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Purification and Characterization of a Novel Inhibitor from *Poinciana pulcherrima* Seeds with Activity towards Pest Digestive Enzymes

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ABSTRACT

Six tree legumes were analysed for trypsin inhibitor (TI) activity and inhibition of trypsin enzyme extracted from the *Pieris brassicae* larvae midgut. TI extracted from *Poinciana pulcherrima* seeds contained maximum TI activity (trypsin units inhibited (TUI) = 17.24) and it also inhibited gut TI of *P. brassicae* larvae to the maximum extent (TUI = 16.61); minimum inhibition of gut TI was observed in seeds of *Poinciana regia* (TUI = 2.46). The inhibitor was purified to 50.98-fold with 66.46% recovery by ammonium sulphate precipitation and gel filtration chromatography through a Sephadex G-100 column: a single band was observed in native-PAGE. The molecular weight of the purified protein was 17,782 Da as determined by SDS-PAGE and it was a monomer. The purified TI was heat stable over a wide range of temperatures (20-60°C) and had a pH optimum of 7.5. The purified TI hence appears to be of the Bowman-Birk type that lost its activity when treated with different concentrations of 2-mercaptoethanol, indicating the role of disulphide linkages in maintaining its three-dimensional structure. The inhibition was non-competitive and had a K_i value of 0.6 μM, indicating the high affinity of the inhibitor towards its substrate, BApNA. The purified inhibitor suppressed the growth of *P. brassicae* larvae, judged solely on the basis of the reduction in size and on the weight of excreta. The inhibitor also inhibited trypsin enzyme extracted from the mid-gut of *Spodoptera littoralis* larvae (36.3 TUI/30 μg of purified inhibitor). The plant genes expressing such TIs can be isolated, cloned and introduced in vegetable crops, thereby conferring resistance and minimizing the devastating crop yield losses caused by various insect pests and pathogens.

Keywords: insect resistance, proteolytic enzymes, plant defense, purification, *Pieris brassicae*, trypsin inhibitor, tree legumes
Abbreviations: BApNA, α-benzoyl–DL–arginine–p–nitroanilide; **Da**, Dalton; $\mathbf{K_i}$, dissociation constant; $\mathbf{K_M}$, Michaelis-Menten constant; **PI**, protease inhibitors; **SDS-PAGE**, sodium dodecyl sulphatepolyacrylamide gel electrophoresis; **TI**, trypsin inhibitor; **TUI**, trypsin units inhibited; **TIA**, trypsin inhibitor activity

INTRODUCTION

Protease inhibitors (PIs) are proteins or peptides capable of inhibiting catalytic activities of proteolytic enzymes (Tian and Zhang 2005). They have been classified as serine, cysteine, aspartic and metallo-proteinase inhibitors on the basis of their specificity towards proteolytic enzymes. Serine PIs are small, stable and abundant proteins showing specificity to trypsin and/or chymotrypsin (Bode and Huber 2000). These inhibitors are widespread and have been reported from various plant families (Lawrence and Koundal 2002). The most thoroughly examined plant serine PIs are found in the species of *Leguminosae* family (Norioka *et al*. 1988; Giri *et al*. 2003; Sattar *et al*. 2004; Macedo *et al*. 2007; Ee *et al*. 2009; Prasad *et al.* 2010; Macedo *et al.* 2011). Serine protease inhibitors have been characterized into two families namely Kunitz and Bowman-Birk-type inhibitors. Members of Kunitz family have a molecular mass of about 20,000 daltons (Da) or more and lower cysteine content while the members of Bowman-Birk family have a lower molecular weight ranging from 7000 to 10000 Da and has a conserved pattern of disulphide bonds (Richardson 1991; Laskowski and Qasim 2000; Mosolov and Valueva 2005; Qi *et al*. 2005).

Many herbivorous *Lepidopteran* pests obtain essential amino acids by using trypsin-like proteases secreted into the midgut lumen to digest food proteins (Inanaga *et al*. 2001; Giri *et al*. 2003; Bhattacharyya *et al*. 2007; Liao *et al*. 2007). For this reason, serine PIs work as an important part of the plant defense system by inhibiting digestive proteases in the midgut of *Lepidopteran* pests, leading to inadequate digestion and absorption of essential amino acids, arrested growth, and eventually death by starvation (Giri *et al*. 2003). Plant PIs do not lead to high selection pressure as compared with the 'wipeout' approach executed by other pesticide control measures. This minimizes the possibility of developing resistance in the insect population against protease inhibitors. Unlike *Bacillus thuringiensis* toxins, which usually results in a high level of pest mortality, but are highly specific, PIs are not actually toxic and have broad spectrum activity. Another merit of this approach lies in the fact that these inhibitors are plants own natural defense response against phytophagous insects, usually present in leaves as well as storage tissues and they are also expressed upon insect attack or mechanical wounding (Green and Ryan 1972; Howe *et al*. 1996; Franco *et al*. 2003; Rai *et al.* 2008).

