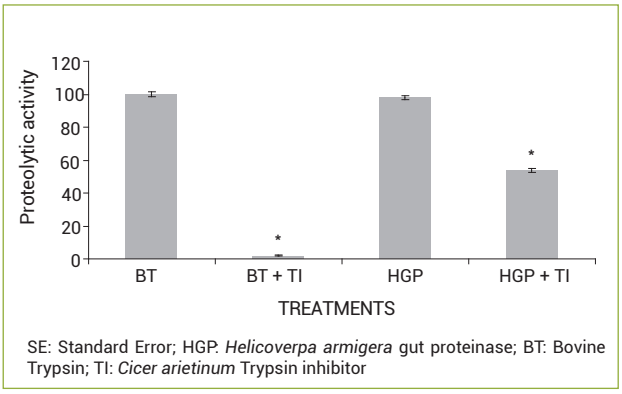

Figure 5. Inhibitory activity of CaTI towards bovine trypsin and proteinases from midgut of *Helicoverpa armigera*. Values are expressed as mean±SM (n=6). Bars with asterisks are significantly different (p>0.05).

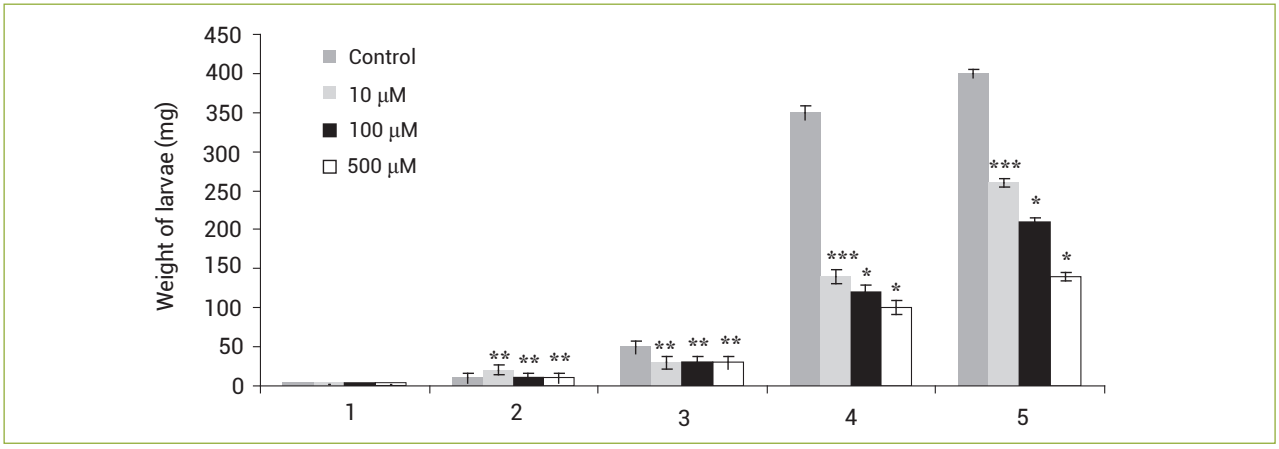

Figure 6. Dose dependent effect of CaTI on the growth and digestive physiology of larval *Helicoverpa armigera*.Each measurement was done in triplicate. Vertical lines represent the confidence intervals (n=10). Significant difference at *p<0.05, significant difference at **p<0.01 and significant difference at ***p<0.001 when compared between the control and inhibitor fed diet.

Resistance due to trypsin inhibitor : Total gut proteinase activity was determined by both the dot blot assay and the proteinase inhibitor assay, in which the proteolytic activity of HGP was determined. The results were in accordance with those obtained by Giri et al. (1998) and Harsulkar et al. (1998).

Inhibition assays using HGP extract and CaTI were also studied at pH 7.8 and pH 10, because two groups of proteinases showing activity at specific pHs were identified earlier in HGP complement (Harsulkar et al. 1998). A 45–50% inhibition of the HGP activity at pH 7.8 and a 30–35% inhibition at pH 10.0, respectively, was noticed indicating the stable existence of a distinct class of inhibitors that does have inhibitory activity against HGP, even at low levels, these data are shown in Table 6. The study was further extended to assess purified CaTI against bovine trypsin and HGP, in which a considerably lower proteolytic activity was seen in treatments, thus indicating a single inhibitory site for trypsin and trypsin-like enzymes (Srinivasan et al. 2005). Furthermore, *in vitro* inhibitory activity of CaTI at different purification levels increased with more purification. Similar results were observed in oryzacystatin (OCI) and Bowman-Birk inhibitors against gut proteinase from *Phaedon cochleariae* (F.) (Girard et al. 1998).

