5. SUMMARY AND CONCLUSIONS

The main aim of present investigation was to isolate and characterize the protease inhibitor proteins and its genes from the Pigeonpea. The PI protein was isolated Pusa-33 variety of Pigeonpea. The PI protein was purified by using ion-exchange chromatography followed by gel filtration. The molecular weight was determined through SDS-PAGE which confirmed by gel filtration. The efficacy of purified PI protein was checked on *Helicoverpa armigera*. The biomass and PI activity also checked for developing seeds. The total RNA was isolated from developing seeds and mRNA used for construction of cDNA library in pGEM-T easy vector. The cDNA library was screened with heterologous tomato T11 cDNA probe. The PI gene was isolated, sequenced and analyzed through bioinformatics tools available on NCBI.

The main findings of present investigation are:

- The PI protein was isolated Pusa-33 variety by using 0.1 M of phosphate buffer (pH 7.6) and subjected to 80% ammonium sulphate fractionation.
- The fraction was dialyzed and PI protein purified by ion-exchange chromatography using DEAE-Cellulose followed by gel-filtration.
- The purified Pigeonpea Protease Inhibitor (PPI) protein showed 26 KD band on SDS-PAGE, which was confirmed on gel filtration as 24.95kD molecular weight proteins.
- The purified PI protein retains its 85% activity at 90°C temperature, but it becomes inactive on boiling or autoclaving within 5 minutes and also remains active on the alkaline pH 8.0-10.0.
- The toxicity of purified PPI protein was checked on development of polyphagous insect *Helicoverpa armigera*. The *in-vivo* assay was conducted on the 6 days old neonates of *H. armigera* under three treatments.
- The purified PPI protein affected the growth and development of *Helicoverpa armigera*. The larvae under three treatments, *viz.* T1 (0.5%), T2 (1.0%) and T3 (1.5%) respectively extended their period from 4, 10 and 12 days up to pupation.
- The body weight of larvae and pupal stage was also adversely influenced by the treatments. The larval mortality was dose dependent and highest mortality was 46% in T3 treatment when 1.5% PPI protein was mixed with diet.
- The adults emerged from the treated pupa were observed that they failed to mate and lay eggs. The adults did not survive more than one week. Some adults were deformed and have crippled wings.
• The cDNA library was constructed in pGEMT easy vector from mRNA of 12 DAF seeds and tomato T1 II cDNA probe was used for screening. Six positive clones were identified after primary, secondary and tertiary screening of cDNA library which named as PPl-1 to PPI-6.

• On restriction digestion the clones gave approximately 600-800 bp size insert of out of which clone PPI-6 gave higher size insert approximately 800 bp long.

• This clone was sequenced and gave 786bp nucleotide sequences, which has 693bp nucleotide open reading frame and showed 93% homology with *Cicer arietinum* mRNA trypsin inhibitor 1 (tpi 1) gene.

• This gave protein product of 230 amino acids with theoretical molecular weight of 24.84 kD which showed 100% homology with Kunitz type protease inhibitor of soybean trypsin inhibitor (STI). It belongs to soybean trypsin inhibitor super family.

From above finding it was concluded that PPI Protein become inactive on boiling and autoclave and remains active in alkaline pH. So this will not harm to human beings on consumption. Because PI remains active in alkaline medium so this will be active in insects which have alkaline pH mid gut and affect insect growth and development. It also concluded that PPI needed 1.0% to 1.5% gene expression levels because pigeonpea has 0.6% of PI protein its own. So it will be good candidate for the development the transgenic for the insect resistance. The gene constructs will be prepared for PPI gene with suitable vector and mobilized in the susceptible crop plant by using established protocols. By the plant transformation transgenic crop plants will be developed. These crop plants will be resistant for insect pests and help in enhancing the crop productivity.

Therefore further research work is required to clone PPI gene and used for genetic transformation to develop transgenic crop plant, which will be resistant for insect pests.
REFERENCES


Michaud D., Nguyen-Quoc C., Vrain T.C., Fong D. and Yelle S. (1996). Response of digestive cysteine proteinases from the Colorado potato beetle (Leptinotarsa decemlineata) and the black vine weevil (Otiorhynchus sulcatus) to a recombinant form of human stefin A. Archives of Insect Biochemistry and Physiology. 31: 451-464.


APPENDIX

List of Buffers and Solutions

Buffer for Protein isolation

1M Phosphate Buffer (pH-7.6)
(Monobasic + Dibasic)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>pH</th>
<th>1M Na$_2$HPO$_4$ (Mono-basic)</th>
<th>1M NaH$_2$PO$_4$ (Di-basic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>6.8</td>
<td>46.3</td>
<td>53.7</td>
</tr>
<tr>
<td>2.</td>
<td>7.0</td>
<td>57.7</td>
<td>42.3</td>
</tr>
<tr>
<td>3.</td>
<td>7.2</td>
<td>68.4</td>
<td>31.6</td>
</tr>
<tr>
<td>4.</td>
<td>7.4</td>
<td>77.4</td>
<td>22.6</td>
</tr>
<tr>
<td>5.</td>
<td>7.6</td>
<td>84.5</td>
<td>15.5</td>
</tr>
</tbody>
</table>

(Data from ISCO-1982)

Protein estimation buffers

Reagent A:
2% Sodium carbonate in 0.1 N sodium hydroxide (freshly prepared)

Reagent B:
0.5% Copper sulphate (CuSO$_4$.5H$_2$O) 1% potassium tetracetic

Reagent C:
Alkaline copper solution (50 ml of A + 1 ml of B).

Folin Ciocalteau Phenol Reagent:
2 N and was diluted to 1 N with distilled water just before use.

