Antimicrobial Activity of *Phaseolus vulgaris* L. Cultivar Baspa Trypsin Inhibitor Protein

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**Abstract**

Antimicrobial activity of partially purified trypsin inhibitor from seed flour of bean (*Phaseolus vulgaris* L.) cultivar Baspa was determined. Maximum per cent (88.12 %) inhibition of growth was against fungal strain *Cercospora punicae* in presence of 300 µg ml⁻¹ inhibitor protein. Inhibition in growth was 84.20 %, 81.00 % and 79.41 % against *Fusarium oxysporum*, *Colletotricum gloeosporioides* and *Alternaria solani*, respectively. The inhibitor protein was highly effective against bacterial strain *Xanthomonas campestris* and less effective against *Ralstonia solanacearum*.

The zone of growth inhibition was 8, 10, 11, 16 and 21 mm in *Xanthomonas campestris*, 6, 8, 12, 13 and 16 mm in *Agrobacterium tumefaciens* and 4, 8, 16, 17 and 19 mm in *Ralstonia solanacearum* in presence of 26, 52, 104, 208 and 416 µg/ml of inhibitor protein loaded into the well. No bacterial growth was observed when clear zone was streaked in control media indicating death of bacterial cells. The studies indicated partially purified inhibitor protein to be effective against plant pathogenic bacterial and fungal strains with varying efficiencies. Inhibitor proteins produced from natural products emerge as potential antimicrobial agents. It can be applied in agricultural sector for development of transgenic resistant to plant diseases.

**Keywords:** Trypsin inhibitor, microbial plant pathogens, antimicrobial agent, transgenic.

**Introduction**

Protease inhibitors play essential role in biological systems, regulating proteolytic processes and participate in defence mechanisms against attack by a large number of insects, fungi and other pathogenic microorganisms³. Protease inhibitors are ubiquitous in nature and most storage organs such as seed and tuber contain 1-10% of total protein as protease inhibitors.⁴ On the basis of specificity towards proteolytic enzymes, protease inhibitors are classified as serine, cysteine, aspartic and metallo-protease inhibitors.⁵ Out of these, most extensively studied protease inhibitors are serine protease inhibitors, found in leguminosae family showing specificity towards trypsin and chymotrypsin.⁶,⁷,³³,³⁴,³⁵

Protease inhibitors control the action of proteases that are indispensable for the growth and development of the organism. They play an important role in the protection of plant tissues from pest and pathogen attack. In plants, fungal infection can kill the plants causing losses of agricultural commodities in many zones of the world. These losses can occur on growing in-field crops as well as harvested commodities, leading to damage ranging from rancidity, odour, flavour changes, loss of nutrients and germ layer destruction.

Crop losses due to pathogens are often more severe in developing countries (e.g. cereals 22%) as compared to those in developed countries (e.g. cereals 6%)⁶. For individual crops, worldwide fungal losses can be 100% if a susceptible cultivar is planted or the climate is favourable in any year and down to 0%, if resistant varieties are planted, fungicides used and good husbandry are employed.⁸

*Alternaria* and *Fusarium* are amongst the most common fungal species associated with growth and damage to food crops in the field. Many phytopathogenic bacteria are known to produce extracellular proteinases which may play an active role in the development of diseases.⁹ In response to such attack by proteinases, plants synthesize inhibitory polypeptides that can suppress the enzyme activities. Inhibitory activity against growth and development of *Pieris brassicae* has been demonstrated by trypsin inhibitor of *Phaseolus vulgaris* L. cultivar Baspa.¹⁰ In this study, we have reported the antimicrobial activity of protease inhibitors against various plant pathogenic microbes.

**Material and Methods**

**Materials:** Seeds of bean (*Phaseolus vulgaris* L.) cultivar Baspa were procured from Sangla valley of Kinnaur, Himachal Pradesh, India. The chemicals viz., Bovine pancreas trypsin, Sephadex G-100, BAPNA (Benzoyl–DL–arginine–p–nitroanilide) and SDS-PAGE reagents were purchased from Sigma Aldrich (USA), the other chemicals were obtained from SRL Pvt. Ltd. (India). The pure cultures of plant pathogenic fungi (*Fusarium oxysporum*, *Colletotricum gloeosporioides*, *Alternaria solani* and *Cercospora punicae*), bacteria (*Ralstonia solanacearum*, *Agrobacterium tumefaciens* and *Xanthomonas campestris*) were procured from the Department of Plant Pathology and Microbiology Laboratory of the Department of Basic Sciences, Dr YSP University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh.

