MATERIALS
AND
METHODS
3. MATERIALS AND METHODS

3.1. MATERIALS USED IN THE STUDY

Seeds of twenty-one genotypes of black gram (*Vigna mungo*) including two popular varieties and nineteen pre-release cultures (Table 4) and other common pulses viz. Bengal gram (*Cicer arietinum*), cowpea (*Vigna unguiculata*), green gram (*Vigna radiata*), horse gram (*Dolichos biflorus*), red gram (*Cajanus cajan*) and soybean (*Glycine max*) were obtained from the Department of Pulses, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore-3 (Table 5). Bengal gram (white) (*Cicer arietinum*), fenugreek (*Trigonella foenum gracum*), field bean (*Dolichos lab lab*), kidney bean (*Phaseolus vulgaris*) and pea (*Pisum sativum*) were purchased from the local market.

3.2. ESTIMATION OF PROTEIN

The protein in the seeds and insect larval extract was quantified by the method of Lowry *et al.* (1951).

3.2.1. Materials

Reagent A: Alkaline sodium carbonate solution (2% sodium carbonate in 0.1N sodium hydroxide).

Reagent B: 0.5% copper sulphate (CuSO₄·5H₂O) in 1% potassium sodium tartarate.

Reagent C: Prepared by mixing 100 mL of reagent A and 2 mL of reagent B.

Reagent D: Folin-Phenol reagent. Commercially available reagent diluted as per the manufacturer's instructions.

Tris-HCl Buffer, pH 8.2, 0.05 M

6.05 g Tris (hydroxymethyl aminomethane) and 2.94 g CaCl₂·H₂O were dissolved in 900 mL water; pH was adjusted to 8.2 with dilute hydrochloric acid and the volume made up to 1000 mL with distilled water.
3.2.2. Seed extracts

One hundred milligram of finely ground seed flour was extracted in 1.0 mL distilled water, centrifuged at 10,000 rpm for 30 min at 4°C; supernatant collected and stored frozen in aliquots until further use.

3.2.3. Extraction of protease from *Callosobruchus maculatus*

The procedure adopted is a modification of the method used by Godbole *et al.* (1994)

Infested Bengal gram seeds were dissected and the active larvae were homogenized in cold 50 mM Tris-HCl buffer, pH 8.2

The homogenate was centrifuged in a microfuge at 10,000 rpm for 20 min at 4°C.

The supernatant after a second centrifugation for 10 min was used as the source of protease without any further dilution and purification.

The supernatant was distributed into 0.5 mL aliquots and stored at -20°C until further use.

3.2.4. Method

- Appropriate aliquots of the seed and larval extracts (in triplicates) were taken and the volume made up to 1mL with distilled water.

- Five-milliliter reagent C was added mixed thoroughly with the extract and the tubes were left in dark for 10 minutes.

- Reagent D (0.5 mL) was added, contents mixed again and left in dark for 30 minutes.

- The color was measured at 660 nm.

- The concentration of protein in each sample was calculated from the standard graph drawn by plotting known concentration of bovine serum albumin (BSA) as standard.
3.3. BIOASSAY

Screening of legumes for bruchid resistance

The feeding experiments were carried out as described by Tomooka et al. (1992) using *C. maculatus*, since it is the most predominant among the three species found in India.

Rearing of insects

3.3.1. Method

- Newly emerged adults of *C. maculatus* were brought from the insectary and put in a jar containing Bengal gram seeds; mouth of the jar was plugged with cotton.
- The jar was incubated at 30°C until the emergence of the first beetle.
- Newly emerged adult beetles were collected; males and females identified based on the body size (males were smaller than females).
- Ten seeds in each of the crop to be tested for bruchid resistance were incubated at room temperature in screw cap tubes with a single pair of the adult beetles and looked for emergence of new adult beetles.
- After the adult emergence the number of insects in each tube was then counted.
- Also the number of seeds damaged and the number of seeds infested with eggs were noted.
Table 4

Black gram (*Vigna mungo*) genotypes screened for trypsin inhibitory activity and resistance to bruchid

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
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<tr>
<td>1</td>
<td>CO 5</td>
<td>12 COBG 637</td>
</tr>
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<td>2</td>
<td>VBN 3</td>
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<tr>
<td>10</td>
<td>COBG 635</td>
<td>21 COBG 646</td>
</tr>
<tr>
<td>11</td>
<td>COBG 636</td>
<td></td>
</tr>
</tbody>
</table>

Table 5

Other common pulses screened for trypsin inhibitory activity and resistance to bruchid

