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

Amarjit K. Nath* • Kanika Chadha • Pratima Sharma

Department of Biotechnology, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni-Solan (H.P.) 173 210 India

**Corresponding author:** amarjitnath@yahoo.com

---

**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 three-dimensional structure. The inhibition was non-competitive and had a $K_i$ value of 0.6 $\mu$M, 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 $\mu$g 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, $\alpha$-benzoyl-DL-arginine-p-nitroanilide; Da, Dalton; $K_d$, dissociation constant; $K_M$, Michaelis-Menten constant; PL, protease inhibitors; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide 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-protease 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; Ouchkourov 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-
suing 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, Leucaena 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 CaCl2, 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 CaCl2 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 [Merek, 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. Vo, the void volume, was calculated by loading Blue dextran (1 mg/ml). The molecular weight of the TI 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), soya bean 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-mercaptoethanol 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 discs 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 larvace 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 x 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 ≤ 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 faecal 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>
<thead>
<tr>
<th>Tree legumes</th>
<th>TUI per mg seed flour weight (using synthetic trypsin enzyme)</th>
<th>TUI per mg seed flour weight (using gut enzyme of Pieris brassicae larvae)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poinciana pulcherrima</td>
<td>17.24 ± 0.10 d</td>
<td>16.61 ± 0.01 a</td>
</tr>
<tr>
<td>Acacia Arabrica</td>
<td>16.04 ± 0.05 b</td>
<td>13.27 ± 0.03 b</td>
</tr>
<tr>
<td>Cassia fistula</td>
<td>11.87 ± 0.08 c</td>
<td>10.62 ± 0.03 c</td>
</tr>
<tr>
<td>Albizia lebbeck</td>
<td>11.02 ± 0.04 c</td>
<td>8.57 ± 0.03 d</td>
</tr>
<tr>
<td>Leucaena leucocephala</td>
<td>9.61 ± 0.02 d</td>
<td>4.61 ± 0.03 e</td>
</tr>
<tr>
<td>Poinciana regia</td>
<td>8.84 ± 0.07 d</td>
<td>2.46 ± 0.02 f</td>
</tr>
<tr>
<td>Chá de café</td>
<td>0.18 ± 0.07 d</td>
<td>0.07 ± 0.05</td>
</tr>
</tbody>
</table>

* 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-
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-mercaptoethanol for 2, 4 and 24 h, respectively.

Susceptibility to reducing agent and thermal stability of purified inhibitor

<table>
<thead>
<tr>
<th>Step</th>
<th>Total TUI</th>
<th>Total soluble protein (mg)</th>
<th>Specific activity</th>
<th>Fold purification</th>
<th>Per cent yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>86062.5</td>
<td>6750</td>
<td>12.75</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulphate precip</td>
<td>72687.5</td>
<td>4661</td>
<td>15.59</td>
<td>1.22</td>
<td>84.45</td>
</tr>
<tr>
<td>Gel filtration chromatography</td>
<td>57200</td>
<td>88</td>
<td>650</td>
<td>50.98</td>
<td>66.46</td>
</tr>
</tbody>
</table>

Table 2 Purification of trypsin inhibitor from *Poinciana pulcherrima*
Novel trypsin inhibitor from *Poinciana pulcherrima* seeds with activity towards pest digestive enzymes. Nath et al.

### Table 3

<table>
<thead>
<tr>
<th>Concentration of trypsin inhibitor (μg)</th>
<th>1st day</th>
<th>2nd day</th>
<th>3rd day</th>
<th>4th day</th>
<th>1st day</th>
<th>2nd day</th>
<th>3rd day</th>
<th>4th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0)</td>
<td>2.54 ± 0.06</td>
<td>4.73 ± 0.05</td>
<td>7.0 ± 0.07</td>
<td>9.60 ± 0.14</td>
<td>1.845 ± 0.06</td>
<td>2.65 ± 0.10</td>
<td>9.70 ± 0.20</td>
<td>19.6 ± 0.45</td>
</tr>
<tr>
<td>120</td>
<td>0.66 ± 0.03</td>
<td>1.47 ± 0.06</td>
<td>2.92 ± 0.16</td>
<td>5.80 ± 0.55</td>
<td>0.45 ± 0.05</td>
<td>1.10 ± 0.20</td>
<td>5.40 ± 0.40</td>
<td>10.3 ± 0.55</td>
</tr>
<tr>
<td>( r ) value</td>
<td>30.08</td>
<td>43.79</td>
<td>23.96</td>
<td>20.554</td>
<td>18.76</td>
<td>13.864</td>
<td>13.878</td>
<td>13.228</td>
</tr>
</tbody>
</table>