Tobacco plants transformed with gene coding for serine PI have been the first transgenic plants developed possessing insect pest resistance (Hilder *et al.* 1987). A number of plant PI genes that were cloned, reconstructed and expressed in some transgenic crops plants (Jouanin *et al.* 1998; Schuler *et al.* 1998) has resulted in enhanced defensive capabilities under field conditions (Graham *et al*. 2002; Wu *et al*. 2005); however, there are some exceptions (Nandi *et al.* 1999; Brunelle *et al.* 2004; Outchkourov *et al.* 2004). The insect-resistant transgenic plants that have reached the market so far express the insecticidal cry genes from *Bacillus thuringiensis* (*Bt*). The use of transgenic plants expres-

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sing a single *Bt* gene could induce appearance of resistant insects. In order to reduce this risk, associating two or more entomopathogenic protein genes, with different modes of action would be beneficial. The availability of such diverse genes from different plant species makes it a possibility to use one or more genes in combination, whose products are targeted at different biochemical and physiological processes within the insect. These packages will not only contain protease inhibitor genes but also lectins, α-amylase inhibitors or other plant genes encoding insecticidal proteins. This technology may not replace the use of chemical pesticides in near future but effectively complement it. Thus, it is important to biochemically characterize the protease inhibitors from various indigenous legumes because the choice of an efficient inhibitor will determine the success of a pest control strategy. In the present paper, we describe the isolation, purification and characterization of a potent protease inhibitor from *Poinciana pulcherrima* seeds having maximum insecticidal activity among the tested tree legumes towards insect pests.

MATERIALS AND METHODS

Collection of seeds and insects

The seeds of six tree legumes namely *Poinciana pulcherrima*, *Poinciana regia*, *Albizia lebbeck*, *Cassia fistula*, *Leuceana leucocephala* and *Acacia arabica* were procured from the Department of Botany, Forest Research Institute, Dehradun, Uttaranchal. The eggs and 4th instar larvae of *Spodoptera littoralis* as well as 4th instar larvae of *Pieris brassicae* were collected from the fields of Department of Entomology and Apiculture, Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan, H.P., India.

Preparation of crude extract

The crude extract was prepared from the seeds of legume trees according to the method described by Deepika and Nath (2010). Seeds were ground to make fine powder. The flour obtained were defatted with acetone (1:10 w/v), air dried 3-4 times and then used for protein extraction. One gram of defatted seed flour was shaken with 10 ml (1:10 w/v) of the extraction media (distilled water, 0.1 M sodium phosphate buffer; pH 7.5 and 0.2% NaCl) in shaking water bath for 4 h at room temperature, separately. The extracts were centrifuged at 10,000 rpm at 4°C for 30 min. The supernatants obtained were used for trypsin inhibition assay and further studies. The results of trypsin inhibition assay showed that the three extraction media could extract TI equally well and distilled water was selected as the extraction medium.

Screening of tree legumes for trypsin inhibitor activity

Preliminary screening of seed extracts of tree legumes for their TI content was performed according to the method described by Hajela *et al.* (1999) with slight modifications. The activity of the seed extracts was measured using α-benzoyl-DL-arginine-*p*-nitroanilide (BApNA, Sigma Aldrich, St. Louis, USA) as substrate and bovine pancreas trypsin (Sigma Aldrich) as source of enzyme. One ml of trypsin solution (prepared by mixing 1 mg of bovine trypsin with 10 ml of buffer-II (prepared fresh by mixing 5 ml of 0.1 M sodium phosphate buffer, pH 7.5, 0.5 ml of 0.1 M $CaCl_2$, 0.036 ml of 3 M NaCl and 4.464 ml of distilled water) was mixed with 0.1 ml of diluted TI and incubated in a metabolic shaking water bath at 37°C for 10 min, followed by the addition of suitable volume of buffer I (prepared fresh by mixing 10 ml of 0.1 M sodium phosphate buffer (pH 7.5), 4 ml of 0.1 M $CaCl_2$ and 6 ml of distilled water) and 0.3 ml of BApNA (4 mg of BApNA dissolved in 0.1 ml of dimethyl sulphoxide (DMSO) and the final volume made to 1 ml by adding 0.9 ml of buffer I). The reaction was stopped after 10 min by adding 0.5 ml of 30% acetic acid. The blank was prepared by adding 0.3 ml of buffer I instead of substrate BApNA whereas, in control TI was not added and the volume of the reaction mixture was adjusted with buffer-I. To measure the trypsin inhibitor activity (TIA), optical density of the test and control reaction mixtures was measured at 410 nm. Trypsin units inhibited (TUI) were calculated by comparing the absorbance measured for test mixture and control mixture. Decline in optical density (OD) monitored at 410 nm as compared to control of 0.01 OD units/min was considered to be one TUI.