1. Bengal gram CO3 (*Cicer arietinum*)
2. Cowpea CO6 (*Vigna unguiculata*)
3. Green gram CO6 (*Vigna radiata*)
4. Horse gram CO1 (*Dolichos biflorus*)
5. Soybean CO1 (*Glycine max*)
6. Red gram CO5 (*Cajanus cajan*)
7. Bengal gram (white) (*Cicer arietinum*)
8. Fenugreek (*Trigonella foenum gracum*)
9. Field bean (*Dolichos lab lab*)
10. Kidney bean (*Phaseolus vulgaris*)
11. Pea (*Pisum sativum*)

# 7 - 11 Variety not known, locally purchased.
3.4. DETERMINATION OF TRYPSIN INHIBITORY ACTIVITY

Trypsin inhibitory activity is measured indirectly by inhibiting the activity of trypsin. (Sadasivam and Manickam, 1996) A synthetic substrate benzoyl-DL-arginine paranitroanilide (BAPNA) is subjected to hydrolysis by trypsin to produce yellow colored p-nitroanilide. The degree of inhibition of the yellow color production by the extract is measured at 410 nm. The trypsin inhibitor activity is expressed as trypsin inhibitor units (TIU) per gram sample or per milligram protein.

3.4.1. Materials

30% Glacial acetic acid (v/v)

Trypsin: Lyophilized trypsin (5 mg) was dissolved and made up to 250 mL with 0.001 M hydrochloric acid.

Tris-HCl Buffer, pH 8.2, 0.05 M

6.05 g Tris (hydroxymethyl aminomethane) and 2.94 g CaCl₂·H₂O were dissolved in 900 mL water; pH adjusted to 8.2 with dilute hydrochloric acid and made up to 1000 mL with distilled water.

Substrate: Benzoyl-DL Arginine-paranitroanilide (BAPNA).

BAPNA (40 mg) was dissolved completely in 1.0 mL of dimethyl sulfoxide and made up to 100 mL with Tris-HCl buffer, pH 8.2.

3.4.2. Source of trypsin inhibitor

Finely ground seed flour (500 mg) was extracted in 25 mL distilled water using a pre-chilled mortar and pestle and kept in the refrigerator for 3 h with occasional shaking for complete extraction of trypsin inhibitor. The homogenate was centrifuged at 10,000 rpm for 30 min at 4°C and 1.0 mL of the supernatant diluted to 10 mL with distilled water and used as trypsin inhibitor source.
3.4.3. Method

- The extract was pipetted out (from 0 to 1mL) in duplicate sets of test tubes, one to serve as endogenous (E) and the other test (T).

- The volume was made up to 2 mL with buffer in the endogenous set. The volume in the test set was made up to 1.0 mL.

- One mL of trypsin solution (20 μg) was added to each tube in the test set.

- One mL of buffer and 1mL of trypsin solution were pipetted out into a separate test tube for standard (S).

- All the tubes were incubated at 37°C.

- After few minutes, 2.5 mL of substrate (1mg BAPNA) was added to each tube.

- The reaction was allowed to proceed for 30 min at 37°C.

- Reaction was stopped by adding 0.5 mL of 30% glacial acetic acid.

- The absorbance was read at 410 nm in a spectrophotometer.

The aliquot size of the extract required to inhibit 50% of the trypsin activity (S/2) was determined; which is considered to be one unit of trypsin inhibitor. One unit activity corresponds to that amount of trypsin inhibitor in μg protein, which gives 50% inhibition of enzyme activity under experimental conditions.

3.5. EXTRACTION AND PURIFICATION OF TRYPSIN INHIBITOR

Extraction and purification were carried out by following the method of Kamalakannan et al. (1981).

3.5.1. Extraction

Five hundred gram of black gram seed flour (100 mesh) was extracted with 500 mL of acetone for 20 min followed by extraction with 500 mL of ethanol for 20 min; filtered and air-dried at room temperature. Extracted with 2500 mL of 1% saline for 2 h using a mechanical stirrer at 4°C, pH adjusted to 4.4 with 10% hydrochloric acid; centrifuged at 10,000 rpm for 30 min. at 4°C. Isolation of the different fractions was carried out as represented in Fig. 2.
300 g seed flour extracted with acetone, ethanol & 1% saline

Centrifuged at 10,000 rpm for 30 min at 4°C

Debris
Not found to contain any considerable amount of trypsin inhibitor activity; Discarded

Residue: Dissolved in water, dialysed and centrifuged at 10,000 rpm for 30 min at 4°C

Supernatant (crude extract) 0-30% ammonium sulphate saturation; kept overnight and centrifuged at 10,000 rpm for 30 min at 4°C

Residue: Dissolved in water, dialysed and centrifuged at 10,000 rpm for 30 min at 4°C

Supernatant 75-100% ammonium sulphate saturation; kept overnight and centrifuged at 10,000 rpm for 30 min at 4°C