\( ^a \) 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

<table>
<thead>
<tr>
<th>Concentration of trypsin inhibitor (μg)</th>
<th>Per cent leaf area eaten(^a)</th>
<th>Faecal matter (mg)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st day</td>
<td>2nd day</td>
<td>3rd day</td>
</tr>
<tr>
<td>Control (0)</td>
<td>10.3 ± 0.20</td>
<td>25.95 ± 0.33</td>
</tr>
<tr>
<td>240</td>
<td>6.86 ± 0.04</td>
<td>10.78 ± 0.28</td>
</tr>
<tr>
<td>( r ) value</td>
<td>16.918</td>
<td>35.052</td>
</tr>
</tbody>
</table>

\( ^a \) By area; \( ^b \) By weight; * Significant differences were observed between the control and treatment according to Student’s \( t \)-test (\( P < 0.05 \)).

### 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; Kloskial 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 *Lepidoptera* 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. Kloskial 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 A25 and gel filtration chromatography on Sephadex G-75 to 58.1 fold with 22.8% recovery. TI from *Pithecocellobium 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 sellosi* Machrîde (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 pulcherrima 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 Dimorphandra 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 Vigna radiata 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). Klotmkio et al. (2011) reported no activity of TI purified from Vigna radiata in the presence of 2-mercaptoethanol. In this study, the potential biosinicipetal 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. Rajdeepta 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 *Albizia kalkora* (Zhou et al. 2008) was also reported. In this study, the purified TI also inhibited gut trypsin enzyme extracted from *S. litto-ralis* larvae gut protease in the presence of 2-mercaptoethanol. By crude TI of was reported by Rajdeepta et al. (2008). 57% inhibition of larval midgut proteases of *Sesi-gua* at 45 μg/ml of inhibitor from *Albizia kalkora* seeds was reported by Zhou et al. (2008). Schuler et al. (1998) observed 100% mortality of the first instar larvae of *S. littor-alis* when fed on transgenic tobacco plants expressing the serine proteinase inhibitor KTI3 gene from soybean. Inhi-

**REFERENCES**


terization and kinetic properties. Phytochemistry 67, 232-241


Calderon LA, Teles RCL, Leite JR, Franco OL, Grossi-de-Sá MF, Medrano FJ, Bloch JC, Freitas SM (2005) Purification of a 6.5 kDa protease inhibitor from *Inga umbratica* seeds effective against serine proteases of boll weevil *Anthonomus grandis*. Protein and Peptide Letters 12, 583-587


Davis BJ (1964) Disc electrophoresis-II: Methods and application to human proteins. Annals of the New York Academy of Science 121, 404-427


Deepika R, Nath A, Nath AK (2008) Toxicity of trypsin inhibitor from *Phaseo-
lus vulgaris* L. cultivar against *Pluta brassicae*. Pest Technology 2, 32-37


Ee KY, Zhao J, Rehman A-at-ur, Agboosa SO (2009) Purification and charac-
terization of a Kunitz-type trypsin inhibitor from *Acacia victoriae* Benthem seeds. Journal of Agricultural and Food Chemistry 57, 7022-7029

ties of a Kunitz type protein inhibitor obtained from *Pithecobolus dolcipes* seeds. Journal of Agricultural and Food Chemistry 52, 6115-6121

Franco OL, Santos RC, Batista JAN, Mendes ACM, Araújo MAM, Mon-

García VA, Freire MG, Novello JC, Marangoni S, Macedo ML (2004) Tryp-
inhibitor from *Poecieanthe parviflora* seeds: Purification, characterization and activity against pest proteases. Protein Journal 23, 343-350


Gomes CE, Barbosa AE, Macedo LL, Pitanga JC, Moura FT, Oliveira AS, Moura RM, Queiroz AF, Macedo FP, Andrade LB, Vidal MS, Sales MP (2005) Effect of trypsin inhibitor from *Crotalaria pallida* seeds on *Callosbro-
chus maculatus* (cowaee weevil) and *Ceratitis capitata* (fly). Plant Physiology and Biochemistry 43, 1095-1102


Gupta P, Dhawan K, Malhotra SP, Singh R (2000) Purification and charac-


*Functional Plant Science and Biotechnology* (Special Issue 1), 75-81 ©2012 Global Science Books

