Chapter 3

MATERIALS AND METHODS

3.1 SCREENING OF PLANTS FOR PROTEASE INHIBITOR

3.1.1 Plants for the study

Plants, which are locally available, and belonging to the families of Leguminosae, Malvaceae, Graminae, Rutaceae and Guttiferae were used as the source material to screen for protease inhibitory activity. Different plant parts including seeds, leaves, flowers and bark were used for the study.

3.1.2 Extraction and recovery of protease inhibitor

Plant materials for the study were washed thoroughly in distilled water and air-dried. A buffer extract was prepared in a 500 ml conical flask by homogenizing 25 g of plant materials in 100 ml of 0.1M phosphate buffer with pH 7.0 in an electrical blender. The homogenate was further mixed thoroughly by incubating the contents at room temperature in a rotary shaker for 30 minutes at 150 rpm. The slurry was then filtered through cheesecloth and the filtrate was centrifuged at 10,000 rpm for 15 minutes at 4°C for removing any cell debris that remains in the preparation (Pichare and Kachole, 1996). The clear supernatant obtained represented the crude extract, and was assayed for protease inhibitory activity and protein content as described in section 3.1.3.1 and 3.1.3.3 respectively.
3.1.3 Analytical methods

3.1.3.1 Protease inhibitor assay

Activity of protease inhibitor against protease was assayed according to the procedure described by Kunitz with slight modifications (Kunitz, 1947). In this method, the TCA soluble fractions formed by action of trypsin on the protein substrate Hammerstein casein was measured by the change in absorbance at 280nm. The residual caseinolytic activity of the trypsin in the presence of inhibitor, at 37°C, was used as a measure of inhibitory activity. Appropriate blanks for enzyme, inhibitor, and substrate were also included in the assay along with the test.

Reagents

1) Trypsin (SRL, India) - 0.5mg/ml (1000 units/mg) (Prepared in 0.1M phosphate buffer pH 7.0)
2) Hammerstein Casein in 0.1M phosphate buffer - 1%
3) Trichloro acetic Acid - 0.44M in Distilled Water
4) Phosphate Buffer (pH-7.0) - 0.1M

The assay procedure included the following steps:

a. One ml aliquot of trypsin was preincubated with 1 ml of suitable dilution of protease inhibitor at 37°C for 15 minutes.

b. To the above mixture 2ml of 1% Hammerstein casein was added and incubated at 37°C for 30 minutes.

c. The reaction was terminated by the addition of 2.5ml of 0.44 M trichloroacetic acid (TCA) solution.
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d. The reaction mixture was transferred to centrifuge tube and the precipitated protein was removed by centrifugation, at 10,000rpm for 15 minutes (Sigma, Germany).

e. The absorbance of the clear supernatant was measured at 280nm in UV-Visible spectrophotometer (Shimadzu, Japan) against appropriate blanks. The TCA soluble peptide fractions of casein formed by the action of trypsin in the presence and absence of inhibitor was quantified by comparing with tyrosine as standard.

f. One unit of trypsin activity was defined as the amount of enzyme that liberated 1 µg of tyrosine per milliliter of the reaction mixture per minute under the assay conditions.

g. One unit of inhibitor activity was defined as the decrease by one unit of absorbance of TCA soluble casein hydrolysis product liberated by trypsin action at 280nm per minute at 37°C in the given assay volume.

The protease inhibitory activity was expressed in terms of percent inhibition and it was calculated as

\[
\text{Inhibitory activity (\%)} = \frac{\text{Amount of tyrosine released without inhibitor} \ - \ \text{Amount of tyrosine released with inhibitor}}{\text{Amount of tyrosine released without inhibitor}} \times 100
\]

3.1.3.2 Residual inhibitory activity

Residual inhibitory activity is the percent inhibitory activity of the sample with respect to the percent inhibitory activity of the control.

\[
\text{Residual Inhibitory Activity} = \frac{\text{Inhibitory Activity of the sample (\%/ml)}}{\text{Inhibitory Activity of the control (\%/ml)}} \times 100
\]
3.1.3.3 Protein

Protein content was determined according to the method of Lowry et al., (1951) using Bovine Serum Albumin (BSA) as the standard and the concentration was expressed in milligram per milliliter (mg/ml).

Reagents

a) Solution I: A stock solution containing 2% (w/v) sodium carbonate (w/v) in 0.1 N sodium hydroxide in distilled water.
b) Solution II: A stock solution of 0.5 % (w/v) cupric sulphate in distilled water.
c) Solution III: A stock solution of 1% (w/v) sodium potassium tartarate in distilled water.
d) Solution IV: Working reagent: To 100 ml of solution (I), 1 ml each of solution (II) and solution (III) was added and mixed well.
e) Solution V: 1:1 Folin and Ciocalteau’s phenol reagent diluted with distilled water was prepared fresh just before use.

Estimation

The sample was made up to 500 µl with distilled water and added with 2 ml freshly prepared working reagent (Solution IV), mixed thoroughly, and incubated for 10 minutes. Later, 250µl of solution (V) was added, incubated for 30 minutes and the absorbance was measured at 750nm in a UV-Visible spectrophotometer (Shimadzu, Japan).

3.1.3.4 Specific activity

Specific activity was calculated using percent inhibitory activity and percent specific activity as per the following formula:

\[ \text{Specific activity} = \frac{\text{percent specific activity}}{\text{percent inhibitory activity}} \]

3.2 SELECTION OF PROTEINS

Those proteins precipitated by ammonium sulphate and selection of potential plant samples with maximum crude protein inhibitory activity are described in the sections.