Residue: Dissolved in water, dialysed and centrifuged at 10,000 rpm for 30 min at 4°C

Supernatant 30-75% ammonium sulphate saturation; kept overnight and centrifuged at 10,000 rpm for 30 min at 4°C

Residue: Dissolved in water, dialysed and centrifuged at 10,000 rpm for 30 min at 4°C

Supernatant 0-30% ammonium sulphate saturation; kept overnight and centrifuged at 10,000 rpm for 30 min at 4°C

Residue (Discarded) Debris Not found to contain any considerable amount of trypsin inhibitor activity; Discarded

Supernatant Lyophilized Trypsin inhibitor fraction I

Supernatant Lyophilized Trypsin inhibitor fraction II

Supernatant Lyophilized Trypsin inhibitor fraction III

Figure 2. Schematic representation of isolation of black gram trypsin inhibitor fractions

(Rotor used is Hitachi RPR-12-2-767, diameter 16.5 cm; 10,000 rpm = 9232 x g)
3.5.2. Purification

The lyophilized black gram trypsin inhibitory fractions I, II and III were stored at 
-20°C until use. Purification of the trypsin inhibitor was achieved through 
diethylaminoethyl cellulose (DEAE-cellulose) column chromatography and finally through affinity chromatography on trypsin agarose affinity column.

3.5.3. DEAE-cellulose chromatography

3.5.3.1. Materials

Column material : DEAE cellulose (SIGMA)
Equilibration buffer : Sodium phosphate, 0.05M, pH 8.0
Elution buffer : Sodium phosphate, 0.05M, pH 8.0 with NaCl gradient 0.1-0.3M.
Black gram trypsin Inhibitor (BGTI) fraction II

3.5.3.2. Method

The column was prepared according to the method of Peterson and Sober (1972) as detailed below

Preparation of adsorbents for use:

Commercial DEAE cellulose adsorbent was thoroughly washed before use, regardless of grade. The dry power/material was soaked in distilled water for 2-3 h; water was changed in between (every 30 min). Then the suspension was filtered and washed with 1N sodium hydroxide, until no more colour was removed. This was followed by washing the material with distilled water thoroughly to remove the sodium hydroxide. Washing with distilled water continued until the pH reached neutral. This was followed by the addition of sufficient 1N hydrochloric acid, to make a strongly acid suspension. Filtered almost immediately and washed free of acid with water until the neutral pH. Then the prepared material was equilibrated with buffer, then poured into the column to a height of 18 cm (bed volume 56 mL).

• The column was washed with 20 volumes of double distilled water and further with 10 volumes of equilibration buffer.
The lyophilized sample of BGTI (fraction II) was dissolved in 0.05 M phosphate buffer (pH 8.0) to give a protein concentration of 2 mg/mL.

One millilitre of the sample was applied to the column with a flow rate of 2.5 mL per min and step-wise elution carried out.

Unbound proteins were eluted using 0.05 M phosphate buffer (pH 8.0).

Bound proteins were eluted with a NaCl gradient in the range of 0.1-0.3 M in 0.05M phosphate buffer (pH 8.0).

Fractions of 5mL were collected and absorbance read at 280 nm.

Fractions containing protein were tested for trypsin inhibitory activity.

The fractions possessing the maximum inhibitory activity were pooled, dialyzed and lyophilized.

3.5.4. Trypsin–agarose affinity chromatography

3.5.4.1. Materials

Insoluble enzyme attached to cross-linked beaded agarose from SIGMA.

Equilibration buffer : 0.1M sodium formate containing 0.02 M CaCl₂ (pH 4.0)

Elution buffer : 0.01M HCl-0.02 M CaCl₂.

Partialy purified BGTI (through DEAE-cellulose column)

Sample extract:

Prepared as described by Chrispeels and Baumgartner (1978) for direct purification using trypsin-agarose affinity column.

3.5.4.2. Method

Sample preparation (Chrispeels and Baumgartner, 1978)

- Seeds of black gram (*Vigna mungo*) (100 g) were homogenized in cold water (400 mL) in a mixer grinder.

- The slurry was stirred for 2 h at room temperature and centrifuged at 9232 x g for 30 min at 4°C.
Trichloroacetic acid was added to the supernatant to make 5% solution: allowed to stand for 3 h in the cold.

Centrifuged at 9232 x g for 30 min at 4°C and pH of the supernatant was adjusted to 4.0 with 1N NaOH.

The solution was passed over the trypsin affinity column, equilibrated with 0.1M sodium formate –0.02M CaCl2 (pH 4.0)

The column was washed extensively with the equilibrium buffer until the A230nm was almost zero, followed by washing with 0.02 M CaCl2.