Screening of tree legumes for the presence of trypsin inhibitor activity using gut enzyme extracted from larvae of *P. brassicae*

The larval midgut proteases were extracted according to the method described by Raj Deepika *et al.* (2008). Midguts of *P. brassicae* larvae were dissected on ice and the extract was prepared by homogenizing it with 10 ml of 0.1 M sodium phosphate buffer (pH 7.5) in prechilled test tube using a glass rod. The homogenate obtained was filtered through Whatman filter paper No. 1 [Merck, Germany] and the filtrate was then used for gut enzyme inhibition assay. One ml of the filtered extract of *S. littoralis* larval midgut was mixed with 0.1 ml of the crude *P. pulcherrima* extract (herein referred to as TI extract) and preincubated in a metabolic shaking water bath at 37°C for 10 min, followed by the addition of buffer I and 0.3 ml of BApNA. The total volume of the reaction mixture was 2 ml. The reaction mixture was incubated at 37°C in a shaking water bath. After 10 min of incubation the reaction was stopped by adding 0.5 ml of 30% acetic acid. The blank was prepared by adding 0.3 ml of buffer I instead of substrate BApNA whereas in the control TI extract was not added and the volume of the reaction mixture was adjusted with buffer I. The O.D. of the test and control mixtures was measured at 410 nm against the blank using a UV/VIS spectrophotometer to measure TIA. BApNA is an artificial substrate that reveals the non-inhibited trypsin as it becomes yellow when it reacts with trypsin (Kakade *et al.* 1974). So, the TUI was calculated by comparing the absorbance measured for test mixture and control mixture. The decline in OD monitored at 410 nm as compared to control of 0.01 OD/min was taken one TUI.

Purification of inhibitor from *P. pulcherrima* seeds

The crude extract prepared from the seeds of *P. pulcherrima* was subjected to ammonium sulphate precipitation. The ammonium sulphate cuts of 0 to 30% and 30 to 80% were prepared at 4°C by adding solid ammonium sulphate with constant stirring using magnetic stirrer. The protein was allowed to precipitate for 3 h at 4°C. The precipitated protein was collected by centrifugation at 10,000 × *g* for 20 min. The pellet thus obtained was dissolved in minimum volume of 0.1 M phosphate buffer (pH 7.5) and dialyzed overnight against diluted (1:10) extraction buffer for the elimination of sulphate ions. The partially purified TI was further subjected to purification by gel filtration chromatography on Sephadex G-100 (Sigma Aldrich) column (30 × 2.5 cm) and eluted with 0.1 M phosphate buffer. A flow rate of 12 ml/h was maintained. Fractions containing TIA were pooled and poured into a dialysis bag placed in a beaker filled with prechilled solid sucrose at 4°C. The concentrated protein was further characterized and used for gel electrophoresis. The soluble protein was estimated after each step of purification as described by Lowry *et al*. (1951).

Molecular weight determination

The molecular weight of the purified TI was determined by gel filtration through Sephadex G-100 column by loading the concentrated purified inhibitor. The elution volume (Ve) of the fraction showing maximum TIA was noted, Ve/Vo was calculated. $V_{O,}$ the void volume, was calculated by loading Blue dextran (1 mg/ml). The molecular weight was determined from the calibration curve prepared by loading standard molecular weight markers, namely phosphorylase b (94,000 Da), bovine serum albumin (67,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (30,000 Da), soyabean trypsin inhibitor (20,100 Da) and α-lactalbumin (14,400 Da) on the Sephadex G-100 column.

Polyacrylamide gel electrophoresis

The purity of purified TI was determined by native-PAGE (Davis 1964). SDS-PAGE was carried out to determine the sub-unit composition of the purified inhibitor.

1. Characterization of purified inhibitor

In all the characterization experiments the trypsin inhibition assay was carried out as described earlier.

2. Effect of inhibitor concentration

Effect of inhibitor concentration on TUI was studied by varying the concentration of inhibitor in the inhibition assay. TI concentration was varied from 9 to 91.3 µg.

3. Effect of temperature on stability of TI

The effect of temperature on stability was studied by incubating inhibitor for 10 min at 30, 37, 40, 50, 60, 70, 80, and 90°C and measuring TIA.

4. Effect of pH

Different buffers viz., acetate buffer (pH 4.0 to 6.0), phosphate buffer (pH 6.0 to 8.0) and Tris buffer (pH 8.0 to 9.0) were used in the reaction mixture and TIA was estimated.

5. Determination of K_M value and nature of inhibition

To determine the nature of inhibition, two different concentrations of inhibitor were used (12.00 and 24.3 µg), and a Lineweaver Burk (1934) plot was plotted using different concentrations of substrate in the presence and absence of inhibitor and K_M value was determined.