**Partial purification of trypsin inhibitor protein:** Trypsin inhibitor protein was extracted from defatted seed flour of bean (*Phaseolus vulgaris* L.) cultivar Baspa and trypsin inhibitory activity (TIA), was estimated as described by
Deepika and Nath. Soluble protein content in the crude extract was estimated as described by Lowry et al. The crude extract was subjected to ammonium sulphate precipitation (20–80%) to precipitate the trypsin inhibitor protein overnight. After centrifugation at 10,000 rpm for 30 min at 4°C, the precipitates were dissolved in distilled water and dialyzed.

The dialyzed extract was subjected to gel filtration chromatography on Sephadex G-100 column at a flow rate of 12 ml h⁻¹. The fractions were collected till the A₂₈₀ approached to zero. The fractions were assayed for the activity of trypsin inhibitor. Fractions containing active inhibitor protein were pooled, concentrated, analysed for trypsin inhibitor activity and stored at 4°C.

In vitro anti-fungal activity of partially purified trypsin inhibitor protein: In vitro anti-fungal activity of the partially purified trypsin inhibitor protein was monitored against plant pathogenic fungal strains in 100 x 15 mm petri plates each containing 25 ml of potato dextrose agar (PDA) medium. Different concentrations of partially purified trypsin inhibitor protein viz., 60, 120, 180, 240 and 300 µg ml⁻¹ were added to 25 ml of potato dextrose agar medium after sterilization through 0.2 µm Millipore filter. The PDA medium was then poured into petri plates and allowed to solidify. In control sterile distilled water was used in place of the inhibitor protein. All the experiments were performed in triplicate. A bit of fungus having diameter 8 mm (using 8 mm borer) was cultured on PDA medium in Petri plates containing different concentrations of inhibitor protein.

The incubation was carried out at 28 ± 2°C. The inhibition of fungal growth was evaluated after one week through the measurement of colony diameter. The mycelia growth in the control plates for each fungal strain after one week had covered the entire medium. The % growth inhibition was calculated as follows:

\[
\text{Colony diameter of control plate} = C \\
\text{Colony diameter of test plate} = T \\
\% \text{ Growth inhibition} = \frac{C - T}{C} \times 100
\]

In vitro anti-bacterial activity of purified trypsin inhibitor protein: In vitro anti-bacterial activity of the partially purified trypsin inhibitor protein was monitored against plant pathogenic bacterial strains. The bacteria were maintained on nutrient broth at 37°C. Fresh overnight cultures of inoculums (0.1ml) of each culture containing 10⁸ cells were spread on Petri plates containing nutrient agar medium (NA). Different concentrations of partially purified trypsin inhibitor protein viz., 26, 52, 104, 208 and 416 µg ml⁻¹ after sterilization through 0.2µm Millipore filter were loaded in one well of the two wells having diameter of 8mm made on each Petri plate (using 8 mm borer) and to the remaining well, sterile distilled water was loaded as a control. The plates were incubated at 37°C±2°C for 24 hrs. The diameter of zone of inhibition as indicated by clear area devoid of microbial growth was measured.

Results and Discussion

Partial purification of protease inhibitor: Trypsin inhibitor protein was extracted and partially purified from the seed flour of bean (Phaseolus vulgaris L.) cultivar Baspa by ammonium sulphate fractionation (20–80%) and gel filtration chromatography on Sephadex G-100 column to 5.5 fold with 76.5% recovery. The specific activity increased from 19.04 TUI / mg proteins in crude extract to 105.98 TUI / mg in partially purified inhibitor protein. Trypsin inhibitor was purified from seeds of Albizia lebbeck seeds to 29.62 folds with 51.43% recovery using ammonium sulphate precipitation, gel filtration chromatography and ion exchange chromatography²¹.

From seeds of Inga vera, the trypsin inhibitor protein was purified to 1.36-fold with 7.14 per cent recovery using DEAE Sepharose and trypsin Sepharose⁴. Protease inhibitor protein from leaves of Cassia fistula was purified to 9.2-fold with 15.4% recovery by ammonium sulphate precipitation and Sephadex G-100¹.