The trypsin inhibitor was then eluted with 0.01 M HCl-0.02 M CaCl2.

Three mL fractions were collected and their absorbance measured at 230 nm.

The fractions showing high activity were pooled, dialyzed for 4 h against water and then lyophilized.

3.6. SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) OF THE PURIFIED INHIBITOR

The polypeptides in the samples were fractionated in sodium dodecyl sulphate-polyacrylamide gel in a discontinuous buffer system as described by Laemmli. (1970).

3.6.1. Materials

Acrylamide monomer solution (30%) :
(29.2% acrylamide, 0.8% bisacrylamide)

Acrylamide solution (30%, w/v)
Thirty g of acrylamide was mixed with 0.8 g of bisacrylamide and made upto 100 mL with deionised water. Stored in dark in a brown bottle at 4°C.

Resolving gel buffer : (1.5 M Tris –HCl, pH 8.8)
Tris base (18.2 g) was dissolved in double distilled water (90 mL); pH adjusted to 8.8 with HCl and volume made upto 100 mL.

Stacking gel buffer : (0.5 M Tris –HCl, pH 6.8 )
Tris base (6.05 g) was dissolved in double distilled water (90 mL) pH adjusted to 6.8 with HCl; volume made upto 100 mL.
Tank buffer: 5x
Five gram of SDS, 72 g of glycine and 15.15 g of Tris base were dissolved in 1 L of double distilled water. Diluted to 1x at the time of running gel (pH 8.3 approximately).

Sodium dodecyl sulphate (SDS): 10% (w/v)
One gram of SDS dissolved in 10 mL of double distilled water.

Ammonium per-sulphate (APS): 10% (prepared fresh)
One hundred milligram of APS dissolved in 1 mL of double distilled water.

Sample buffer: 5x (0.5 M Tris-HCl, 20% glycerol, 10% SDS, 10% β-mercapto-ethanol, 0.5% bromophenol blue, pH 6.8).
Two hundred milligram of SDS, 0.152 g Tris base, 0.2 mL glycerol and 0.2 mL of β-mercapto-ethanol were dissolved in 5 mL of double distilled water. Adjusted the pH to 6.8 with HCl.

TEMED (N,N,N,’N’-tetra methyl ethylene diamine): Fresh from the refrigerator.

Staining solution 0.1% Coomassie brilliant blue – R-250, 40% methanol, 10% acetic acid.

Destaining solution: (40% methanol, 10% acetic acid).

Slab gel electrophoresis and power supply units.

3.6.2. Gel casting

- The gel casting unit was assembled- following the manufacturer’s instructions, using 1 mm spacer set.

- In a side-arm flask, (as per the percentage and volume of the gel) 30% monomer, separating gel buffer, 10% SDS and 10% APS were mixed degassed and TEMED was added. (Table.6)

- The mixture was poured in the gel plate sandwich up to a level of about 5 cm from the top.

- The gel was overlaid with distilled water and left to polymerize for 3 h.

- The water was removed and traces of water on top of the gel was blotted out.

- In a side-arm flask, (as per the volume of the gel) 30% monomer, stacking gel buffer, 10% SDS and 10% APS were mixed.

- The solution was degassed, TEMED was added with gentle swirling and poured over the separating gel
- An appropriate comb was inserted in place and the gel was allowed to polymerize for one hour

Table 6. Solutions for separating and stacking gel preparation.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Separating gel (18 %)</th>
<th>Stacking gel (4 %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mini gel</td>
<td>Big gel</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>3.0 mL</td>
<td>12.0 mL</td>
</tr>
<tr>
<td>Resolving gel buffer</td>
<td>1.3 mL</td>
<td>5.2 mL</td>
</tr>
<tr>
<td>Stacking gel buffer</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>0.6 mL</td>
<td>2.4 mL</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>50 µL</td>
<td>200 µL</td>
</tr>
<tr>
<td>10 % APS</td>
<td>40 µL</td>
<td>160 µL</td>
</tr>
<tr>
<td>TEMED</td>
<td>2 µL</td>
<td>8 µL</td>
</tr>
</tbody>
</table>

3.6.3. Sample preparation, loading and running

- Samples were mixed with equal volume of sample buffer and boiled in a water bath for 3 min and kept in ice till loading.

- Centrifuged in a microfuge at 10,000 rpm for 1 min just before loading.

- The comb was removed and the wells were repeatedly washed with tank buffer to remove any unpolymerized monomer.

- The gel assembly was placed in the lower buffer chamber with a heat exchanger in place and filled with tank buffer.

- Appropriate amounts of protein samples were loaded in each well, power supply was turned on and the gel was run at 10 mA constant current until the dye front reached the resolving gel and then to 30 mA.