6. Determination of K_i value

A Dixon's plot was plotted using different concentrations of inhibitor at two fixed concentrations of substrate (2.44 and 4 mM). The K_i value was determined from the plot as described by Dixon (1953).

7. Effect of 2-Mercaptoethanol

TI was incubated with different concentrations (2.5 to 25 mM) of 2-mecrcaptoethanol for 2, 4 and 24 h. Trypsin inhibition assay was carried out using the above TI extracts as described earlier.

8. Feeding bioassay: Effect of TI on P. brassicae larvae

Cabbage leaf dics were coated with 120 µg of purified TI. Feeding assay was conducted by feeding 10 larvae of *P. brassicae* immediately after hatching on treated leaf discs. The experiment was conducted in three sets. In control, leaves were coated with 0.1 M sodium phosphate buffer instead of purified TI. All the larvae were given fresh leaves after 24 h. The per cent leaf area eaten and weight of faecal matter were recorded after 24 h for 4 days. Similarly, feeding bioassays were conducted using three-days-old larvae of *P. brassicae*. The experiment was carried out in three replications. Ten larvae were used per replication and 240 µg of purified TI was coated on leaf discs. Larvae were given fresh leaves after 24 h. The per cent leaf area eaten and weight of faecal matter were recorded after 24 h for 4 days.

9. Effect of purified TI on gut trypsin extracted from S. littoralis larvae

Five midguts of *S. littoralis* larvae were surgically removed and placed in a prechilled test tube containing 5 ml of 0.1 M phosphate buffer. Guts were homogenized and centrifuged at 10,000 × *g* at 4°C for 30 min. The supernatant was used as the source of trypsin for the measurement of *in vitro* TIA, as described earlier.

Statistical analysis

The experiment on measurement of TIA in different tree legumes was carried out in a completely randomized design (CRD). Each observation was repeated thrice with duplicate estimations. Critical differences between mean values of TIA were calculated at $P \leq 0.05$ using ANOVA. The data presented in the purification table are the mean of three experiments. The percent yield after each purification step did not vary more than 10% and hence only mean values are given in the purification table. The student's *t*-test was applied to the data on feeding bioassays because there was only one treatment and student's *t*-distribution is applicable. In the above mentioned experiment a paired *t*-test (Agarwal 2003) was used for testing the mean difference of the samples. The experiments were carried out to find the effect of feeding TI on percent leaf area eaten and weight of feacal matter. Appropriate controls (larvae fed leaf discs without inhibitor coating) were set for comparing with the treatment. The experiment was conducted in three replications. The use of paired samples enabled us to perform a more precise and accurate analysis. To test the significance the calculated *t* values were compared with tabulated *t* values at the 5% level of significance. If the calculated *t* values were significantly higher than the tabulated *t* values at the 5% level of significance, the treatment was considered to be significantly different from the control.

RESULTS

Screening of tree legumes for trypsin inhibitor activity and inhibition of gut trypsin enzyme extracted from larvae of *P. brassicae*

Considerable variations were observed among tree legumes for TIA (**Table 1**). The TUI/mg seed flour weight was maximum in *P. pulcherrima* (17.24) followed by *A. arabica*, *C. fistula*, *A. lebbeck*, *L. leucocephala* and *P. regia* which had TUI values of 16.04, 11.87, 11.02, 9.61 and 8.84, respectively (**Table 1**). Similarly, *P. pulcherrima* seeds flour (TUI 16.61) showed maximum inhibition of gut trypsin enzyme extracted from larvae of *P. brassicae* as compared to minimum inhibition shown by *P. regia* with TUI 2.46/mg seed flour weight (**Table 1**). Since *P. pulcherrima* exhibited maximum TUI/mg seed flour in both screening experiments, further purification and characterization of the TI was carried out from *P. pulcherrima* seeds.

Table 1 Trypsin inhibitor activity in tree legumes.

Tree legumes	TUI per mg seed flour weight (using synthetic trypsin enzyme)	TUI per mg seed flour weight (using gut enzyme of *Pieris brasicae* larvae)
Poinciana pulcherrima	17.24 ± 0.10 a	16.61 ± 0.01 a
Acacia Arabica	16.04 ± 0.05 b	13.27 ± 0.03 b
Cassia fistula	11.87 ± 0.08 c	10.62 ± 0.03 c
Albizia lebbeck	11.02 ± 0.04 c	8.57 ± 0.03 d
Leuceana leucocephala	9.61 ± 0.02 d	4.61 ± 0.03 e
Poinciana regia	8.84 ± 0.07 d	2.46 ± 0.02 f
$CD_{0.05}$	0.184	0.0754

* Different letters within a column indicate significant differences at 5 % level of significance; *n* = 3 or more, mean values are shown, SD was always less than 20%

Purification of *P. pulcherrima* TI

The TI was purified to 50.98 fold with 66.46% recovery using ammonium sulphate precipitation and gel filtration chromatography (**Table 2**). The apparent homogeneity of the purified inhibitor as judged by native-PAGE revealed a single band. The molecular weight and subunit composition, as determined by SDS-PAGE, indicated the purified TI to be a monomer with a molecular weight of 17,782 Da.

