CHAPTER II

PRELIMINARY STUDIES ON THE WINGED BEAN PLANT PROTEINASE INHIBITORS
Proteinase inhibitors are widely distributed in the plant kingdom, particularly in the seeds and other storage organs of the plants. The seeds of legumes have been investigated most extensively in this respect. Read and Hass (1938) were the first to recognise and report the presence of a proteinase inhibitor in soybeans. Subsequently Kunitz (1945) isolated a protein in crystalline form from soybeans which inhibited trypsin along with an inactive trypsin – inhibitor complex also in crystalline form. After the isolation of soybean trypsin inhibitor, a large number of proteinase inhibitors have been isolated and characterised from various legumes such as lima bean, double bean, navy bean, Adzuki bean, chickpea and black eyed pea, etc.

Sohonie and Bhandarkar (1954) were the first to report the presence of a heat labile proteinase inhibitor in the winged bean seeds. Kortt later isolated and characterised three trypsin and one chymotrypsin inhibitor from winged bean seeds. The presence of proteinase inhibitors in the winged bean tubers has been reported by deLumen and Belo Jr. (1954).

This chapter deals with the distribution of proteinase inhibitory activity in different parts of the plant viz-tubers, developing seeds (pods) and mature seeds.
The effect of heat treatment on the proteinase inhibitory activities of winged bean seeds and tubers is also discussed.

Materials

Winged bean seeds and tubers of the variety IHR-60, were procured from the University of Agricultural Sciences, Hebbal, Bangalore. Winged bean pods were harvested from the winged bean plants (IHR-60) grown at the College of Agricultural Sciences, Dharwad. Bovine pancreatic trypsin (type-III 2xcrystallised) and bovine pancreatic Cα-chymotrypsin (type II, 3xcrystallized) were obtained from Sigma Chemical Co. St.Louis (USA). Casein, soluble in alkali was obtained from E. Merck AG Darmstadt’s (German). Bovine serum albumin crystalline 100% (pentex) was purchased from Fluka AG, Buchs SG. Folin-ciocolteu reagent was prepared by the method of Folin and Cioccolteu.

Methods

Extraction of inhibitor from seeds

The mature seeds were ground to fine powder in a laboratory mill and passed through a standard sieve of 100 mesh size. The fine powder thus obtained was defatted using acetone. The acetone powder was dried at room temperature till the powder was completely free of acetone vapours. In a typical lot, 10 gm seeds yielded 4.15 gm of defatted
seed powder. Defatted seed powder was extracted with 0.1M sodium phosphate buffer, pH 7.6 in the ratio of 1:20 (w/v) by stirring magnetically at 4°C, overnight. The extract obtained was then centrifuged at 3000 rpm in a refrigerated centrifuge (Janetzky K-70) for 20 min. The clear supernatant was assayed for proteinase inhibitor activity against trypsin and chymotrypsin.

Extraction from developing winged bean pods

Winged bean plants were grown at the college of Agricultural Sciences, Dharwad. Flowers were tagged on the day of flowering. After the onset of pods (2-3 days after flowering), they were harvested every third day. The harvested pods were sun dried and then homogenized in a waring blender with 0.1M sodium phosphate buffer, pH 7.6 for about 1-2 min using a pod to buffer ratio of 1:20 (w/v). The homogenate was then kept stirring magnetically at 4°C, overnight. The extract was then centrifuged at 3000 rpm for 20 min in a refrigerated centrifuge. Proteinase inhibitory activity against trypsin and \( \alpha \)-chymotrypsin was assayed in the clear supernatant.

