CHAPTER III

PURIFICATION AND PHYSICOCHEMICAL PROPERTIES OF PROTEINASE INHIBITORS
Introduction

After the discovery of soybean trypsin inhibitor independently by Ham and Sandstedt and Bowmann on the basis of the growth retardation effect of soybean and the purification and crystallisation of the proteinase inhibitor of soybean and the inhibitor-trypsin complex, a large number of proteinase inhibitors from different sources have been isolated and characterised.

Till the advent of affinity chromatography using immobilised proteinases as a method of purification of proteinase inhibitors, ion exchange chromatography and gel filtration techniques were commonly used for the isolation and purification of proteinase inhibitors. Several proteinase inhibitors have now been isolated using affinity chromatography in which proteinases attached to insoluble resin matrix such as sepharose, act as the affinity ligands. The proteinase inhibitors bound specifically to the ligand bound resin matrix on the affinity column can be eluted at acidic pH or at high ionic strength of the salt in the eluting buffer. Because of the relatively high speed and higher yields, affinity chromatography is gaining popularity as a method of protein purification.

Several proteinase inhibitors are often present in the same tissue of which a few are closely related.
isoinhibitors and others are unrelated that their properties differ significantly. The presence of multiple inhibitors in the same source or tissue, often presents difficulties in their isolation particularly by affinity chromatography methods. Affinity chromatography is followed by ion-exchange chromatography for the separation of iso-inhibitors in many cases.

This chapter deals with the purification of winged bean tuber proteinase inhibitors and some of the physicochemical properties of the purified inhibitors.

Materials

Winged bean tubers (IHR-60 variety) were procured from the University of Agricultural Sciences, Hebbal, Bangalore (India).

The enzymes bovine pancreatic trypsin (type III2X crystallised) and bovine pancreatic α-chymotrypsin (Type II 3Xcrystallised), standard proteins—egg albumin, chymotrypsinogen-A, myoglobin, Lysozyme, R-Nase, SDS-molecular weight markers (MW-SDS-70 kit), Dansyl chloride, standard dansyl-amino acids, and 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB) were from sigma Chemical Co., St. Louis, U.S.A. Sepharose-4B, Sephadex G-75 superfine, QAR-Sephadex A-25 (3 meq/gm) and blue dextran were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.
Acrylamide was from Hayashi pure Chemical Industries Ltd, Japan, \(N,N\)-methylene bisacrylamide and \(N,N,N',N'\)-tetramethylene diamine, were from Eastman Kodak Co. Tris (hydroxy methyl)-amino methane was from E-Merck, German.

All other chemicals used were of analytical reagent grade.

**METHODS**

**Isolation of proteinase inhibitors**

**Extraction**

100 gm of the winged bean tubers were cut to fine pieces and were homogenized in a waring blender using 1.5 l of pre-cooled distilled water. The fine slurry thus obtained was kept stirred on a magnetic stirrer over night at \(4^\circ C\) and then it was filtered through cheese cloth. The residue was further washed with additional 500 ml of pre-cooled distilled water. The washings obtained were combined with the bulk filtrate obtained earlier. The resulting cloudy extract was then centrifuged at 3000 rpm in a refrigerated centrifuge (K-70, Janetuki) for 30 min. The clear supernatant obtained was used as the starting material for further purification of the proteinase inhibitors.
Ammonium sulfate fractionation

The supernatant obtained from the previous step was subjected to 0-60% ammonium sulfate fractionation. Saturated ammonium sulfate solution was used and the volume of ammonium sulfate solution required for obtaining the desired saturation was calculated using the formula:

$$\text{Volume of ammonium sulfate solution needed} = \frac{\text{Vol.of the extract} \times [\text{Final saturation} - \text{Initial saturation}]}{100 - \text{Final saturation}}$$

To 1880 ml of the aqueous extract 2820 ml of saturated ammonium sulfate solution was added slowly with constant stirring in an ice bath to obtain 60% ammonium sulfate saturation. After one hour of continued stirring, the resulting precipitate was separated by centrifugation at 0°C for 30 min at 3000 rpm. The residue thus obtained was dissolved in 100 ml of 0.1M sodium phosphate buffer, pH 7.6 and dialysed against three litres of 0.05M sodium phosphate buffer, pH 7.6, at 4°C with three change of buffer. The clear yellowish dialysed extract was lyophilized and the powder was preserved in a desiccator at -20°C till further use.

preparation of affinity matrices

Cyanogen bromide required for the preparation of activated Sepharose was prepared according to the procedure
Trypsin and chymotrypsin affinity resins

The activation of Sepharose-4B and the coupling of the trypsin or α-chymotrypsin was carried out at 4°C according to the method of March et al. as follows.

Sepharose-4B was thoroughly washed with double distilled water on a sintered glass funnel (G2) to remove the sodium azide used as preservative. 22.5 ml of the washed (settled volume) resin was taken in a silanized 250 ml beaker and mixed with an equal volume of distilled water with slow stirring on a magnetic stirrer. Further, 45 ml of 2M sodium carbonate buffer, pH 11.5 was added with continued slow stirring. To this mixture, 2.0 ml of cyanogen bromide in acetonitrile (2 gm/ml) was added all at once. The slurry was kept stirred vigorously for 2 min, and then filtered through a sintered glass funnel with gentle suction. The gel in the sintered glass funnel was kept stirred constantly without allowing it to settle down using a glass rod having a blunt end, and then washed sequentially with precooled (1) 500 ml of 0.1M sodium bicarbonate, pH 9.5 (2) 500 ml of double distilled water and finally with 250 ml of 0.1M tris-HCl buffer, pH 8.0 (for coupling trypsin) or 0.1M sodium phosphate buffer, pH 7.6 (for coupling chymotrypsin). After the last wash, the slurry was filtered under vacuum to get a moist compact cake.
The cake was then transferred into a 150 ml silanized round bottom flask containing 45.0 ml of 0.2M sodium bicarbonate buffer, pH 9.5 and the resulting slurry was kept stirred on a magnetic stirrer slowly at 4°C. To this, 300 mg of solid trypsin or chymotrypsin was added in one lot and the stirring continued for two hours. This coupling was further continued over night by transferring it onto a cyclomixer kept at minimum speed in the cold room.

The unbound trypsin or chymotrypsin was removed by washing with 500 ml each of 0.1M sodium acetate buffer, pH 4.0, 2M urea and 0.1M sodium bicarbonate buffer, pH 10.0. All these washing solutions contained 0.5M NaCl. Finally, the enzyme bound Sepharose was washed with excess of the buffer which is to be used in the subsequent steps.