Protease Inhibition assay buffer

Reaction Buffer (100 ml)

2M NaCl 01.00ml
0.5M CaCl$_2$ 01.00ml
0.1M Phosphate Buffer 50.00ml
0.001M DDT 00.10ml
Water 48.00ml
Total volume 100.00ml

BAPNA (1mM) (6 ml)

BAPNA 26.04 gm

Dissolve the in minimum volume of DMSO and make the final volume 6 ml with distilled water.
Trypsin stock (10mg/ml) 9590U/mg
Trypsin 20mg
Dissolve the trypsin in 2ml of 1mM HCl and stored at -80°C for future use. The trypsin has 9590 Unit / mg. The working concentration as 1mg/ml will be used.

Acetic acid (30%) 100ml
Acetic acid 30ml
Water 70ml

PAGE buffers
Stock acrylamide solution (30%)
Acrylamide 29.2 gm
Bis-acrylamide 0.8 gm

Buffer for SDS-PAGE
(i) Resolving gel buffer
Tris base 1.5 M
Adjusted pH to 8.8 with HCl
(ii) Stacking gel buffer
Tris base 1.0 M
Adjusted pH to 6.8 with HCl
(iii) Electrode buffer (1 liter)
Tris base 6.0 g
Glycine 14.4 g
SDS 1.0 g
(iv) 5X SDS loading dye
1M Tris-HCl (pH 6.8) 0.50 ml
SDS 0.05 gm
Sucrose 0.50 gm
β-mercaptoethanol 0.025 ml
0.5% Bromophenol blue 0.1 ml
Water to 1.0 ml

Buffer for native PAGE
(i) Resolving gel buffer
Tris base 1.5 M
Adjusted pH to 8.8 with HCl
(ii) Stacking gel buffer
Tris base 0.8 M
Adjusted pH to 6.8 with HCl

(iii) **Electrode buffer (1 liter)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>6.0 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>14.4 g</td>
</tr>
</tbody>
</table>

(iv) **2X sample loading dye**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8 M Tris-HCl (pH 6.8)</td>
<td>0.125 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.200 ml</td>
</tr>
<tr>
<td>0.5% Bromophenol blue</td>
<td>0.050 ml</td>
</tr>
<tr>
<td>Water</td>
<td>0.625 ml</td>
</tr>
</tbody>
</table>

**Protein staining solution (1 liter)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>500 ml</td>
</tr>
<tr>
<td>Methanol</td>
<td>400 ml</td>
</tr>
<tr>
<td>Glacial Acetic Acid</td>
<td>100 ml</td>
</tr>
<tr>
<td>Coomassive Blue (R-250)</td>
<td>1.0 gm</td>
</tr>
</tbody>
</table>

**Protein destaining solution (1 liter)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
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<tr>
<td>Methanol</td>
<td>400 ml</td>
</tr>
<tr>
<td>Glacial Acetic Acid</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

**DNA extraction buffer (100ml)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% CTAB</td>
<td>20 ml</td>
</tr>
<tr>
<td>4M Sodium chloride</td>
<td>35 ml</td>
</tr>
<tr>
<td>0.5M EDTA (pH 8.0)</td>
<td>4 ml</td>
</tr>
<tr>
<td>1M Tris-Cl (pH 8.0)</td>
<td>10 ml</td>
</tr>
<tr>
<td>DD Water</td>
<td>31 ml</td>
</tr>
<tr>
<td>β-ME</td>
<td>20 μl</td>
</tr>
</tbody>
</table>

**RNA extraction buffer**

**Lysis Buffer (100ml)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>8M</td>
</tr>
<tr>
<td>LiCl₂</td>
<td>5M</td>
</tr>
</tbody>
</table>

Mixed both content and makeup the final volume with distilled water.

**Resuspension Buffer (100ml)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS</td>
<td>0.5 %</td>
</tr>
<tr>
<td>Sod. Chloride</td>
<td>100mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>25mM</td>
</tr>
<tr>
<td>Tris (pH 7.5)</td>
<td>10 mM</td>
</tr>
</tbody>
</table>
PVP 2.0 %  
Mixed all the content and makeup the final volume with distilled water.

Other requirements

Phenol (saturated) with 10mm Tris pH-8
Phenol: chloroform: Isoamyl alcohol (25:24:1)
Chloroform: Isoamyl alcohol (24:1)
95%Ethanol with 2% Acetate
70% Ethanol

10 X MOPS buffer

MOPS 0.20 M
Sodium citrate (pH 7.0) 0.05 M
EDTA 0.01 M

10X RNA loading dye

Formamide 0.72 ml
10 X MOPS 0.16 ml
Formaldehyde 0.26 ml
H2O 0.18 ml
80% glycerol 0.10 ml
Bromophenol blue (saturated) 0.08 ml

Denaturing solution

Sodium chloride 1.5 M
Sodium hydroxide 0.5 M

Neutralizing solution

Sodium chloride 1.5 M
Tris-HCl pH 7.2 0.5 M
EDTA 1 mM

20X SSC

Sodium chloride 3.0 M
Sodium citrate 0.3 M

Pre-hybridization buffer

Phosphate buffer pH 7.2 0.5 M
SDS 7 %
EDTA 10 mM

50X TAE buffer (1 liter)

Tris base 242.0 gm
Glacial Acetic Acid 57.1 ml
EDTA 100.0 ml

Adjusted pH to 8.0

**LB Broth** (1 liter)

- Sodium chloride 10.0 gm
- Tryptone 10.0 gm
- Yeast extracts 5.0 gm

For LB Agar, added 15 gm of agar per liter

**Antibiotics**

Ampicillin sodium salt 100 μg/μl in sterile distilled water