Extraction from winged bean tubers

Unsprouted (dormant) winged bean tubers were harvested before the onset of monsoon showers and preserved in deep freeze in plastic bags till use. Tubers were washed
thoroughly under running water to remove the adhering soil and pressed between filter paper pads to remove water. Later, the tubers were cut into small pieces and homogenized with prechilled 0.1M sodium phosphate buffer, pH 7.6 in a Waring blender using a ratio of tuber to buffer of 1:20 (w/v). The homogenate thus obtained was passed through a cheese cloth to remove the fibrous debris. This clarified extract was then centrifuged at 3000 rpm for 20 min in a refrigerated centrifuge. Proteinase inhibitory activity against trypsin and α-chymotrypsin was determined in the clear supernatant.

**Determination of Proteinase Activity**

Proteinase activity of bovine trypsin or bovine α-chymotrypsin was routinely determined by the caseinolytic method of Kakade et al. with a slight modification. To 0.1 ml of trypsin or α-chymotrypsin (containing 10 μg of enzyme) in 0.001 M HCl, 0.1M sodium phosphate buffer, pH 7.6 was added to make up the volume to 1.0 ml and the assay tube was incubated in a water bath maintained at 37°C for 5 min. After incubation, 1.0 ml of 1% casein in 0.1 M phosphate buffer (pH 7.6) was added. The enzymatic reaction was allowed to continue for 20 min at 37°C. After exactly 20 min, the reaction was terminated by the addition of 3.0 ml of 5% trichloro acetic acid (TCA), and allowed to stand at room temperature for 30 min. Then the precipitate was centrifuged in a table top centrifuge at 3000 rpm for 20 min. The
absorbance of the supernatant (TCA soluble fraction) was read at 280 nm in a Bausch and Lomb spectronic-2000 uv-vis spectrophotometer, against a suitable control containing all other components except the enzyme.

One unit of trypsin (TU) or chymotrypsin (CU) is Arbitrarily defined as the increase in absorbance by 0.01 unit at 280 nm under the above assay condition.

**Determination of proteinase inhibitory activities**

For the assay of proteinase inhibitory activities, a suitable aliquot of inhibitor extract was preincubated with 10 μg of trypsin or Cu-chymotrypsin (in 0.001 M HCl) for 10 min at 37°C. The residual proteinase activity of trypsin or chymotrypsin was determined by the caseinolytic method as described earlier. The decrease in the proteolytic activity of the enzyme was taken as the index of the inhibitory activity. Suitable controls containing all the components except the enzyme were used, to correct for the endogenous proteinase activity in the extracts used. The inhibitory activity was calculated at 50% inhibition of proteinase used for assay since the linearity of proteinase inhibitor activity lies up to a range of 70-90% generally.¹⁵

One unit of trypsin inhibitor activity (TIU) or one unit of chymotrypsin inhibitor activity (CIU) is arbitrarily defined as the number of proteinase units (TU or CU) inhibited under the above mentioned assay conditions.
Estimation of protein

Protein concentration in the various extracts was determined by the method of Lowry et al.\textsuperscript{239} using bovine serum albumin as the standard protein. The total protein content in the winged bean seeds and tubers was calculated from nitrogen content determined by micro-Kjeldahl's method\textsuperscript{240} the N values were multiplied by the conversion factor, 6.23 to get the protein content of the samples.

Effect of heat treatment on proteinase inhibitory activities of winged bean seeds and tubers

1.0 ml aliquots (containing 500 \( \mu \text{g} \) protein) of winged bean seed and tuber extracts were taken in different test tubes (covered with sand bulbs to avoid evaporation) and were kept in a boiling water bath. After definite time intervals, the tubes were taken out and cooled to room temperature. Proteinase inhibitory activity was determined against trypsin and chymotrypsin as described before. The percentage of residual proteinase activities (trypsin or chymotrypsin) were plotted against the duration of heat treatment.