A rough estimation of trypsin or chymotrypsin bound to Sepharose-4B was done by determining the bound protein by micro-kjeldahl's method and also by determining trypsin or chymotrypsin activities by caseinolytic method. An aliquot of the homogeneous gel suspension (about 0.2 ml) was taken in a silanized graduated centrifuge tube and centrifuged at 1000 rpm to get the settled volume of the gel. The supernatant was removed carefully. To this settled gel in the centrifuge tube, 0.1M sodium phosphate buffer, pH 7.6 was added to make up the volume to 1.0 ml. Further, 1.0 ml of 1% casein was
added and the mixture was incubated for 20 min at 37°C with slow shaking frequently. After 20 min, the reaction was stopped by adding 5% TCA. After 30 min, the precipitate was centrifuged and the absorbance of the TCA soluble supernatant was read at 280 nm, which gave a rough estimate of trypsin or chymotrypsin per ml of settled gel.

**Affinity chromatography of the crude inhibitor on trypsin-Sepharose-4B column**

The lyophilized ammonium sulfate fraction (0-60% saturation) containing trypsin and chymotrypsin inhibiting activity was subjected to affinity chromatography on trypsin-Sepharose column.

Trypsin-Sepharose 4B (prepared as described earlier) suspended in 0.1M tris-HCl buffer, pH 8.0 was packed into a column 1.5x16 cm) in the cold room and was then equilibrated with the same buffer. The eluate was then checked for absorbance at 280 nm to see whether any protein is desorbed. 100 mg of lyophilized powder from step 2 (ammonium sulfate fraction 0-60% saturation) containing 61.8 mg protein was dissolved in 5.0 ml of 0.1M tris-HCl buffer, pH 8.0 and was loaded on to the column carefully. The initial development and subsequent washing of the column was carried out with 100 ml of starting buffer to remove the unbound protein. Further, nonspecifically adsorbed proteins were removed by washing the column with the starting buffer containing
0.5M NaCl. This second washing was continued until the absorbance of the eluted fractions read zero at 280 nm.

Finally, the bound trypsin inhibitors were eluted with 0.1M HCl, pH 2.0 containing 0.5M NaCl. Fractions (3.0 ml) were collected at a flow rate of 30 ml/hr and assayed for trypsin and chymotrypsin inhibitory activities by caseinolytic method. Fractions having similar activities were pooled, dialysed against 0.01M HCl and lyophilized. All the affinity column operations were carried out in the cold room at 4°C.

The non-specifically adsorbed protein fraction eluted from the affinity column with the starting buffer containing 0.5M NaCl was designated as 'chymotrypsin inhibitor' fraction and the specifically adsorbed protein fraction obtained by elution with 0.1M HCl, pH 2.0, was designated as 'trypsin inhibitor' fraction.

**Fractionation of chymotrypsin inhibitor on chymotrypsin-Sepharose-4B column**

The chymotrypsin inhibitor fraction bound on the trypsin-Sepharose column, eluted from the trypsin-Sepharose column with the starting buffer containing 0.5M NaCl was fractionated by affinity chromatography using chymotrypsin-Sepharose column.

Chymotrypsin-Sepharose 4B suspended in 0.1M sodium phosphate buffer, pH 7.6, containing 0.5M NaCl was packed
into a column (1.5x30 cm) in the cold room and was equili-
brated with the same buffer. Lyophilized chymotrypsin in-
hibitor fraction (60.2 mg protein) in 5.0 ml of starting
buffer was loaded onto the column. The column was developed
with the starting buffer and washing was continued with the
same buffer till the absorbance of the eluted fractions read
zero at 280 nm. Finally, the elution of the adsorbed chymo-
trypsin inhibitor was achieved by eluting with 0.1M HCl,
pH 2.0. 3.0 ml fractions were collected as described
previously. Chymotrypsin inhibitory activity was assayed
in the eluted fractions, and the fractions containing the
active material were pooled, dialysed against 0.01M HCl and
lyophilized.

Ion-exchange chromatography of trypsin inhibitor fraction on
QAE-Sephadex

The trypsin inhibitor fraction obtained from trypsin-
Sepharose column was further fractionated on a QAE-Sephadex
column.

QAE-Sephadex A-25, Cl form was suspended in 0.05M
tris-HCl buffer, pH 8.0 containing 0.025M NaCl. The gel was
allowed to swell for 24 hours at room temperature in a
vacuum desiccator. The supernatant buffer was changed
several times during swelling and deaeration. The swollen
gel was then packed into a column (0.6x45 cm) under gravity
and equilibrated with the starting buffer.

The freeze dried trypsin inhibitor fraction (40 mg protein) in 5.0 ml of starting buffer was carefully loaded onto the column. The column was developed with the starting buffer. The flow rate of the column was maintained at 30 ml per hour using a peristaltic pump (Pharmacia) and 3 ml fractions were collected. The fraction eluted with the starting buffer (Peak 1) had only trypsin inhibitory activity and was designated WBT-TI-I. Elution with the starting buffer was continued until all the weakly adsorbed components were eluted out. The adsorbed proteins were eluted by a linear salt gradient obtained by mixing 200 ml of 0.05 M tris-HCl containing 0.025M NaCl in the mixing chamber and 200 ml of 0.05M tris-HCl containing 0.5M NaCl in the reservoir. The large protein peak eluted with salt gradient (Peak 4) had both chymotrypsin and trypsin inhibitory activities and was designated as WBT-TI-II. Absorbance of the eluted fractions were measured at 280 nm. Trypsin and chymotrypsin inhibitory activities of the fractions were determined by the usual caseinolytic method and those containing similar inhibitory activities were pooled separately, dialyzed against 0.025M tris-HCl buffer, pH 8.0 and lyophilized.
Analytical techniques

Estimation of protein: Protein was routinely estimated by Lowry's method using bovine serum albumin as standard. Protein present in the column effluents was measured by reading the absorbance at 280 nm. Protein concentration of trypsin or chymotrypsin dissolved in 0.001M HCl were determined spectrophotometrically using $A_{1\%}^{1cm}$ at 280 nm of 15.6 for trypsin and 20.0 for chymotrypsin respectively.

Assay of the inhibitory activities

Trypsin and chymotrypsin inhibitory activities during the column operations were routinely assayed by the Kunitz's casainolytic method according to Kakade et al., as described in Chapter II.