Characterization of TI

The TI purified from *P. pulcherrima* seed flour was charac-

Table 2 Purification of trypsin inhibitor from *Poinciana pulcherrima*.

Step	Total TUI	Total soluble protein (mg)	Specific activity	Fold purification	Per cent yield
Crude extract	86062.5	6750	12.75	1	100
Ammonium sulphate precipitation	72687.5	4661	15.59	1.22	84.45
Gel filtration chromatography	57200	88	650	50.98	66.46

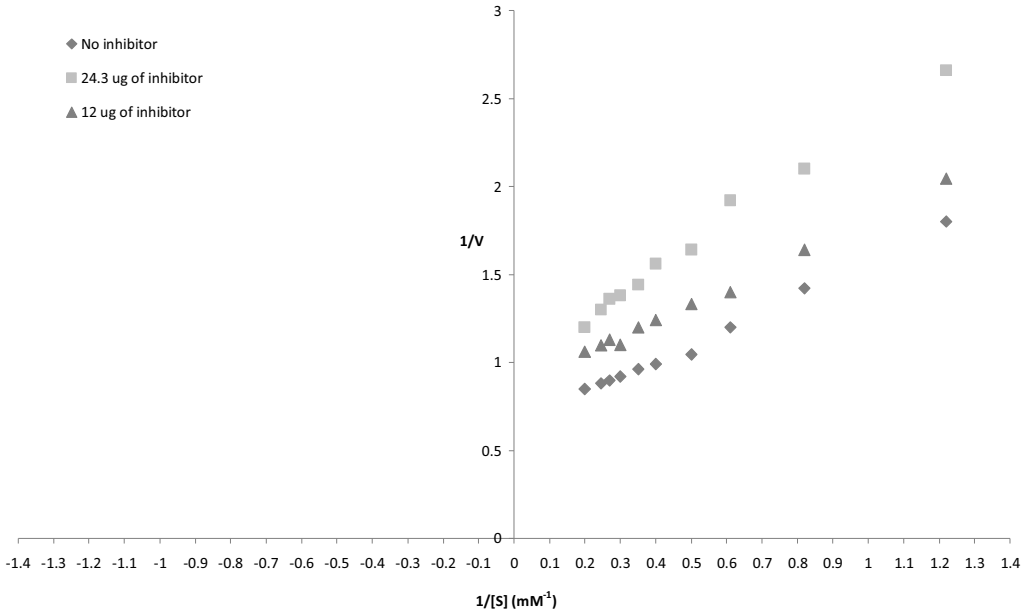

Fig. 1 Lineweaver-Burk plot of trypsin activity in the presence of different concentrations of inhibitor. The standard reaction mixture contained 0, 12 and 24.3 µg of inhibitor protein. Each point in the graph represents the mean of three estimations with duplicates.

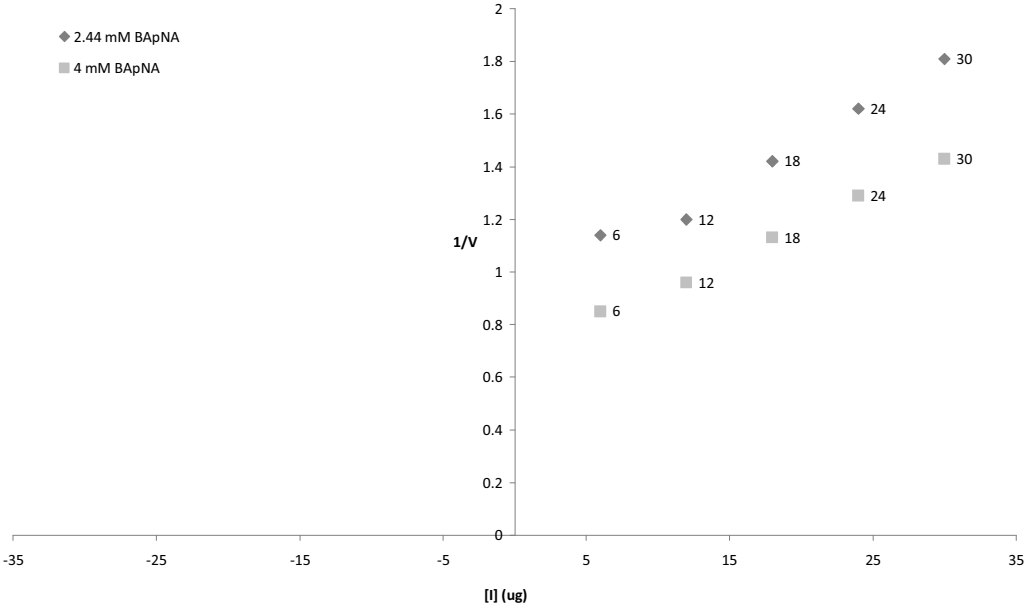

Fig. 2 Dixon's plot of trypsin activity in the presence of different concentrations of substrate i.e., 2.44 and 4 mM BApNA. Each point in the graph represents the mean of three estimations with duplicates.