- When the dye reached the bottom of the gel, the power supply stopped, and the gel assembly was removed.
3.6.4. Staining and destaining

The gels were stained in 100 mL staining solution overnight with constant gentle shaking and destained with several changes of destaining solution (with gentle shaking) until the bands were clearly visible.

3.7. CHARACTERIZATION OF BLACK GRAM TRYPsin INHIBITOR

3.7.1. Determination of molecular weight

Molecular weight of the purified trypsin inhibitor was determined by SDS-PAGE as described previously under section 3.6.

Time course of purification of trypsin inhibitor was checked through SDS-PAGE. Crude extract, ammonium sulfate precipitate fractions, DEAE-cellulose column fractions and fractions collected from trypsin-agarose affinity column were analyzed for their protein content by Lowry's method, then subjected to SDS-PAGE along with standard marker proteins. (viz. Phosphorylase b (97.4 kDa), Bovine serum albumin (66 kDa), Ovalbumin (43 kDa), Carbonic anhydrase (29kDa), Soybean trypsin inhibitor (20.1 kDa), Lysozyme (14.3 kDa). The molecular weight of the black gram trypsin inhibitor was calculated from the semi-log graph plotted with molecular weight against Rm values.

3.7.2. Glycoprotein staining

To check whether black gram trypsin inhibitor is glycoprotein in nature, glycoprotein staining was done according to the method of Kapitany and Zebrowski (1973) with modifications.

3.7.2.1. Materials

<table>
<thead>
<tr>
<th>Purified black gram trypsin inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixing solution : 12.5 % Trichloroacetic acid.</td>
</tr>
<tr>
<td>Oxidizing solution : 1.0 % Periodic acid.</td>
</tr>
<tr>
<td>Destaining solution : 15 % acetic acid.</td>
</tr>
<tr>
<td>Schiff's reagent : Commercially available.</td>
</tr>
</tbody>
</table>
3.7.2.2. Method

- PAGE of the purified trypsin inhibitor was carried out on 18% gel.
- The gel was incubated in the fixing solution for 1 h.
- The gel was then subjected to sample oxidation with 1 % periodic acid for 2 h.
- Followed by washing the gel in 15 % acetic acid for 2 h (with 4-5 changes).
- All the above steps were carried out at room temperature.
- The gel was then placed in Schiff's reagent and stored in the refrigerator (in the dark) for 2 h.
- Destaining was carried out using 15% acetic acid with shaking.
- Bands corresponding to glycoproteins were visualized.

3.7.3. Carbohydrate analysis

Total sugar content in the purified black gram trypsin inhibitor was measured by phenol-sulphuric acid method of Du Bois et al. (1956)

3.7.3.1. Materials

- Purified black gram trypsin inhibitor
- Phenol: 5 % (Re-distilled reagent grade)
- Sulphuric acid: 96 % Analar Reagent grade.

3.7.3.2. Method

- Appropriate aliquot of the purified trypsin inhibitor was taken and the volume was made up to 1 mL with distilled water. One milliliter of phenol solution was added and thoroughly mixed.
- Five milliliter of 96% sulphuric acid was added.
- After mixing the contents again, the tubes were placed in a water bath at 25-30°C for 20 min.
- A blank was set using 1mL distilled water.
- The absorbance was read at 490 nm.
• The total sugar content in the sample was calculated from the standard graph constructed with known concentrations of glucose.

3.7.4. Detection of trypsin /trypsin inhibitor interactions

Native-PAGE was done according to the method of Blanco-Labra et al. (1996) to follow the enzyme /inhibitor interaction by negative staining method.

3.7.4.1. Materials

Stock acrylamide solution : 30% (w/v)
Running gel buffer : 1.5 M Tris-HCl (pH 8.8)
Stacking gel buffer : 0.5 M Tris-HCl (pH 6.8)
APS : 10% (w/v)
TEMED : Fresh from the refrigerator.
Electrode buffer : 0.05 M Tris – HCl, 0.192M glycine. (pH 8.3)
Sample loading buffer 5x : 0.5 M Tris–HCl, 20 % glycerol,
0.5 % bromophenol blue, (pH 6.8).

3.7.4.2. Method

Twelve percent native gel was cast with the following gel recipe (Table7). Casting the gel, sample loading and electrophoretic separation was done as described previously under SDS-PAGE.