Analytical electrophoresis on polyacrylamide gels

The homogeneity of the pooled fractions obtained after each purification step was tested by polyacrylamide gel electrophoresis according to the method of Davis et al. at pH 8.3, using 7.5% gels. After complete polymerisation of the gels, the lower ends of the gel tubes were tied with cellophane membrane. In some cases, the gels were preelectrophoresed for 1-6 hours using 1mA per tube (without samples) to remove excess ammonium persulfate. Later, the buffer on the top of the gel was removed before the protein
samples were loaded. Generally 10 to 30 μg of sample protein was loaded. The electrophoresis was carried out for 1.5 hour at a current strength of 3mA per tube; bromophenol blue was used as tracking dye. The gels were stained overnight with 0.1% coomassie brilliant blue-R-250 in methanol:Acetic acid:water (5:1:4 v/v). 7% acetic acid was used for destaining the gels and also for their preservation.

Polyacrylamide gel electrophoresis of the purified trypsin inhibitors was also carried out at pH 4.3 according to Reisfield et al. using 7.5% gels, p-alanine-acetic acid, pH 4.3, was used for electrode buffer and pyromin-G as the tracking dye. Staining and destaining of the gels were done similarly as described above.

Physical and chemical characterisation

Molecular weight

a) SDS-polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis of the purified trypsin inhibitors WBT-TI-I and WBT-TI-II were carried out according to Laemmli's procedure using 10% gels.

Samples containing 100 μg protein in 0.5 ml volume were made up in a solvent containing 0.0625M tris-HCl buffer pH 6.8, 2% SDS, 20% sucrose and 0.001% bromophenol blue
tracking dye. In order to ensure the optimum binding of SDS, the samples were heated in a boiling water bath for 2-3 minutes. The electrode buffer, tris-glycine buffer, pH 8.3, containing 0.025M tris and 0.192 M glycine with 0.1% SDS was used. The electrophoresis was run at 4°C using a current strength of 3mA per tube until the tracking dye reached about 0.5 cm away from the bottom of the tube. Similarly a standard protein mixture of known molecular weights containing bovine serum albumin, egg albumin, ß-lactoglobulin, lysozyme, trypsinogen and pepsin (Dalton mark VI, Sigma) was run simultaneously.

The mobility of the proteins were calculated by using the formula

\[
\text{Mobility} = \frac{\text{Dist. of protein migration}}{\text{length of the gel after staining}} \times \frac{\text{length of the gel before staining}}{\text{Dist. of the tracking dye migration}}
\]

A calibration curve was obtained by plotting the mobility values of standard proteins in the mixture versus their respective log molecular weights. From this calibration curve the molecular weights of the inhibitors WBT-TI-I and WBT-TI-II were calculated.
b) **Gel Filtration** method using *Sephadex*-G-75 column

Molecular weight of the trypsin inhibitor WBT-TI-II was also determined by molecular sieve chromatography on *Sephadex* G-75 column according to the method of Andrews.  

*Sephadex* G-75 was suspended in 0.05M sodium phosphate buffer, pH 7.6 and the gel was swollen by heating on a boiling water bath for one hour. Further the gel was kept in a vacuum desiccator to ensure complete deaeration. The swollen gel was then packed into a column (1.5x132 cm) and was equilibrated with the same buffer. The flow rate of the column was maintained at 12.5 ml per hour. The void volume ($V_0$) of the column was determined by using one ml of blue dextran (2 mg blue dextran containing 2% sucrose) in 0.05M phosphate buffer, pH 7.6.

The protein sample (≈5 mg) in 1.0 ml of starting buffer containing 2% sucrose was layered on the top of the gel bed and elution was carried out with the same buffer. 3 ml fractions were collected and absorbance of the eluted fractions was read at 280 nm. The total eluted volume up to the fraction having maximum absorbance was considered as the elution volume of the protein ($V_e$).

In a similar way, the elution volumes for different standard proteins of known molecular weights were determined. A calibration curve was obtained by plotting $V_e/V_0$ for each
of the standard protein against their respective log molecular weights. Egg albumin, chymotrypsinogen-A, Myoglobin were used for obtaining the calibration curve. From this calibration curve, the molecular weight of the inhibitor, WBT-TI-II was estimated.

II Determination of sedimentation coefficient and molecular weight by ultra centrifugation

Analytical ultra centrifugal studies of the trypsin inhibitors WBT-TI-I and WBT-TI-II were carried out in a Beckmann model-E, Analytical ultracentrifuge equipped with schlieren optics and RTIC unit for temperature measurement. For sedimentation value, the centrifugation was carried out in a single sector, 12 mm cell at 21.5°C housed in Antt Ti rotor at 68,000 rpm. Distances of the protein boundary on the schlieren patterns were measured in a micro comparator.

Sedimentation coefficient of WBT-TI-I was determined by using 0.1% protein solution in 0.1M sodium phosphate buffer, pH 7.6. The centrifugation was followed for 40 min, photographs were taken every 10 min after reaching a final speed of 68000 rpm. For WBT-TI-II, 0.5% protein solution in 0.1M sodium phosphate buffer, pH 7.6, was used and the centrifugation was followed for 100 min, taking photographs at every 20 min time intervals. A plot of logr-versus the time interval was drawn and from which \( \frac{d \log x}{dt} \) was calculated.
The molecular weight of WBT-TI-II was calculated by Archibald's approach to equilibrium method using An D rotor at 20,000 rpm.

III. Ultra-violet absorption spectra and the determination of $1\%_{\text{Alm}}$

Ultra-violet absorption spectra of the trypsin inhibitors WBT-TI-I and WBT-TI-II were determined in a Bausch and Lomb's spectronic 2000, UV-Vis recording spectrophotometer, using 0.1M sodium phosphate buffer, pH 7.6.

Exactly 3.0 ml (0.1% protein as determined by Lowry's method) inhibitor protein solution was taken in the quartz cuvette. The base line was adjusted to zero using 0.1M sodium phosphate buffer and the samples were scanned for absorbance versus wavelength.

IV. Amino acid analysis

The amino acid composition of the trypsin inhibitor WBT-TI-II was carried out according to Moore and Stein's procedure using LKB amino acid analyzer.

Preparation of the sample

Lyophilized and dried inhibitor protein (1 mg protein as estimated by Lowry's method) was taken in a pyrex test tube. To this, 1.0 ml 6N HCl (electrolytic grade) was added
and the solution in the tube was allowed to freeze in a liquid nitrogen bath. The tube was then sealed under nitrogen. Hydrolysis of the protein was carried out for 24 hrs by keeping the tube in an oven maintained at 105 ± 1°C. The hydrolysate was dried in a vacuum desiccator over sodium hydroxide pellets and sulfuric acid; the residue obtained was dissolved in double distilled water and transferred quantitatively to a ground glass joint tube along with the washings and dried in a rotary flash evaporator. Washing the residue with water and drying was repeated 2-3 times to ensure the complete removal of HCl. Finally, the dried material was dissolved in 2.0 ml of 0.2M citrate buffer, pH 2.2, and purified by passing through millipore filter (0.45 μm). An aliquot of it was used for amino acid analysis.