Effect of partially purified inhibitor protein on growth of plant pathogenic fungal strains: Antifungal activity of partially purified trypsin inhibitor was tested against four plant pathogenic fungal strains viz., Fusarium oxysporum, Colletotricum gloeosporioides, Alternaria solani and Cercospora punicae. The pathogenic fungi and disease symptoms caused by them are enlisted in table 1 and fig. 1. All these fungi showed high growth in PDA (potato dextrose agar) medium not supplemented with inhibitor protein. As the concentration of inhibitor protein was increased in PDA medium, the growth of fungal strain decreased (Fig. 2. 3, 4, 5). Maximum per cent (88.12%) inhibition of growth was observed in fungal strain Cercospora punicae grown in medium supplemented with 300 µg ml⁻¹ of inhibitor protein. While at same concentration of inhibitor protein in medium, 84.20%, 81.00% and 79.41% inhibition in growth was found in case of Fusarium oxysporum, Colletotricum gloeosporioides and Alternaria solani respectively.

Minimum per cent inhibition in growth (9.52%) was observed in fungus Alternaria alternata followed by Fusarium oxysporum (15.21 %), Cercospora punicae (17.14%) and Colletotricum gloeosporioides (29.16%) cultured on the medium supplemented with 60 µg ml⁻¹ of inhibitor protein. The results obtained are presented in table 2.

Protease inhibitors inhibit the growth of phytopathogenic fungi by inhibiting extracellular and intracellular proteases that display important roles in nutrition and infection processes since the invasion of host tissue and fungal...
development depends on the degradation of membrane and cell wall proteins. Thus, the protease inhibitors act directly on the protease produced by pathogenic fungi and reduce their pathogenicity. A strong antifungal activity has been reported in vitro by inhibiting hyphal growth of *Septoria tritici*, *Fusarium graminearum* and *Fusarium culmorum* in presence of wheat kernels inhibitor protein⁹.

A peptide with antifungal activity against *Fusarium oxysporum* and *Mycosphaerella arachidicola* was isolated from *Phaseolus vulgaris* cv. ‘Spotted Bean’¹². Morphological changes including cellular agglomeration and formation of pseudohyphae in *Candida tropicalis* were reported in presence of inhibitor protein isolated from *Capsicum chinense*⁹. Antifungal activity of trypsin inhibitor protein isolated from *Abelmoschus moschatus* seeds was reported against *Candida albicans*, *Candida tropicalis*, *Aspergillus flavus*, *Saccharomyces cerevisiae*, *Candida glabrata* and *Aspergillus niger*¹⁰.

Antifungal activity of crude inhibitor extract isolated from testa of *Citrullus lanatus* Linn. was reported against the growth of *Aspergillus niger* and *Candida albicans*². Antifungal activity of nontoxic trypsin inhibitor protein isolated from *Albizia amara* Boiv. was reported against *Alternaria alternata*, *Alternaria tenuissima* and *Candida albicans*³.

**Effect of partially purified inhibitor protein on growth of plant pathogenic bacterial strains:** Effect of partially purified *Phaseolus vulgaris* L. cultivar Baspa trypsin inhibitor protein was seen against three plant pathogenic bacterial strains viz., *Xanthomonas campestris*, *Agrobacterium tumefaciens* and *Ralstonia solanacearum*. The disease symptoms caused by them are shown in table 3.

The protein was highly effective against bacterial strain *Xanthomonas campestris* and less effective against *Ralstonia solanacearum*. With increase in the amount of inhibitor protein in well, the zone of growth inhibition increased in case of each bacteria (Fig. 6, 7, 8). The zone of growth inhibition in bacterial strain *Xanthomonas campestris* (8, 10, 11, 16 and 21mm), *Agrobacterium tumefaciens* (6, 8, 12, 13 and 16mm) and *Ralstonia solanacearum* (4, 8, 16, 17 and 19mm) was observed in presence of 26, 52, 104, 208 and 416 µg/ml of inhibitor protein loaded into the well. The results obtained are presented in table 4. When colony was picked from the clear zone formed in presence of inhibitor protein loaded in well, no growth was observed in control media indicating death of bacterial cells (Fig. 9).