It was a matter of interest to examine the effect of CaTI ingestion on larval digestive proteases, since preliminary *in vitro* studies showed inhibition in proteolytic activity at the presence of both bovine trypsin and HGP. The 5th instar larvae reared on a diet containing CaTI showed decreased trypsin proteinase activities of the midgut, as confirmed by the trypsin inhibitor assay, suggesting that the toxic effect of the inhibitor complicates its digestion activity by blocking the enzymes involved in digestion. Lee et al. (1999) reported the inhibitory effect of soybean trypsin inhibitor (SBTI) gene on the brown plant hopper in transgenic rice plants. Similarly, SKTI (soybean trypsin inhibitor) and CpTI (cow pea trypsin inhibitor) inhibited serine proteinases from larvae of tomato moth (*Lacanobia oleracea*) (Gatehouse et al. 1999).

It is likely that the consumption of CaTI led to the overproduction of sulfur-deficient primary larval proteolytic enzyme, which was detrimental to larval health (Headey et al. 2010). It has been reported that serine proteinase inhibitor KTi_3 from the soybean resulted in up to 100% mortality of first instar *Spodoptera littoralis* (Schuler et al. 1998). Wu et al. (1997) reported a 13% decrease in total proteinase activity in *H. armigera* larvae, fed on transgenically expressed PI from giant taro. Moreover, Bown et al. (1997) also reported decrease in the overall level of proteinases and mRNAs encoding trypsin-like proteinases of *H. armigera* fed on soybean trypsin inhibitor (TI), suggesting that the decrease in activity was at transcriptional level. Parde et al. (2010) evaluated 22 different hosts and non-host plant protease inhibitors (PIs) for *in vivo* inhibition of *Helicoverpa armigera* gut pro- and proteinases. *In vivo studies* reported stunted growth of *H. armigera* larvae using non-host plant PIs, indicating the latter as good candidates for genetically engineered plants to confer resistance against *H. armigera.* Likewise, Saadati and Bandani (2011) reported a decrease in tryptic activity in digestive tract extracts from *H. armigera* larvae fed with SKTI.

Our results were concurrent with those by Wang and Qin (1996) and Johnston et al. (1993), as soybean kunitz type trypsin inhibitor and soybean Bowman-Birk type trypsin-chymotrypsin inhibitor both showed reduction in the larval weight of *H. armigera* in trypsin inhibitor amended diet. Gomes et al. (2005) observed a 45% mortality rate when 1.5% (w/w) of chickpea trypsin inhibitor was fed to *Anthonomus grandis* larvae, and a 33% mortality rate of *H. armigera* was observed in feeding mung bean TI impregnated diet. Similar insecticidal response against *Spodoptera litura* (Fabricus) was observed using protease inhibitor from leaves of *Coccinia grandis* (L.) Voigt. (Satheesh and Murugan 2012).

By gathering the present and previously reported data (Kansal et al. 2008), it appears that CaTI acts as an antimetabolite and has an insecticidal property, since it works on the insect's intestinal tract or interferes with digestion. We may interpret that the use of trypsin inhibitor to protect plants against insect pests is complicated due to the ability of insects to circumvent plant defenses. Thus, the action of CaTI on the development of *H. armigera* indicates its detrimental potential as it significantly increased mortality. The defensive activity may be related to the presence of a large number of trypsin-like proteases in their midgut. The results of *in vitro* trypsin inhibitory assay and ingestion studies revealed its antimetabolite activity as it affected both the growth and digestive physiology of *H. armigera*, leading to starvation and death.

Additional studies of CaTI in Central India concerning other insects are required to confirm the biotechnological potential of this inhibitor as an agent against phytophagus insects. As India is the largest chickpea producing country, therefore continuous efforts are carried out by

J.N.K.V.V. and FBIPL to provide best seeds to farmers in the Jabalpur region. To sum up, CaTI can be used as a marker for generating resistant cultivars as future research should be addressed to using trypsin inhibitor for marker assisted selection.

ACKNOWLEDGEMENTS

The authors would like to thank the Head of the Department of Biological Sciences, Rani Durgavati University, Jabalpur (India) for laboratory facilities and University Grant Commission Project (Govt. of India), New Delhi for financial assistance vide project no. UGC (32-370/2006) (SR) dated. 24/04/07. We would also like to thank the Head of the Department of Plant Breeding and Genetics for providing his excellent co-operation during the field studies carried out in J.N.K.V.V., Jabalpur fields. The author would also like to thank Mr. Sukhrat for his work in authenticating plant cultivars and for providing *Helicoverpa armigera* samples.

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