Analysis of the hydrolysate

The amino acid analysis of the protein hydrolysate was carried out on LKB amino acid analyser 4150 model, attached with a recording LKB integrator (Bromma). The sample (corresponding to 20 μgm protein) hydrolysate was loaded on to the analytical column (55x0.6 cm) containing cation exchange resin with a particle size of 11±0.5. Elution of the amino acids was done using a buffer sequence of pH 3.20, 4.24, 6.45 and NaOH 0.4N. Eluted amino acids were detected spectrophotometrically after ninhydrin reaction. The column was calibrated with a standard amino acid mixture.
Determination of tryptophan content

Tryptophan content in the intact protein of WBT-TI-II was estimated spectrophotometrically from the alkaline spectra according to the method of Bencze and Schmid. The UV spectrum of the trypsin inhibitor-WBT-TI-II in 0.1N NaOH was obtained by scanning in a Spectronic 2000 spectrophotometer as described previously. The slope of the tangent drawn to the two characteristic maxima of the absorption curve in the range between 278 and 295 nm was determined. The slope is indicative of the ratio of tyrosine and tryptophan content of the protein. The reference slope value was calculated using the formula

\[ S = \frac{a/b \times 10^3}{\lambda_{\text{max}}} \]

where 'a' is the absorbance units, 'b' is the wavelength in nm and \( \lambda_{\text{max}} \) is the maximum absorption in the range 278 and 295 nm in the alkaline spectra.

N-terminal amino acid determination

Analysis of the N-terminal amino acid of winged bean tuber trypsin inhibitor WBT-TI-II was carried out by the dansyl chloride method of Hartley.
250 μg of WBT-TI-II in 25 μl of 0.1M sodium phosphate buffer, pH 7.6 was mixed well with 10 μl 0.2M sodium bicarbonate. The pH of the mixture was more than 9.0 and the solution was found to be clear. To this, 5 μl Dansyl chloride (5 mg/ml in acetone) was added and the tube was covered with parafilm. Reaction was allowed to continue in dark at room temperature for an hour. A colourless solution (indicating the completion of the reaction) obtained was dried in a vacuum desiccator over NaOH pellets. To this 25 μl of 6N HCl was added and the tube was sealed. Hydrolysis of dansylated protein was carried out for 18 hrs by keeping the tube in an oven maintained at 105±1°C and the hydrolysate was dried in vacuum over NaOH and P₂O₅. Residue obtained was mixed thoroughly with 100 μl of 96% ethanol on a cyclomixer in order to take out the adhering sample on the walls of the tube, and dried in vacuum. Washing with 96% ethanol and drying was repeated 2-3 times to ensure the complete removal of HCl. Finally the residue obtained was taken in 25 μl of 96% ethanol. Dansylated amino acid was identified by spotting an aliquot (1 μl) on a polyamide sheet (8x8 cm). A standard mixture of dansylated amino acids, containing DNS-Pro, DNS-Ileu, DNS-Phe, DNS-Ser, DNS-Val, and DNS-Arg was spotted on the other side of the polyamide plate corresponding to sample spot. The plate was developed using different solvent systems as follows.
Solvent I: 1.5% (v/v) aqueous formic acid (1.76 ml formic acid in 100 ml)

Solvent II: Benzene : acetic acid (9:1)

Solvent III: Ethylacetate : MeOH : Acetic acid (20:1:1)

Solvent IV: Acetic acid : pyridine : water : ethanol (1.6ml:0.9ml:100ml:37.5 ml)

Solvent V: 0.05M Na₃PO₄ : Ethanol (3:1) (modified according to Hartley).

The first direction run was carried out with solvent system (I). Solvents II, III, IV and V were used for the 2nd direction run at right angles to the 1st direction run. After each run the plate was dried with a cold stream of air. The separated dansylated amino acids were identified by observing under an UV lamp.

Results

The flow chart summarizes the purification steps involved in the isolation and purification of winged bean tuber proteinase inhibitors.

The recovery of proteinase inhibitory activities (both trypsin and chymotrypsin inhibitor proteins) by extraction with water was almost similar to that of 0.1M sodium phosphate buffer (pH 7.6) extraction. Hence, water extraction was preferred for the preparation of crude extract.
FLOW CHART FOR THE PURIFICATION OF WINGED BEAN TUBER PROTEINASE INHIBITORS

Winged bean tuber water extract

0-60% (NH₄)₂SO₄ precipitation, centrifuged at 3000 rpm

<table>
<thead>
<tr>
<th>Supernatant</th>
<th>Residue</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>dissolved in 0.1M sodium phosphate buffer (pH 7.6) and dialyzed against 0.05M sodium phosphate buffer, pH 7.6 and lyophilized</td>
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<table>
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<tr>
<th>Lyophilized powder</th>
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Trypsin-Sepharose affinity column

<table>
<thead>
<tr>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Peak 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>eluted C washing buffer</td>
<td>eluted C washing buffer containing 0.05M NaCl (Chymotrypsin inhibitory activity)</td>
<td>eluted C 0.1M HCl pH 2.0 containing 0.5M NaCl (Trypsin and chymotrypsin inhibitory activity)</td>
</tr>
</tbody>
</table>

Chymotrypsin-Sepharose column

<table>
<thead>
<tr>
<th>Peak 1</th>
<th>Peak 2</th>
<th>WBT-TI-I</th>
<th>WBT-TI-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>devoid of inhibitory activity eluted C starting buffer</td>
<td>chymotrypsin inhibitor (eluted C) (eluted C 0.1M HCl pH 2.0)</td>
<td>WBT-TI-I (eluted C gradient)</td>
<td>WBT-TI-II</td>
</tr>
</tbody>
</table>
Ammonium sulfate precipitation (0-60%) led to 98% recovery of trypsin inhibitory activity and about 83.7% recovery with respect to chymotrypsin inhibitory activity. Approximately 1.75 and 1.5 fold purification was achieved by this step with regard to trypsin and chymotrypsin inhibitors respectively. Starting from 100 gm winged bean tubers, 2.763 gm of lyophilized powder (2.270 gm protein) was obtained after ammonium sulfate fractionation.