It has been proposed that the protein with antibacterial action forms a channel on the cell membrane and the cell dies as a result of the out flowing of cellular contents. Antibacterial activity of nontoxic trypsin inhibitor isolated from *Albizia amara* Boiv. has been reported against *Pseudomonas aeruginosa* and *Bacillus subtilis*. Antimicrobial activity of trypsin inhibitor isolated from seeds of *Abelmoschus moschatus* has been demonstrated against *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris*, *Bacillus subtilis*, *Streptococcus pneumonia* and *Bacillus aureus*. It was reported to be moderately active against *Klebsiella pneumonia*¹⁰.

**Table 1**

<table>
<thead>
<tr>
<th>Plant pathogenic fungi</th>
<th>Disease caused</th>
<th>Disease symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fusarium oxysporum</em></td>
<td>Fusarium wilt of tomato</td>
<td>Yellowing on one side of the plant or leaf, followed by wilting, browning and defoliation</td>
</tr>
<tr>
<td><em>Colletotrichum gloeosporioides</em></td>
<td>Mango anthracnose</td>
<td>Infected fruit develop black spots, shrivel and drop off, cause considerable loss during storage and marketing</td>
</tr>
<tr>
<td><em>Alternaria solani</em></td>
<td>Early blight of potato</td>
<td>Potato tuber develop dark, sunken lesions, under these lesions, the tissue is dry, leathery and brown</td>
</tr>
<tr>
<td><em>Cercospora punicae</em></td>
<td>Cercospora leaf and fruit spot on pomegranate</td>
<td>Light brown spots appears on the leaves and fruits, Black and elliptic spots appear on the twigs</td>
</tr>
</tbody>
</table>

**Table 2**

<table>
<thead>
<tr>
<th>Inhibitor concentration (µg)</th>
<th><em>Fusarium oxysporum</em></th>
<th><em>Colletotrichum gloeosporioides</em></th>
<th><em>Alternaria Solani</em></th>
<th><em>Cercospora punicae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>15.21±0.21</td>
<td>29.16±0.12</td>
<td>9.52±0.20</td>
<td>17.14±0.18</td>
</tr>
<tr>
<td>120</td>
<td>27.42±0.13</td>
<td>36.03±0.09</td>
<td>20.17±0.15</td>
<td>34.03±0.12</td>
</tr>
<tr>
<td>180</td>
<td>43.25±0.12</td>
<td>52.28±0.12</td>
<td>41.52±0.14</td>
<td>53.20±0.31</td>
</tr>
<tr>
<td>240</td>
<td>60.00±0.20</td>
<td>63.14±0.16</td>
<td>52.64±0.09</td>
<td>67.75±0.16</td>
</tr>
<tr>
<td>300</td>
<td>84.20±0.17</td>
<td>81.00±0.42</td>
<td>79.41±0.19</td>
<td>88.12±0.18</td>
</tr>
</tbody>
</table>

Data represents mean values ± standard error of three values.
Table 3
Disease symptoms of pathogenic bacteria on plants and other organisms

<table>
<thead>
<tr>
<th>Plant pathogenic bacteria</th>
<th>Disease caused</th>
<th>Disease symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthomonas campestris</td>
<td>Black rot of cabbage</td>
<td>V-shaped yellow lesions to margin of leaves, vascular blackening, stem rot and wilting</td>
</tr>
<tr>
<td>Agrobacterium tumefaciens</td>
<td>Crown gall disease of apple</td>
<td>Galls form on the roots or stems and have a rough convoluted surface</td>
</tr>
<tr>
<td>Ralstonia solanacearum</td>
<td>Bacterial wilt of tomato</td>
<td>Wilting of tomato leaves without their yellowing and stunted growth</td>
</tr>
</tbody>
</table>

Table 4
Effect of partially purified trypsin inhibitor on bacterial growth

<table>
<thead>
<tr>
<th>Inhibitor concentration (µg)</th>
<th>Zone of Bacterial growth inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Xanthomonas campestris</td>
</tr>
<tr>
<td>26</td>
<td>8±0.61</td>
</tr>
<tr>
<td>52</td>
<td>10±0.55</td>
</tr>
<tr>
<td>104</td>
<td>11±0.52</td>
</tr>
<tr>
<td>208</td>
<td>16±0.45</td>
</tr>
<tr>
<td>416</td>
<td>21±0.46</td>
</tr>
</tbody>
</table>

Data represents mean values ± standard error of three values.