terized to ascertain how it differs from the already known inhibitors and to classify it.

Inhibitory properties, K_i and K_M determination

The inhibition was linear up to 82.3% with increasing concentrations of purified TI in the reaction mixture and the inhibition pattern deviated from linearity at higher concentrations of inhibitor. The K_M value of the substrate BApNA for trypsin enzyme was 1.4 mM. The Lineweaver Burk plot (**Fig. 1**) and Dixon's plot (**Fig. 2**) revealed non-competitive type of inhibition with K_i value of 0.6 µM. Low K_i value indicated high affinity of the inhibitor for trypsin enzyme.

Studies on stability of the purified inhibitor

TIA was determined after incubating purified TI at temperatures ranging from 20 to 100°C. The purified inhibitor was found to be heat stable over a wide range of temperature (20-60°C). Purified TI was found to have an optimum pH of 7.5. The inhibitory activity of the purified TI in the presence of reducing agent 2-mercaptoethanol showed the inhibitor to be susceptible to varying concentrations of 2-mercaptoethanol. TIA reduced to 37.28, 28.44 and 0% after incubation with 25 mM 2-mercaptocthanol for 2, 4 and 24 h, respectively.

Susceptibility to reducing agent and thermal stability of

Table 3 Per cent leaf area eaten and faecal matter (mg) of *Pieris brassicae* larvae (immediately after hatching).

Concentration of trypsin inhibitor (µg)	Per cent leaf area eaten[a]				Faecal matter (mg)[b]			
	1st day	2nd day	3rd day	4th day	1st day	2nd day	3rd day	4th day
Control (0)	2.54 ± 0.06	4.73 ± 0.05	7.0 ± 0.07	9.60 ± 0.14	1.845 ± 0.06	2.65 ± 0.10	9.70 ± 0.20	19.6 ± 0.45
120	0.66 ± 0.03	1.47 ± 0.06	2.92 ± 0.16	5.80 ± 0.55	0.45 ± 0.05	1.10 ± 0.20	5.40 ± 0.40	10.3 ± 0.55
t value	30.08	43.79	23.960	20.554	18.76	13.864	13.878	13.228

[a]: By area; [b]: By weight; * Significant differences were observed between the control and treatment according to Student's *t*-test ($P < 0.05$).

Table 4 Per cent leaf area eaten and faecal matter (mg) of *Pieris brassicae* larvae (after three days of hatching).

Concentration of trypsin inhibitor (µg)	Per cent leaf area eaten[a]				Faecal matter (mg)[b]			
	1st day	2nd day	3rd day	4th day	1st day	2nd day	3rd day	4th day
Control (0)	10.3 ± 0.20	25.95 ± 0.33	41.10 ± 0.36	75.5 ± 0.26	9.95 ± 0.15	29.3 ± 0.40	48.85 ± 0.25	70.8 ± 0.75
240	6.86 ± 0.04	10.78 ± 0.28	20.59 ± 0.14	27.14 ± 0.17	4.40 ± 0.40	8.95 ± 0.25	21.05 ± 0.65	41.9 ± 0.10
t value	16.918	35.052	52.478	163.903	12.992	43.142	39.918	38.261

[a]: By area; [b]: By weight; * Significant differences were observed between the control and treatment according to Student's *t*-test ($P < 0.05$).