Affinity chromatography of the winged bean tuber proteinase inhibitors

Elution profile of the affinity chromatography of the lyophilized crude inhibitor preparation on trypsin-Sepharose 4B column is given Fig. III.1.

Initial washing with the starting buffer resulted in the elution of unbound proteins constituting a large proportion of the protein applied to the affinity column. This peak (I) showed neither antitryptic nor antichymotryptic activities. A non-specifically adsorbed protein peak (II) was then eluted by washing the column with the starting buffer containing 0.5M NaCl. Fractions of the descending portion of this peak exhibited antichymotryptic activity and no antitryptic activity. Fractions having chymotrypsin-inhibitory activities were pooled and this fraction was designated as "chymotrypsin inhibitor fraction". This
inhibitor fraction constituted approximately 70% of the total chymotrypsin inhibitory activity and about 44% of the total protein loaded onto the column.

The protein specifically bound to trypsin-Sepharose matrix was dissociated and eluted as a sharp peak (III) by elution with 0.1M HCl, pH 2.0 containing 0.5M NaCl. It is interesting to note that this peak was found to contain both antitryptic and antichymotryptic activities. Approximately 62% of the trypsin inhibitory activity and about 18% of the chymotrypsin activity loaded onto the column was present in this fraction. The protein recovered in this fraction amounts to 29% of the total protein loaded on to the column. This peak fraction was designated as "trypsin inhibitor fraction". The percentage recoveries, with regard to protein and inhibitory activities were fairly reproducible with different preparations of ammonium sulfate fraction. The affinity column was found to be active for more than a year when preserved at 4°C.

Polyacrylamide gel electrophoretic patterns of the trypsin inhibitor and chymotrypsin inhibitor fractions are shown in the inset of the respective elution profiles. These patterns revealed the presence of multiple components in both the fractions.

The chymotrypsin inhibitor fraction obtained from the trypsin-Sepharose column was further purified on a
### Table III.1

Fractionation of proteinase inhibitors of winged bean tubers

<table>
<thead>
<tr>
<th>Purification step.</th>
<th>Total protein (mg)</th>
<th>Total inhibitory activity (units)</th>
<th>Specific inhibitory activities</th>
<th>Activity recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Trpysin Chymotrypsin</td>
<td>Trpysin Chymotrypsin</td>
<td>TIu</td>
</tr>
<tr>
<td>A) Water extract</td>
<td>4042</td>
<td>22,42,486 31,20,254</td>
<td>554 772</td>
<td>100% 100%</td>
</tr>
<tr>
<td>B) 0-60% Ammonium sulfate fraction*</td>
<td>2270</td>
<td>21,91,556 30,25,910</td>
<td>965 1333</td>
<td>98 97</td>
</tr>
<tr>
<td>C) Trypsin affinity chromatography of crude inhibitor†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) Chymotrypsin inhibitor fraction</td>
<td>1004</td>
<td>- 24,58,356</td>
<td>- 2448</td>
<td>- 78</td>
</tr>
<tr>
<td>b) Trypsin inhibitor fraction</td>
<td>651.2</td>
<td>13,52,633 5,66,581</td>
<td>2077 870</td>
<td>61.75 18.2</td>
</tr>
</tbody>
</table>

* The values obtained for 100 gm tubers
† The results are average values from three batch experiments and were scaled up for 100 gm tuber's preparation of ammonium sulphate fraction
Chymotrypsin-Sepharose 4B affinity column. Elution pattern on chymotrypsin-Sepharose column is shown in the Fig. III.2. Lyophilized chymotrypsin inhibitor fraction (60.2 mg protein) was loaded on to the column in 0.1M sodium phosphate buffer, pH 7.6 containing 0.5M NaCl. Washing the column with the starting buffer led to the elution of a protein peak which was devoid of any antichymotryptic activity. Later, a sharp protein peak was eluted by using 0.1M HCl, pH 2.0. This peak contained chymotrypsin inhibitory activity. Approximately 57% of the total activity was recovered from this peak and about two fold purification was obtained compared to the original loaded material.

Polyacrylamide gel electrophoresis of this fraction at pH 8.3, gave three distinct bands and on SDS PAGE and it was found to be a mixture of two proteins with very little difference in their mobility. Further attempts to purify the chymotrypsin inhibitor using CM-Sephadex, QAE-Sephadex column chromatography and by preparative polyacrylamide gel electrophoresis were unsuccessful and hence, further attempts were not made to characterize this inhibitor.

Ion-exchange chromatography of the trypsin inhibitors on QAE-Sephadex

The trypsin inhibitor fraction obtained from trypsin-Sepharose column was found to be heterogeneous on PAGE at pH 8.3. Further fractionation was achieved by anion exchange
chromatography on QAE-Sephadex-A25 column. The elution profile of QAE Sephadex column chromatography of the trypsin inhibitor fraction is shown in Fig. III.3. The elution profile clearly revealed, two major (I & IV) and two minor (II & III) peaks. Data relating to the amount of protein, inhibitory activities, specific activity of the inhibitors and the recoveries of each peak is summarised in Table III.2.

Peak I represents the unadsorbed material eluted with the starting buffer. It exhibited only trypsin inhibitory activity and accounts for 18% of the total trypsin inhibitory activity applied to the column. The pooled fractions corresponding to this protein peak are referred to as WBT-TI-I. Peaks II, III and IV were eluted by using the buffer with a linear salt gradient of 0.025M to 0.5M sodium chloride. Peaks II and III contain very small amount of protein and exhibit trypsin inhibitory activity only and they accounted for 2.7% and 1.42% of the total trypsin inhibitory activity loaded. Peak IV which forms the major fraction, exhibits both trypsin and chymotrypsin inhibitory activities. This fraction accounts for 31% of the total trypsin inhibitory activity loaded. This peak fraction was designated as WBT-TI-II. An overall recovery of 53% of the trypsin inhibitory activity was achieved by this fractionation step.