80

**ACKNOWLEDGMENTS**

Financial assistance provided by ASPEE, Agricultural Research and Development Foundation, Malad (W), Mumbai 400064 in the form of scholarship and contingency grant is duly acknowledged. The authors also thank Dr. Jaime A. Teixeira da Silva for making significant improvements to style and language.
Norioka N, Hara S, Ikenaka T, Abe J
Mosolov VV, Valueva TA
Lawrence PK, Koundal KR
Nandi AK, Basu D, Das S, Sen SK
Laskowski M Jr., Qasim MA
(2009) Kunitz-type trypsin inhib-
Kakade ML, Rackis JJ, McGhee JE, Puski G
Lineweaver H, Burk D
Maggo S, Malhotra SP, Dhawan K, Singh R
Macedo MLR, Matos DGG, Machado OLT, Marangoni S, Novello JC
445-452
444-452
81

mutant of tomato is compromised in signaling for defence against insect
attack. Plant Cell 8, 2067-2077
Inanaga H, Kobayashi D, Kouzuma Y (2001) Protein engineering of novel proteinase inhibitors and their effects on the growth of Spodoptera exigua lar-
Kouzuma Y (2001) Protein engineering of novel proteinase inhibitors and their effects on the growth of Spodoptera exigua larvae. Biotechnology 39, 2559-2264
Kakade ML, Rackis JJ, McGhee JE, Puski G (1974) Determination of tryp-
with high stability from Spinacia oleracea L. seeds. Biochemistry 74, 102-109
rum 30, 761-768
Klomkla S, Benjakul S, Kishimura H, Chaijan M (2011) Extraction, puri-
fication and properties of trypsin inhibitor from Thai mung bean (Vigna radi-
ata (L.) R. Wilczek). Food Chemistry 129, 1348-1354
Laskowski M Jr., Qasim MA (2000) What can the structures of enzyme–
inhibitor complexes tell us about the structures of enzyme substrate com-
plexes? Biochimica et Biophysica Acta 1477, 324-337
Lawrence PK, Koundal KR (2002) Plant protease inhibitors in control of phy-
Lineweaver H, Burk D (1934) The determination of enzyme dissociation cons-
tants. Journal of the American Chemical Society 56, 658-666
Lowry OH, Rosebough NJ, Farr AK, Randall RJ (1951) Protein measure-
ments with Folin’s phenol reagent. Journal of Biological Chemistry 193, 265-
275
Maggo S, Malhotra SP, Dhanaw K, Singh R (1999) Purification and charac-
terization of trypsin inhibitor from rice bean (Vigna unguiculata L.) seeds. Journal of Plant Biochemistry and Biotechnology 8, 61-64
and Biological Chemistry 52, 1245-1252
Ouchchourov NS, de Kogel WJ, Wiegers GL, Abrahamsson M, Jongsma MA (2004) Engineered multidomain cysteine protease inhibitors yield resis-
tance against western flower thrips (Frankliniella occidentalis) in greenhouse trials. Plant Biotechnology Journal 2, 387-397
Paiva PM, Oliva ML, Fritz H, Coelho LC, Sampaio CA (2006) Purification and primary structure determination of two Bowman-Birk type trypsin isooin-
hibitors from Cratylia mollis seeds. Phytochemistry 67, 545-552
Prasad ER, Dutta-Gupta A, Padmasree K (2010) Purification and characteri-
zation of a Bowman-Birk protease inhibitor from the seeds of black gram (Vigna mungo). Phytochemistry 71, 363-372
Qi RF, Song ZW, Chi CW (2005) Structural features and molecular evolution of Bowman–Birk protease inhibitors and their potential application. Acta Biochimica et Biophysica Sinica 37, 283-292
Rai S, Aggarwal KK, Babu CR (2008) Isolation of a serine Kunitz trypsin inhib-
hitor from leaves of Terminalia arjuna. Current Science 94, 1509-1512
11355
Saini HS, Weder JKP, Knights EJ (1992) Inhibitor activities of chickpeas (Cicer arietinum L.) against bovine, porcine and human trypsin and chymo-
trypsin. Journal of the Science of Food and Agriculture 60, 287-295
nism of enzyme inhibition: Prediction of the three dimensional structure of the dimeric trypsin inhibitor from Lenscuma leucocephala by homology
modelling. Biochemical and Biophysical Research Communications 314, 755-765
Schuler TH, Poppy GM,erry BR, Denholm I (1998) Insect resistant trans-
genic plants. Trends in Biotechnology 16, 168-175
Sharma U, Suresh CG (2011) Purification, crystallization and X-ray charac-
terization of a Kunitz-type trypsin inhibitor protein from the seeds of chick-
pea (Cicer arietinum). Acta Crystallographica Section F Structural Biology and Crystalization Communications 67, 714-717
Shee C, Sharma AK (2007) Purification and characterization of a trypsin inhi-
hitor from seeds of Marraya koenigii. Journal of Enzyme Inhibition and Medicinal Chemistry 22, 115-120
nomica Sinica 31, 53-57
Yoshizaki L, Troncoso MF, Lopes JL, Hellman U, Beltramini LM, Wolfen-
2634
tion of Kunitz inhibitor from Albizia kailaura and its inhibitory effect against pest midgut proteases. Biotechnology Letters 30, 1495-1499