Results and discussion

The total protein content and the proteinase inhibitory activities present in the extracts of winged bean seeds and tubers are presented in Table II.1. The total
## Table II.1

Protein content and proteinase inhibitory activity in winged bean seeds and tubers

<table>
<thead>
<tr>
<th></th>
<th>Crude protein (%)</th>
<th>Protein in extract (mg/ml)</th>
<th>Inhibitory activity gm</th>
<th>Specific activity&lt;sup&gt;5&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>TIU</td>
<td>CIU</td>
</tr>
<tr>
<td>Winged bean seeds</td>
<td>36.0&lt;sup&gt;1&lt;/sup&gt;</td>
<td>2.0</td>
<td>4,472</td>
<td>7,700</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>187</td>
<td>212</td>
</tr>
<tr>
<td>Winged bean tubers</td>
<td>9.8&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.5</td>
<td>7,060&lt;sup&gt;3&lt;/sup&gt;</td>
<td>17,258&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>240</td>
<td>586</td>
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</tbody>
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1. For whole dry seeds
2. For fresh tubers
3 & 4 For defatted seed powder
5. Inhibitory units expressed per mg extractable proteins
protein content of the winged bean seeds (36%) is much higher when compared to other legume seeds except the soybeans. Like soybeans, the winged bean seeds are also rich in lipid (17%). This high lipid content interferes with the extraction of the inhibitors, by forming a lipid scum on the supernatant. Hence, it was essential to defat the seed powder before extraction of the inhibitors.

The winged bean tubers were found to contain 9.8% protein which is fairly high when compared to the protein content of other wild edible tubers like Yam (2.8%), cassava (2%) and colocasia (3%). Our results were slightly higher when compared to earlier reports. This may be due to the differences in soil fertility in which they are grown and variety. The high protein content of the tubers has been attributed to the remarkable ability of this plant to fix atmospheric nitrogen.

Proteinase inhibitory activity of the winged bean tubers were found to be higher (per gm weight basis) compared to that of seeds. This observation is in contrast to the earlier report. This may be due to the maturity state of tubers used as was observed in the case of potato tubers by Ryan et al. (1968).

The proteinase inhibitory activities in the winged bean pods harvested on different days is summarised in
Fig.II.1. The inhibitor activities against both trypsin and chymotrypsin were observed only after the 9th day of pod setting (the period required for the seed storage proteins to accumulate). The proteinase inhibitory activities against trypsin and chymotrypsin were found to increase steeply up to 21st day of pod setting in parallel with the increase in total protein content of the pods. A similar observation has been made by Birk and Waldman in the case of soybeans. The accumulated proteinase inhibitors may probably have a role in keeping the endogenous proteases inactive during the development of seeds. Since the proteinase inhibitory activity increases with the total protein in the developing pods, the proteinase inhibitors may also serve as storage proteins.

The effect of heat on the proteinase inhibitory activities of winged bean seeds and tubers against trypsin and chymotrypsin are shown in Fig.II.2. The rate of inactivation of proteinase inhibitory activities of seeds and tubers is somewhat similar. However, the rate of inactivation of antichymotryptic activity is faster compared to that of antitryptic activity. Heating in a boiling water bath for 60 min destroys both trypsin and chymotrypsin inhibitory activities of both winged bean seeds and tubers. The differences in the thermal denaturation pattern of antitryptic and antichymotryptic activities (Fig.II.2) suggest that the trypsin and chymotrypsin inhibitors are different.
FIG. II.2
It is seen from the above heat treatment data that winged bean proteinase inhibitors are destroyed during the cooking process and hence may not require any special attention. However, proper prior treatment are required when raw tubers, seeds and pods are used for cattle or poultry feeds. The loss of proteinase inhibitory activity upon heat treatment has been shown to vary considerably for different legumes. For example, the inhibitors from chick peas retained all their activity after being heated at 80°C for 5 min and were only 50% inactivated at 100°C for 5 min or roasting at 130°C for 8 min. Similarly the trypsin inhibitors in Faba beans still retained 20% of their original activity after heating in a boiling water bath for 60 min. The loss of proteinase inhibitory activity by heat in vivo is a function of several variables such as particle size and moisture content of the seeds.