The electrophoretic patterns of these peak fractions are given in Fig. III.4, WBT-TI-I showed three distinct
FIG. III.3
Table III.2
Summary of purification of trypsin inhibitors on QAE-Sephadex column

<table>
<thead>
<tr>
<th>Peak</th>
<th>Protein (mg)</th>
<th>Total trypsin inhibitory activity (units)</th>
<th>Total chymotrypsin inhibitory activity (units)</th>
<th>Specific inhibitory activity</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak I</td>
<td>3.77</td>
<td>14780</td>
<td>Nil</td>
<td>3920</td>
<td>17.8</td>
</tr>
<tr>
<td>Peak II</td>
<td>1.575</td>
<td>2250</td>
<td>Nil</td>
<td>1458</td>
<td>2.7</td>
</tr>
<tr>
<td>Peak III</td>
<td>1.350</td>
<td>1180</td>
<td>Nil</td>
<td>874</td>
<td>1.42</td>
</tr>
<tr>
<td>Peak IV</td>
<td>10.875</td>
<td>25665</td>
<td>20,066</td>
<td>2360</td>
<td>31%</td>
</tr>
</tbody>
</table>

Total protein loaded = 40 mg
Total trypsin inhibitory units loaded = 83,060
Total chymotrypsin inhibitory units loaded = 34,800
closely spaced bands on PAGE at pH 8.3 (Fig. III-4-(1)). However, the sample showed a single band when it was run on pre-electrophoresed gel (Fig. III-4-(2)) (to remove ammonium persulfate). This inhibitor was also found to give a single protein band on PAGE at pH 4.3 (Fig. III-4-(7)). SDS-polyacrylamide gel electrophoresis of this inhibitor revealed a single band (Fig. III-4-(10)). From the ultracentrifugal data, this inhibitor, WBT-TI-I was found to be homogeneous. Similarly WBT-TI-II showed a faint band in addition to a prominent band, on PAGE at pH 8.3; (Fig. III-4-(3)) the intensity of the faint band diminished gradually depending on the time interval of the preelectrophoretic run (Fig. III-4-(4) & (5)). However, it did not vanish completely even when the gel prerun for six hours, was used. Further at pH 4.3, it gave a single protein band (Fig. III-4-(8)). On the other hand, it was found to be highly homogeneous based on SDS-PAGE, Gel filtration and sedimentation studies.

**physico-chemical properties**

The molecular weight of the winged bean tuber trypsin inhibitors WBT-TI-I and WBT-TI-II were estimated by SDS polyacrylamide gel electrophoresis using 10% gels. SDS-PAGE electrophoresis of the chymotrypsin inhibitor was carried out to find out its homogeneity and its molecular weight. SDS-PAGE electrophoretic patterns of these inhibitors are given in Fig. III-4-(10,11,12). Winged bean tuber
trypsin inhibitors, WBT-TI-I and WBT-TI-II, were found to be homogeneous by this method. However, the chymotrypsin inhibitor showed two bands with very close mobility. The calibration curve of mobility versus the log molecular weight of standard proteins is given in Fig. (III.5). From this curve, the molecular weight of WBT-TI-I and WBT-TI-II were found to be 27,000 and 24,000 respectively and the chymotrypsin inhibitor had a molecular weight around 20,000.

Winged bean tuber trypsin inhibitors WBT-TI-I and WBT-TI-II were subjected to analytical centrifugation to obtain information regarding their sedimentation pattern. The sedimentation patterns of WBT-TI-I and WBT-TI-II are shown in the Fig. (III.6a & 6b). The sedimentation constant (s^w_{app}) has been calculated from the measurements of distances moved by the Schlieren peaks at various time intervals (Figs. III.7 & III.8) and s^w_{20} was calculated using the equation.

\[ s^w_{20} = \frac{2.303}{60w^2} \times \frac{d \log r}{dt} \]

where

- \( s \) = sedimentation constant
- \( w \) = angular velocity in radians per second
- \( r \) = the distance moved in centimeter by the protein boundary
- \( t \) = time in min
The sedimentation patterns of WBT-TI-I (Fig. III-6a) revealed the presence of a small amount of a low molecular weight protein, which settled slower compared to the main component. The protein did not go into solution at higher concentrations above 0.5%. The sedimentation constant was found to be 3.54s°. On the other hand, WBT-TI-II sedimented as a single symmetrical peak in 0.1M sodium phosphate buffer, pH 7.6, at 1% concentration as could be seen from the sedimentation patterns (Fig. III.6b). The sedimentation constant (S^w_20) was found to be 2.1s.

The molecular weight of WBT-TI-II was calculated by Archibald's approach to equilibrium method using the formula:

$$
\frac{RTF \left( \frac{dc}{dx} \right) x_o}{(1-\nu_p) w^2 x_o \Delta R \left( \frac{dc}{dx} - \frac{1}{x_o^2} \frac{x^2 dc}{dx} \right)}
$$

where $R = \text{Gas constant, } 8.317 \times 10^7 \text{ (erg/degree/gm molecule)}$

$T = \text{Absolute temperature in °K, } (21.5°C + 273°K)$

$F = \text{Magnification factor, } 2.1$

$\nu_p = \text{Partial specific volume, } 0.736$

$w^2 = 4.387 \times 10^6$

$\left( \frac{dc}{dx} \right) x_o$ is $Z - Z_{\text{min}}$ value at $R = 1.500$

$x_o = 6.5 - \frac{RC-R}{F}$. 
A molecular weight of 24,600 was estimated by this method for WBT-TI-II.

Further confirmation regarding homogeneity and molecular weight of WBT-TI-II was obtained by molecular sieve chromatography on Sephadex G-75 column. The elution pattern of the inhibitor WBT-TI-II on a calibrated column of Sephadex G-75 is given in Fig. (III,9). The inhibitor eluted as a single symmetrical Gaussian peak indicating its homogeneity. The calibration curve of $V_e/V_o$ versus the log molecular weights of standard proteins is given in Fig.(III,10). From this plot, the molecular weight of the inhibitor WBT-TI-II was estimated to be 24,000 (±200) daltons.

The ultra-violet absorption spectrum of WBT-TI-I and WBT-TI-II is given in Fig. III,11. It is seen that both the inhibitors exhibit typical protein spectrum with absorption maxima in the region 280 nm and minimum maxima in the region 250 nm. However, the inhibitors slightly differ in their absorption maxima. No absorption was seen in the visible region.
Absorbance (280 nm) vs. Fractions

FIG. III.9
FIG. III. 11
The results of the amino acid analysis of the trypsin inhibitor WBT-TI-II is given Table III.3. The amino acid analysis showed that WBT-TI-II contains all the amino acids which are normally found in most proteins. The inhibitor contains fewer histidines (3) and only one methionine. The inhibitor was found to be rich in acidic amino acids. Aspartic and glutamic acids accounted for 27% when compared to 16% of basic amino acids (lysine, histidine and arginine). The tryptophan content of WBT-TI-II in the intact protein was determined spectrophotometrically from alkaline spectra shown in Fig. III.12. The ratio of tyrosine and tryptophan was found to be 2.7. Therefore, the number of tryptophan residues per mole of the inhibitor is 3.0. From the analysis it was found that the WBT-TI-II contains a total of 192 amino acids and this corresponds to a molecular weight of 25,400. This value is in fair agreement with the molecular weight determined by sedimentation analysis, SDS PAGE and gel filtration.