Table 7. Solutions for native gel preparation

<table>
<thead>
<tr>
<th>Gel mixture-composition</th>
<th>Resolving gel</th>
<th>Stacking gel</th>
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</thead>
<tbody>
<tr>
<td>Acrylamide, 30%</td>
<td>2.0 mL</td>
<td>0.67 mL</td>
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<tr>
<td>Tris buffer, pH 8.8</td>
<td>1.3 mL</td>
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</tr>
<tr>
<td>Tris buffer, pH 6.8</td>
<td>-</td>
<td>0.50 mL</td>
</tr>
<tr>
<td>APS, 10% (w/v)</td>
<td>0.05 mL</td>
<td>0.04 mL</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.002 mL</td>
<td>0.002 mL</td>
</tr>
<tr>
<td>Water</td>
<td>1.65 mL</td>
<td>2.79 mL</td>
</tr>
<tr>
<td>Total volume</td>
<td>5.0 mL</td>
<td>4.00 mL</td>
</tr>
</tbody>
</table>
3.7.4.3. Materials (Sample loading and negative staining)

Trypsin
Purified trypsin inhibitor
Casein solution : 2% solution.
Staining solution : 0.1% Coomassie Brilliant Blue-R-250, 40% methanol, 10% acetic acid.
Destaining solution : 40% methanol, 10% acetic acid.

3.7.4.4. Method

- Trypsin solution (20 µg) was incubated at 37°C with 20µg, 40µg and 60 µg of the inhibitor for 30 min.
- Twenty microgram of the enzyme and inhibitor alone were taken as control.
- Sample buffer was added and the samples were loaded in the wells and electrophoresis carried out as described for native PAGE.
- After electrophoresis, the gel was incubated in 100 mL casein solution for 90 minutes at room temperature.
- The gel was then stained in 100 mL staining solution overnight with constant gentle shaking.
- Followed by destaining the gel (with gentle shaking) until the bands were clearly visible in a blue background.

3.7.5. N-terminal amino acid sequence analysis

The purified trypsin inhibitor was transferred to a Poly Vinylidene Difluoride (PVDF) membrane and the N-terminal amino acid sequence was determined.

3.7.5.1 Western transfer

The standard procedure of Laemmli for SDS-PAGE was suitably modified following the instruction of BIO-RAD (PVDF membrane manufactures). The materials used for SDS-PAGE were the same as those described elsewhere.
3.7.5.2. Method

SDS-PAGE was carried out as described previously after loading the purified black gram trypsin inhibitor into the wells (60 µg per well)

3.7.5.3. Materials (for transfer to PVDF membrane)

Trans-Blot SD Semi-Dry transfer Cell (Bio-RAD)
PVDF protein sequencing membrane (Bio-RAD)
Transfer buffer (10 x)
Glycine : 72.10 g
Tris-base : 15.15 g

Volume was made upto 250 mL with sterile distilled water. At the time of use, 10 mL of 10 x transfer buffer, 20 mL methanol and 70 mL sterile distilled water were mixed and used for saturating the filter paper.

Ponceau S (10 x)
2.0 % Ponceau S (SIGMA) in 30% trichloroacetic acid and 30% sulphosalicylic acid.

Methanol : 100 %

3.7.5.4. Method

After electrophoresis, the separated proteins were transferred to PVDF membrane by blotting the gel and the membrane between pieces of Whatman No.3 paper. The gel and the transfer media were sandwiched between layers of buffer-saturated Whatman No.3 filter papers serving as ion reservoirs during transfer; the sandwich was prepared by laying the membrane and the gel carefully without entrapping air bubbles between them. A corner of the membrane was marked to know the orientation of the gel. The transfer was carried out at a current of 15V for 50 min. after completion of transfer, the membrane stained with Ponceau S stain for two minutes to check the resolution and confirm the transfer. The membrane was then destained with several changes of double distilled water. The destained membrane was dried at room temperature by placing between folds of filter paper.
3.7.5.5. Amino acid sequencing

The N-terminal amino acid sequence was determined at the Department of Biochemistry, Kansas State University, USA, following the Edman degradation method.

3.7.6. Determination of inhibitor stability

3.7.6.1. Heat stability of black gram trypsin inhibitor

To determine the stability of black gram trypsin inhibitor at different temperatures, purified black gram trypsin inhibitor was incubated at different temperatures and residual inhibitory activity was measured.

3.7.6.2. Materials

- Purified black gram trypsin inhibitor
- Tris-HCl buffer
- Trypsin
- BAPNA
- 30% acetic acid

Assay method is as in determination of trypsin inhibitory activity. (Sec.3.4.3.)

3.7.6.3. Method

- The purified black gram trypsin inhibitor (100 µg/mL) was added to 50 mM Tris-HCl buffer, pH 8.2 containing 20 mM CaCl₂ and incubated at 20°, 40°, 60°, 80° and 100°C for 20 min in different test tubes.
- The tubes were cooled and an aliquot was added to the assay mixture for measuring the residual inhibitor activity.
- Absorbance was measured at 410 nm.