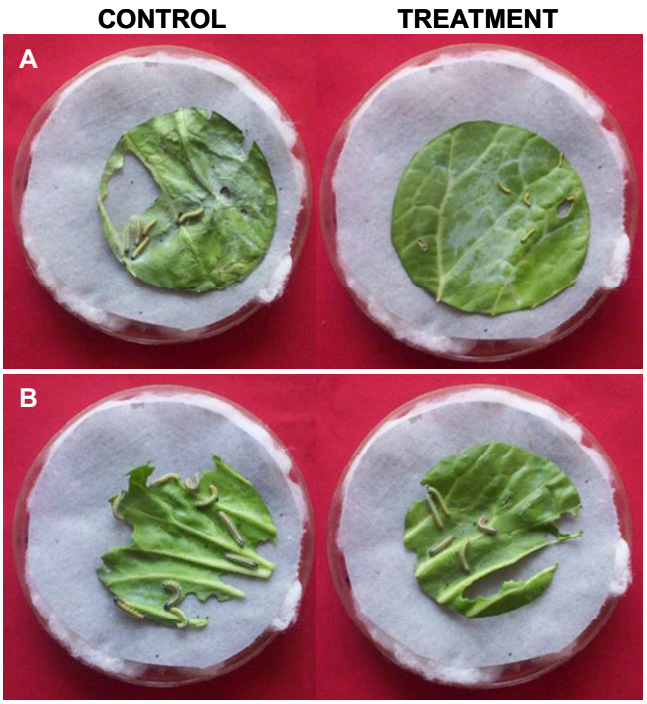

Fig. 3 Effect of purified trypsin inhibitor on larvae of *Pieris brassicae*. Larvae immediately after hatching (**A**) or 3 days after hatching (**B**).

purified TI indicated it to be of the Bowman-Birk type. Generally, Bowman-Birk type inhibitors have lower molecular weights (7,000-10,000) than to Kunitz-type inhibitors (>20,000). Although the molecular weight 17,782 Da of *P. pulcherrima* TI appears to be higher than usually expected for Bowman-Birk type, there are various reports of high molecular weight (above 15,000) Bowman-Birk inhibitors (Maggo *et al*. 1999).

Inhibitory assay against gut protease extracted from *S. littoralis*

Trypsin inhibitor was assayed against gut protease extracted from the gut of larvae of *S. littoralis.* Trypsin units inhibited per ml trypsin was found to be 36.3 ± 0.08/30 µg of TI.

Insect bioassay

Feeding bioassays were carried out at two different concentrations of TI to assess the potential bioinsecticidal effect of purified TI towards the pest, *P. brassicae.* The feeding bioassay showed the larvae (immediately after hatching) of *P. brassicae* to be sensitive to the TI and the growth of the larvae were significantly reduced as compared to the control. The larvae fed on leaf discs coated with 120 µg of inhibitor resulted in % leaf area of treated leaves to be reduced to 26% as compared to the control. Similarly, the amount of faecal matter produced also decreased by 24% (**Table 3**). In the second set of treatment one-week-old larvae showed a reduction of 40 and 59% (**Table 4**) in percent leaf area eaten and the amount of faecal matter produced, respectively (**Fig. 3**) when fed on leaf discs coated with 240 µg of TI.

DISCUSSION

Serine protease inhibitors such as related TIs have been purified and characterized from a variety of plant sources (Mello *et al.* 2001; Kansal *et al.* 2008; Rai *et al.* 2008; Chaudhary *et al.* 2008; Ramos *et al.* 2008; Ee *et al.* 2009; Klomklao *et al*. 2011; Macedo *et al*. 2011). In the present study, a TI from *P. pulcherrima* seed flour was purified and characterized and its potential insecticidal activity against two *Lepidopteran* insects was examined.

The inhibitor was purified by ammonium sulphate precipitation and Sephadex G-100 chromatography to near homogeneity with 50.98-fold purification and 66.46% recovery. Klomklao *et al.* (2011) purified a trypsin inhibitor from mung bean seeds to 13.51-fold 30.25% yield. The inhibitor was purified by heat-treatment at 90°C for 10 min, followed by ammonium sulphate precipitation with 30-65% saturation and gel filtration on Sephadex G-50. Trypsin inhibitor from seeds of *Tamarindus indica* was purified to 127.7-fold with 2.2% recovery by Araújo *et al*. (2005). Kansal *et al.* (2008) purified TI from mung bean by heat denaturation followed by ammonium sulfate precipitation, ion exchange chromatography on DEAE Sephadex A_{25} and gel filtration chromatography on Sephadex G-75 to 58.1 fold with 22.8% recovery. TI from *Pithecellobium dumosum* seeds was purified to 217.6-fold with 4.7% recovery by Oliveira *et al*. (2007). Macedo *et al.* (2011) purified a TI (SSTI) to 124-fold with 14.8% yield from the seeds of *Sapindus saponaria* by ammonium sulfate precipitation, gel filtration (Sephacryl S-100), ion-exchange chromatography (DEAE-Sepharose) and affinity chromatography using trypsin-Sepharose. TI purified during this study was found to be composed of a single polypeptide chain having molecular mass of 17,782 Da, as revealed by Native-PAGE and SDS-PAGE, which is consistent with the molecular masses of other TIs (Devaraj *et al*. 1999; Francisco *et al*. 2004; Paiva *et al*. 2006; Macedo *et al.* 2007; Oliveira *et al*. 2007; Ramos *et al.* 2008; Sharma *et al.* 2011).