Analysis of the NH$_2$-terminal group of WBT-TI-II was carried out by the Dansyl chloride method. Dansylated amino acid was identified by TLC on a poly amide sheet. Five different solvent systems were used. The chromatogram obtained is shown in Fig. III.13. With the first solvent system, a single fluorescent spot was found on the solvent front. When the plate was run in the second direction using
Amino acid composition of winged bean tuber trypsin inhibitor
WBT-TI-II

Weight of the protein taken for analysis = 200 ug.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>% of amino acid recovered</th>
<th>No. of residues per molecule*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>13.80</td>
<td>21</td>
</tr>
<tr>
<td>Threonine</td>
<td>5.24</td>
<td>8</td>
</tr>
<tr>
<td>Serine</td>
<td>10.64</td>
<td>17</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>19.10</td>
<td>30</td>
</tr>
<tr>
<td>Proline</td>
<td>10.38</td>
<td>16</td>
</tr>
<tr>
<td>Glycine</td>
<td>12.46</td>
<td>19</td>
</tr>
<tr>
<td>Alanine</td>
<td>8.70</td>
<td>13</td>
</tr>
<tr>
<td>Half-cystine†</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Valine</td>
<td>11.35</td>
<td>18</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.644</td>
<td>1</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4.97</td>
<td>8</td>
</tr>
<tr>
<td>Leucine</td>
<td>12.63</td>
<td>20</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>6.62</td>
<td>10</td>
</tr>
<tr>
<td>Phenyl alanine</td>
<td>4.45</td>
<td>7</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.81</td>
<td>3</td>
</tr>
<tr>
<td>Lysine</td>
<td>10.84</td>
<td>17</td>
</tr>
<tr>
<td>Arginine</td>
<td>6.86</td>
<td>11</td>
</tr>
<tr>
<td>Tryptophan+</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td><strong>Total no. of residues</strong></td>
<td>192</td>
<td></td>
</tr>
</tbody>
</table>

Molecular weight by amino acid analysis = 25,135.

* Calculated for one methionine residue
† Half cystine was not determined as most of it appears to be destroyed during acid hydrolysis. However, a short peak corresponding to cysteic acid was observed much earlier to elution of Aspartic acid. Performic acid oxidation could not be done due to paucity of sample.
+ Tryptophan estimated spectrophotometrically from the alkaline spectra (Fig. III.12).
FIG. III. 12

[Graph showing absorbance vs. wavelength (λ) with two curves labeled 'a' and 'b'.]
FIG. III.13(b)
FIG. III.13(c)
the 2nd solvent, the spot did not move totally from the baseline. With the third solvent in the same direction, the spot was found to spread as a streak without clear separation indicating it is not DNS-His. Finally with 4th and 5th solvents, two spots were clearly separated, the top one being a faint spot which corresponded to DNS -Lys and the bottom bright fluorescent spot corresponding to DNS-Arg. These studies have revealed that the N-terminal group as 'Arginine'.

Discussion

From the purification results it is evident that the winged bean tubers contain at least three different proteinase inhibitors, one inhibiting solely trypsin, the second one inhibiting solely chymotrypsin and the third inhibiting both trypsin and chymotrypsin. Amongst these, two trypsin inhibitors, WBT-TI-I and WBT-TI-II have been purified to homogeneity. WBT-TI-I inhibits only trypsin and WBT-TI-II inhibits both trypsin and chymotrypsin. WBT-TI-II was also found to bind to chymotrypsin-Sepharose column and could be eluted only with 0.1M HCl, pH 2.0. However, we have preferred to isolate it by using trypsin-Sepharose column. By using trypsin-Sepharose affinity chromatography, WBT-TI-II was separated along with other trypsin inhibitors present in the winged bean tubers. The trypsin affinity adsorbed fraction was further resolved into WBT-TI-I and WBT-TI-II
by QAE-Sephadex column chromatography. Besides these two major trypsin inhibitors, two minor inhibitor fractions were also obtained during this fractionation procedure.

The chymotrypsin inhibitor fraction obtained as a nonspecifically adsorbed fraction of trypsin-sepharose column, was further purified by affinity chromatography on chymotrypsin-sepharose column. Further purification of this inhibitor by other methods were unsuccessful.

The presence of multiple forms of inhibitors in the same source material is well documented in many systems\textsuperscript{16,104,105,119,166,261-265}. It is now very well established in the case of potato tubers which contains as many as 13 inhibitor species\textsuperscript{104}. The multiple forms of these proteinase inhibitors have been ascribed by Feeney and Allison\textsuperscript{16} as due to (i) differences in primary structure (ii) differences in assembly of identical or non-identical subunits in the polymers and (iii) differences in conformational forms. Further, many proteinase inhibitors isolated by affinity chromatography were found to be heterogeneous\textsuperscript{266-258}. Heterogeneity observed in such cases may be due to the probable modifications on the affinity columns\textsuperscript{269} due to peptide bond cleavage and this possibility cannot be ruled out entirely for the winged bean tuber iso-inhibitors. However, in such modifications, the modified inhibitors reacted with their target enzymes more slowly than the native
inhibitors \(^{269,107,152}\). Modification occurs because of the cleavage of an Arg-x or lys-x bond on the inhibitor \(^{148,271}\), leading to two or more different forms without altering the biochemical characteristics and amino acid compositions. This modification is reversible under certain conditions \(^{150,272}\).

The major inhibitors obtained in chick peas \(^{51}\), winged bean seeds \(^{229}\) by affinity chromatographic methods which exhibited different enzyme specificities are truly the different native forms. The minor fractions obtained can be regarded as modified forms. From winged bean seeds, as many as six trypsin inhibitor fractions were obtained by Kortt \(^{229}\) in addition to one chymotrypsin inhibitor \(^{230}\). Of these trypsin inhibitors, three have been purified to homogeneity and characterized, the other three (minor) trypsin inhibitor fractions were probably the modified inhibitors on the affinity column. The trypsin inhibitors of winged bean seeds inhibit trypsin strongly but they were also shown to inhibit chymotrypsin weakly. However, in our studies on the proteinase inhibitors from winged bean tubers, it is only the major inhibitor WBT-TI-II which inhibits trypsin strongly but chymotrypsin only weakly. The other major inhibitor WBT-TI-I, which has been purified to near homogeneity, inhibits trypsin only.