3.7.7. Stability of black gram trypsin inhibitor at different pH

To test the pH stability of the inhibitor, different buffer systems were used and residual inhibitor activity was measured.
3.7.7.1. Materials

Purified black gram trypsin inhibitor

Different buffer systems (50 mM) (pH range 2 - 10)

i. KCl-HCl buffer, pH 2.0

ii. Citrate-phosphate buffer pH 3.0, 4.0, 5.0 & 6.0

iii. Phosphate buffer pH 7.0

iv. Tris-HCl pH 8.0

v. Glycine –NaOH pH 9.0 and 10.0

Reagents and assay method are as in determination of trypsin inhibitory activity.
(See .3.4.3.)

3.7.7.2. Method

- Purified black gram trypsin inhibitor was dissolved in different buffer systems at a concentration of 1 mg per mL and pre-incubated at room temperature for 24 h.

- Aliquots (0.1mL) of the inhibitor in the different buffers were added individually to the assay mixture containing the buffered substrate and the enzyme.

- Residual inhibitor activity was determined by measuring the absorbance at 410nm.

3.7.8. Effect of inhibitor concentration

Effect of increasing concentrations of the inhibitor in the assay medium was studied by adding different levels of the inhibitor.

3.7.8.1. Materials

Purified black gram trypsin inhibitor

Reagents and assay method are as in determination of trypsin inhibitory activity.

3.7.8.2. Method

- Purified black gram trypsin inhibitor was dissolved in 50 mM tris-HCl buffer (pH 8.2) containing 20 mM CaCl₂.
3.7.9. Effect of Enzyme Concentration

Effect of concentration of the enzyme against inhibitory activity of the purified protein was determined by adding increasing the levels of the enzyme in the assay mixture.

3.7.9.1. Materials

Purified black gram trypsin inhibitor
Reagents and assay method are as in determination of trypsin inhibitory activity.

3.7.9.2. Method

- Purified black gram trypsin inhibitor was dissolved in 50 mM Tris HCl buffer, (pH 8.2) containing 20 mM CaCl₂.
- Different amount of the enzyme (5 - 30 µg) was incubated with fixed concentration of inhibitor (25 µg) and BAPNA (1 mg)
- The inhibition (%) was measured as mentioned earlier.

3.7.10. Antifungal activity

Antifungal assay of the black gram trypsin inhibitor was carried out by following the method described by Roberts and Stelitrennikoff (1986).

3.7.10.1. Materials

100 x 15 mm Petri-plates
Sterile filter paper discs

3.7.10.2. Fungal strains

Trichoderma viride
Macrophomina phaseolina
3.7.10.3. Carrot juice agar medium (CJA medium)

Thirty grams of 1-2 mm thick carrot slices were added to 120 mL of water and autoclaved for 20 min. One hundred mL of the resulting broth was added to 400 mL of water with 10 g of agar and autoclaved for 20 min. About 20 mL was poured per plate.

3.7.10.4. Method

Small cubes of agar containing mycelia of the fungi were placed in the centers of carrot juice agar medium plates and kept at room temperature until colonies were 2-3 cm in diameter. Sterile discs were placed 1-2 mm from the growing front. Crude extract, partially purified and purified trypsin inhibitor fractions were spotted on the distinct discs. Plates were incubated at room temperature for 24-72 h depending on the fungus until the growing front covered the disc spotted with sterile water as a negative control. Mycelia growth was examined visually and inhibition zones were noted.

3.8. LOCALIZATION OF BLACK GRAM TRYPsin INHIBITOR IN MATURE GRAINS

Trypsin inhibitory activity in different parts of the black gram grains was determined to assess the localization of the inhibitor.

3.8.1. Materials

CO5 black gram grains

Reagents and assay method are as in determination of trypsin inhibitory activity.

3.8.2. Method

- Black gram grains were soaked for 1h in distilled water.
- Soaked grains were separated into seed coat, cotyledons and embryonic axes.
- Separated portions were ground and extracted with known volume of assay buffer.
- Ground samples were kept in the refrigerator for 30 min with occasional shaking.
• Homogenate was centrifuged at 10,000 rpm for 30 min at 4°C in a microfuge.

• Aliquots from the supernatant were used for trypsin inhibitor activity assay.

• Soluble protein content was also estimated in the supernatant by the method of Lowry et al. (1951).

3.9. PROFILE OF TRYPsin INHIBITOR ACTIVITY DURING SEED GERMINATION AND POD DEVELOPMENT

Germination and pot culture studies were conducted to follow the fate of trypsin inhibitor during seed germination and accumulation pattern during pod development.