The linear inhibition pattern was observed almost up to 82.3% with the increasing levels of purified TI in the reaction mixture during this study. Similarly, deviation from linearity at a level higher that 80% was also observed by Saini *et al.* (1992), whereas Maggo *et al.* (1999) observed linearity up to 60% in case of rice bean.

The K_i value of purified TI during present studies was found to be 0.6 µm, the results are in agreement with low K_i values of 2.21×10^{-7} M, 4×10^{-8} M 2.4×10^{-9} and 0.3 µM, reported from *Calliandra selloi* Macbride (Yoshizaki *et al.* 2007), *Plathymenia foliolosa* (Ramos *et al.* 2008), *Sapindus saponaria* (Macedo *et al.* 2011) and *Cassia obtusifolia*

(Liao *et al.* 2007), respectively. The purified TI was found to be a non-competitive inhibitor like other reported TIs from *Tamarindus indica* (Araújo *et al.* 2005) and *Vigna mungo* (Prasad *et al.* 2010). This opposes the findings of Macedo *et al*. (2011), Oliveira *et al.* (2007), Shee and Sharma (2007) and Bhattacharyya *et al.* (2006), who proposed that serine proteinase inhibitors to be competitive inhibitors. The purified inhibitor was found to be heat stable over a wide range of temperature (20-60°C). TI purified from *Caesalpinia bonduc* seeds exhibited tolerance to extreme temperature viz., 0-60°C (Bhattacharyya *et al*. 2007). The instability of TI purified from *Cassia obtusifolia* above 70°C was reported by Liao *et al*. (2007). Protease inhibitors purified from *Vigna mungo* and mung bean were found to be heat stable up to 80°C (Kansal *et al*. 2008; Prasad *et al.* 2010). TI isolated from *Spinacia oleracea* retained 80% of its activity after boiling for 20 min (Kang *et al.* 2009). Optimum pH of the purified TI from *P. pulcherrima* seeds was 7.5. The TI from *Dimorphondra mollis* seeds had a pH optimum of 7.6 (Macedo *et al*. 2000). Purified TI from *Inga umbratica* and *Murraya koenigii* seeds was stable at pH 5.0-7.0 and 7.5-8.0, respectively (Calderon *et al*. 2005; Shee and Sharma 2007). TI from *Vigna mungo* was active over a pH range of 2-12 (Prasad *et al.* 2010). The TI purified from *P. pulcherrima* seeds was susceptible to varying concentrations of reducing agent. This could be explained by the fact that the three-dimensional structure of the inhibitor is stabilized by disulfide bonds which get reduced in the presence of reducing agents. A significant reduction in TIA after incubation with 2-mercaptoethanol was also reported by Hajela *et al*. (1999), Maggo *et al*. (1999) and Gupta *et al.* (2000). Klomklao *et al*. (2011) reported no activity of TI purified from *Vigna radiata* in the presence of 2-mercaptoethanol. In this study, the potential bioinsecticidal effect of purified TI was tested in feeding bioassays against *P. brassicae* larvae. Results showed deleterious effects on the growth and larval development, when the larvae were fed on leaf discs coated with different concentrations of inhibitor. This may be attributed to the direct inhibition of digestive enzyme and depletion of essential amino acids. Rajdeepika *et al*. (2008) observed 78.67% mortality after four days in feeding bioassay studies with larvae of *P. brassicae* fed on leaf discs coated with 150 µg purified TI and 90% mortality after five days when fed with 300 µg of purified TI from seeds of local yellow cultivar of *Phaseolus vulgaris*. Inhibition of gut trypsin of *P. rapae* larvae by TI purified from *Cassia obtusifolia* (Liao *et al.* 2007) and *Albizzia kalkora* (Zhou *et al*. 2008) was also reported. In this study, the purified TI also inhibited gut trypsin enzyme extracted from *S. littoralis* larvae. Larval gut protease inhibition of *S. littoralis* by crude TI of was reported by Rajdeepika *et al*. (2008). 57% inhibition of larval midgut proteases of *Sexigua* at 45 µg/ml of inhibitor from *Albizzia kalkora* seeds was reported by Zhou *et al*. (2008). Schuler *et al.* (1998) observed 100% mortality of the first instar larvae of *S. littoralis* when fed on transgenic tobacco plants expressing the serine proteinase inhibitor KTi3 gene from soybean. Inhibition of gut protease of *S. frugiperda* by TI from *Poecilanthe parviflora* was also reported (Garcia *et al*. 2004), *Crotalaria pallida* (Gomes *et al*. 2005) and *Tamarindus indica* (Araújo *et al*. 2005). Purified TI from *Sapindus saponaria* seeds also showed significant inhibitory activity against trypsin-like proteases present in the larval midguts of *Anagasta kuehniella*, *Corcyra cephalonica*, *Diatreae saccharalis* and *Anticarsia gemmatalis* (Macedo *et al*. 2011).

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