Fig. 1: Disease symptoms of plant pathogenic fungi
(a) Tomato plant infected by Fusarium oxysporum
(b) Mango fruit infected by Colletotrichum gloeosporioides
(c) Potato tuber infected by Alternaria solani,
(d) Leaves and fruits of pomegranate infected by Cercospora punicae
Fig. 2: Antifungal activity of partially purified inhibitor protein on growth of *Fusarium oxysporum* at different concentrations of inhibitor protein in PDA medium (1:25 v/v)
(a) Control = 0 µg (b) Inhibitor conc. = 60 µg (c) Inhibitor conc. = 120 µg
(d) Inhibitor conc. = 180 µg (e) Inhibitor conc. = 240 µg (f) Inhibitor conc. = 300 µg
Fig. 3: Antifungal activity of partially purified inhibitor on growth of *Colletotrichum gloeosporioides* at different concentrations of inhibitor protein in PDA medium (1:25 v/v)
(a) Control = 0 µg (b) Inhibitor conc. = 60µg (c) Inhibitor conc. = 120µg (d) Inhibitor conc. = 180µg (e) Inhibitor conc. = 240µg (f) Inhibitor conc. = 300µg
Fig. 4: Antifungal activity of partially purified inhibitor on growth of *Alternaria alternata* at different concentrations of inhibitor protein in PDA medium (1:25 v/v)

(a) Control = 0 µg (b) Inhibitor conc. = 60 µg (c) Inhibitor conc. = 120 µg
(d) Inhibitor conc. = 180 µg (e) Inhibitor conc. = 240 µg (f) Inhibitor conc. = 300 µg
Fig. 5: Antifungal activity of partially purified inhibitor on growth of *Cercospora punicae* at different concentrations of inhibitor protein in PDA medium (1:25 v/v)
(a) Control = 0 µg (b) Inhibitor conc.= 60µg (c) Inhibitor conc.= 120µg (d) Inhibitor conc.= 180µg (e) Inhibitor conc.= 240µg (f) Inhibitor conc.= 300µg
Fig. 6: Antibacterial activity of partially purified inhibitor on growth of *Xanthomonas campestris* at different concentrations

(a) V-shaped yellow lesions to margin of leaves of cabbage caused by *Xanthomonas campestris*

Control = 0µg/ml (b) Inhibitor conc.= 26µg/ml (c) Inhibitor conc.= 52µg/ml
(d) Inhibitor conc.=104µg/ml (e) Inhibitor conc.=208µg/ml (f) Inhibitor conc.= 416µg/ml
Fig. 7: Antibacterial activity of partially purified inhibitor on growth of *Agrobacterium tumefaciens* at different concentrations

a) Crown gall disease of apple caused by *Agrobacterium tumefaciens*
Control = 0µg/ml (b) Inhibitor conc. = 26µg/ml (c) Inhibitor conc. = 52µg/ml (d) Inhibitor conc. = 104µg/ml (e) Inhibitor conc. = 208µg/ml (f) Inhibitor conc. = 416µg/ml
Fig. 8: Antibacterial activity of partially purified inhibitor on growth of *Ralstonia solanacearum* at different concentrations
(a) Wilting of tomato leaves without their yellowing caused by *Ralstonian solanacearum*  
Control = 0µg/ml (b) Inhibitor conc.= 26µg/ml (c) Inhibitor conc.= 52µg/ml  
(d) Inhibitor conc.=104µg/ml (e) Inhibitor conc.=208µg/ml (f) Inhibitor conc.= 416µg/ml
Xanthomonas campestris
Agrobacterium tumefaciens
Ralstonia solanacearum

Fig. 9: Plant pathogenic bacteria cells cultured from zone of inhibition in presence of partially purified inhibitor protein showing no growth in treatment (T) and growth in control (C) cultured on media without inhibitor protein

Conclusion
Present investigation indicated the partially purified inhibitor protein from local bean cultivar to be effective against plant pathogenic bacterial and fungal strains with varying efficiencies. These inhibitor proteins emerge as potential antimicrobial agents produced from natural products and can be applied in agricultural sector for development of transgenic resistant to plant diseases caused by pathogenic bacteria and fungus.

Acknowledgement
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References


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