3.9.1. Variation in trypsin inhibitor activity during germination in black gram

3.9.1.1. Materials

Mature CO5 black gram seeds

Petri-plates

3.9.1.2. Method

• Black gram seeds were sterilized by soaking in 0.1 per cent mercuric chloride solution for 30 min and washed thoroughly with sterile water.

• Seeds were spread on Petri-plates layered with moist absorbent cotton and filter paper pads for germination at room temperature under sterile conditions.

• Germinated samples were withdrawn at 12 h interval.

• Germinated samples (with plumule) were extracted with 50 mM Tris-HCl buffer (pH 8.2)

• Homogenate was stirred for 30 min and centrifuged at 10,000 rpm for 30 min at 4°C in a microfuge.

• Aliquots from the supernatant was assayed for soluble protein and trypsin inhibitory activity.
3.9.2. Accumulation of trypsin inhibitor during pod development

3.9.2.1. Materials

Mature CO5 black gram seeds

3.9.2.2. Method

- Pot culture studies were carried out with black gram seeds sown in the pots after surface sterilization.
- Each pot contained 3 - 4 plants and plant protection measures were followed.
- Flowers were tagged to collect the developing pods at regular intervals.
- Developing pod and seed samples were collected at 3 days intervals and extracted with assay buffer starting from flowering till maturity and harvest.
- Aliquots were analyzed for soluble protein and trypsin inhibitory activity.

3.10. IMMUNOLOGICAL STUDIES OF TRYPsin INHIBITOR

3.10.1. Production of polyclonal antibody

Making use of customer service offered by Genei, Bangalore, polyclonal antibodies were produced in rabbits against purified black gram trypsin inhibitor supplied by us.

3.10.2. Western blotting

This highly sensitive immuno detection technique was done to detect the presence of the black gram trypsin inhibitor in different samples and to find out the cross-reactivity of the polyclonal antibody (raised against the black gram trypsin inhibitor) with trypsin inhibitor and homologous proteins present in other pulses. The polypeptides after SDS-PAGE were transferred to nitrocellulose membrane from the gel according to method prescribed by Gallanger et al. (1995).
3.10.2.1. Materials
   Transfer-Blot SD Semi-Dry Transfer Cell
   Nitrocellulose membrane
   Transfer buffer (10 x)
   Glycine 72.1 g
   Tris base 15.15 g
   Final volume was made upto 250 mL with sterile distilled water

3.10.2.2. Method
As described under Western transfer for N-terminal amino acid sequence analysis in Sec. 3.7.5.

3.10.3. Immunostaining
The proteins on the blot were allowed to react with the antibody. The antigen antibody complex bound to the nitrocellulose membrane was allowed to react with the secondary antibody and the color developed by reaction with BCIP/NBT substrate was visualized by the appearance of the purple colored bands.

3.10.3.1 Materials
Phosphate buffered saline (PBS) (10 x)
   KH$_2$PO$_4$ 2.0 g
   Na$_2$HPO$_4$ 11.5 g
   KCl 2.0 g
   NaCl 80 g
   Final volume was made upto 1 L with sterile distilled water.

Alkaline phosphatase buffer (pH 9.5)
   Tris-base 1.21 g
   NaCl 580 mg.
   MgCl$_2$ 100 mg
Constituents were dissolved in 80 mL of sterile distilled water, pH adjusted to 9.5 with HCl and volume made up to 100 mL.

Antiserum buffer: 1 L
- Skimmed milk powder: 50 g
- 10 x PBS: 100 mL
- Tween 20: 1 mL

Blocking solution.
- 5% skimmed milk powder (Sagar, India) in 1x PBS with 1% Tween 20

Primary antibody
- Antibodies raised in rabbit against purified black gram trypsin inhibitor (1:5000 dilution)

Secondary antibody
- Anti-rabbit IgG-alkaline phosphatase conjugate (Genei, Bangalore).

Alkaline phosphatase substrate (Color developing agent)
- 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitro blue tetrazolium (NBT) - ready mix from Sigma.

3.10.3.2. Method
- The separated proteins were transferred to nitrocellulose membrane by blotting. The blot was incubated in blocking solution for 2 h with gentle shaking at room temperature.
- Followed by incubating the blot with primary antibody in antiserum buffer for 2 h at room temperature with gentle shaking.
- The unbound primary antibody was removed by rinsing the blot in antiserum buffer thrice for 5 min each.
- Incubated the blot in secondary antibody (1:5000) in antiserum buffer for 2 h at room temperature with gentle shaking.
- The unbound secondary antibody was removed with three washes in 1x PBS, 0.1% Tween - 20 for 10 min each.
• Equilibrated the blot briefly in alkaline phosphatase buffer and protein was detected using BCIP/NBT substrate

• The protein polypeptides recognized by the antibody appeared as purple bands were visualized.