The electrophoretic pattern of WBT-TI-I at pH 8.3 is of interest. This protein when run on gels which are
preelectrophoresed to remove excess ammonium persulfate showed a single band; on the contrary, the protein gave three closely spaced bands when it was run on gels without pre-electrophoresis. Further, this protein moves as single band on SDS-polyacrylamide gel electrophoresis and it was found to be homogeneous on ultra-centrifugation. Similar phenomenon was observed in clostridiopeptidase B, a proteolytic enzyme by Jovin et al. Further, Mitchell has showed that the variable bands observed in this case was due to the artifacts generated by the excess of ammonium persulfate used for the polymerization of the gels. Even in winged bean seed trypsin inhibitors, Kortt has observed heterogeneity with some inhibitor fractions on the non-denaturing polyacrylamide gels.

Molecular weight of the WBT-TI-I was found to be 27,000 daltons as determined by SDS-PAGE. Sedimentation coefficient of 3.54s obtained for this inhibitor by ultracentrifugation analysis, is rather high for a globular protein of molecular weight 27,000. The possibility of aggregation of the inhibitor protein molecule in the solvent used cannot be ruled out. This is further supported by the fact that the sedimentation pattern of WBT-TI-I showed a minor component which settled slowly compared to the fast settling major component. This minor component may be monomeric and nonaggregated form and the major component may be in the aggregated form. The inhibitor WBT-TI-I does not go
into solution in 0.1M sodium phosphate buffer if the concentration exceeds 0.5% even in the presence of 0.5M NaCl. Therefore, determination of the molecular weight of WBT-TI-I by sedimentation analysis was not attempted.

WBT-TI-II was found to be highly homogeneous as established by SDS-PAGE, gel filtration and ultracentrifugation studies. This inhibitor gave two bands on PAGE at pH 8.3, a major prominent band and a minor faint band. The intensity of this faint band changed under identical conditions from one electrophoretic run to the other. Pre-electrophoresis of the gels to remove the excess ammonium persulfate did not eliminate the appearance of this additional band completely. Further, rechromatography of this inhibitor on QAE-sephadex using a steeper gradient did not resolve the protein into two peaks. Thus it may be concluded that the minor band which appeared on PAGE may be due to enzymatic modification of the native inhibitor similar to those observed in other cases involving affinity chromatography.

The molecular weight of WBT-TI-II was found to be 24,000 as determined by SDS-PAGE and by gel filtration on Sephadex G-75. This inhibitor was shown to have a sedimentation coefficient of 2.13 and a molecular weight of 24,600 based on sedimentation analysis. Unlike WBT-TI-I, WBT-TI-II sediments as a single symmetrical peak. Most of the proteinase inhibitors isolated from the plant sources...
have molecular weights ranging from 8000–10,000\(^1\), with a few notable exceptions—trypsin inhibitor from sweet potato (23,000–24,000)\(^9\), oats (43,500)\(^95\). Soybean (Kunitz) (19,900)\(^126\), and the kallikrein inhibitors from potatoes (25,200–27,100)\(^128\). Even in the case of winged bean seeds, two trypsin inhibitors with molecular weights approximately 20,000 have been obtained\(^229\). The largest plant inhibitor known so far is the pepsin inhibitor from potato tubers\(^129\), with a molecular weight of approximately 80,000.

The amino acid composition of WBT-TI-II has revealed the presence of 192 amino acid residues comprising entirely of all the natural amino acids. The amino acid composition is characterised by high contents of acidic amino acids compared to basic amino acids. Only traces of cystine/cysteine could be detected by amino acid analysis of WBT-TI-II probably due to destruction of cystine/cysteine during acid hydrolysis. Performic acid oxidation of the protein to convert cystine to cysteic acid before hydrolysis was not carried out due to paucity of the purified proteinase inhibitor sample. The minimal molecular weight of the inhibitor was found to be 25,400 based on amino acid composition, and is in fair agreement with the molecular weight determined by other methods.

Soybean trypsin inhibitor (Kunitz) is the only inhibitor whose complete amino acid sequence has been determined
hitherto for the Kunitz type legume proteinase inhibitors, and contains 181 amino acid residues with only two disulfide bridges. Winged bean seed trypsin inhibitors 2 and 3 isolated by Kortt contain 184 and 180 amino acid residues with molecular weights 20,500 and 20,000 respectively. Further, it has been reported that these two inhibitors have low half cystine (4) content. Yamamato et al. have recently isolated a trypsin inhibitor from winged bean seeds, by conventional methods using ion exchange and gel filtration chromatographic methods. This inhibitor contains 172 amino acids with a molecular weight of 19,200 and resembles the trypsin inhibitor-3 of Kortt, in its amino acid composition. This inhibitor (WBT-1) was found to be a mixture of two iso inhibitors, after the amino acid sequence studies. These two iso inhibitors were separated by isoelectric focusing and the amino acid sequence studies have established that these two iso inhibitors contain different amino acid residues in two positions viz. 73 and 152.

In many respects, the amino acid composition of winged bean tuber trypsin inhibitor WBT-TI-II is almost identical with that of winged bean seed trypsin inhibitors 2 and 3 of Kortt, and the soybean trypsin inhibitor (Kunitz) (SBI), which are characterised by low cystine content and high molecular weights (> 20,000). The pattern of amino acid distribution is almost similar for these two
Inhibitors. On the basis of molecular weight and amino acid composition data, WBT-TI-II may be classified under soybean trypsin (Kunitz) inhibitor.

Till recently soybean trypsin (Kunitz) inhibitor was regarded as a unique example of the Kunitz family. Very recently the winged bean seed trypsin inhibitors were added to this group. This fact is further supported by the sequence studies of Yamamoto et al. At least eight Kunitz type protease inhibitors have been identified in winged bean seeds. In order to include the trypsin inhibitors of winged bean tubers in this group (Kunitz family) some more data is needed.

Amino terminal analysis of WBT-TI-II has led to the identification of 'Arginine' as its N-terminal residue. Diversity in N-terminal residue is found among many plant proteinase inhibitors. In the same family of Kunitz inhibitors, the N-terminal residue was found to be different amongst different inhibitors. Lysine was found for Acacia elata and Albizia julibrissin A-II, serine for Albizzia julibrissin B-II, and valine for Erythrina latissima DR-280, and caffra DR-281. Even among the two different inhibitors from the same source, Winged bean seed trypsin inhibitors WBT-1A and WBT-1B were found to contain glutamic acid and aspartic acid as N-terminal
residue respectively\textsuperscript{114}. However, Arginine was also found as N-terminal residue in some of the proteinase inhibitors like bovine pancreatic trypsin inhibitors\textsuperscript{34}, and for some potato tuber trypsin inhibitors\textsuperscript{104}. 