3.2.1 Extraction and precipitation of proteins

The crude<button>button</button> protein was precipitated by ammonium sulfate as described under section 3.1.3.1 and 3.1.3.3 respectively.
Materials and Methods

3.1.3.4 Specific activity

Specific activity of the sample was calculated by dividing the percent inhibitory activity with the protein content and was expressed as percent specific activity/mg protein.

\[
\text{Specific activity} = \frac{\text{Inhibitory activity (\%)} }{\text{Protein (mg/ml)}}
\]

3.2 SELECTION OF POTENTIAL SOURCE AND ISOLATION OF PROTEASE INHIBITOR FROM *MORINGA OLEIFERA*

Those protease inhibitors from various sources that could be precipitated by ammonium sulphate salts were selected for further screening and selection of potential source. For this, the strategy adopted was that the plant samples with more than 60% protease inhibition were selected and the crude protein inhibitor extract was prepared as described in the following sections.

3.2.1 Extraction and recovery of protease inhibitor

The crude buffer extract from the selected plants was prepared as described under section 3.1.2. The samples were assayed for protease inhibitory activity and protein content as described earlier under sections 3.1.3.1 and 3.1.3.3 respectively.
3.2.2 Ammonium sulphate precipitation

Ammonium sulphate precipitation of the prepared sample was done according to the method described by Englard and Seifter (1990). The fractionation using ammonium sulphate precipitation has the advantage of intermediate removal of unwanted proteins and simultaneously the protein of interest could be concentrated. Ammonium sulphate (SRL, India) required to precipitate the protease inhibitor was optimized by adding varying concentrations (30%, 60% and 90%) to the crude extract as detailed below.

1) To precipitate the protein, ammonium sulphate was slowly added initially at 30% (w/v) saturation to the crude extract while keeping in ice with gentle stirring.

2) After complete dissolution of ammonium sulphate, the solution was kept at 4°C for over night precipitation.

3) Protein precipitated was collected by centrifugation at 10,000 rpm for 15 minutes at 4°C.

4) To the supernatant, required ammonium sulphate for next level of saturation was added and the procedure mentioned above was repeated. The precipitation was continued up to 90% (w/v) of ammonium sulphate saturation.

3.2.3 Dialysis

The precipitate obtained after ammonium sulphate precipitation was further dialyzed against 0.01M phosphate buffer (pH 7.0), in order to remove the ammonium sulphate from the precipitate as detailed below.
3.2.3.1 Pretreatment of dialysis tube

Dialysis tube (Sigma-Aldrich) was treated to remove the humectants and protectants like glycerin and sulfur compounds present in it, and to make the pores of the tube more clear. The treated tube retain most of the proteins of molecular weight 12 kDa or greater. The method followed for the treatment of the dialysis tube was as follows.

a) Washed the tube in running water for 3-4 hrs.
b) Rinsed in 0.3% (w/v) solution of sodium sulfide, at 80°C for 1 minute.
c) Washed with hot water (60°C) for 2 minutes.
d) Acidified with 0.2% (v/v) sulphuric acid.
e) Rinsed with hot water (60°C).

3.2.3.2 Dialysis procedure

a) The precipitated protein was resuspended in minimum quantity of 0.1M phosphate buffer (pH 7.0).
b) The solution was taken in the pretreated dialysis tube (Section 3.2.3.1) (Sigma-Aldrich, cut off value 12 kDa) against 0.01M solution of phosphate buffer pH 7.0 for 24 hrs, at 4°C with frequent changes of buffer and assayed for protease inhibitory activity, protein content and specific activity as described under sections 3.1.3.1, 3.1.3.3 and 3.1.3.4 respectively.
3.2.4 Distribution of protease inhibitor in different plant parts of *Moringa oleifera*

Distribution of protease inhibitor in various parts of the mature plant was evaluated by preparing buffer extracts of seeds, leaf, flower, root and bark. Buffer extracts were prepared as described in section 3.1.2 and were assayed for protease inhibitory activity, protein content and specific activity as described under section 3.1.3.1, 3.1.3.3 and 3.1.3.4 respectively.

3.2.5 Selection of suitable solvent for extraction of protease inhibitor from *Moringa oleifera*.

Ideal solution that enables maximal extraction of the protease inhibitor from the sample was optimized by preparing crude extract of leaves with different solutions. 25 g of fresh leaves from the mature plant was dissolved in 100 ml each of sodium chloride 15% (w/v) (Wu and Whitaker, 1990), sodium hydroxide 0.2% (w/v), hydrochloric acid 0.05M (Tawde, 1961), phosphate buffer 0.1M (pH 7.0) (Wu and Whitaker, 1990) and distilled water as described in section 3.1.2. The extracts thus prepared were assayed for protease inhibitory activity, protein content and specific activity as described in section 3.1.3.1, 3.1.3.3 and 3.1.3.4 respectively.

3.3 PROTEASE INHIBITOR PURIFICATION

Protease inhibitor, proteinaceous in nature, isolated from *Moringa oleifera* was purified by standard protein purification methods which included ammonium sulphate precipitation, followed by dialysis, Ion exchange chromatography and preparative polyacrylamide gel
Materials and Methods

electrophoresis as detailed below. All purification steps were carried out at 4\(^\circ\)C unless otherwise mentioned.

3.3.1 Ammonium sulphate precipitation

Ammonium sulphate precipitation (Englard and Seifter, 1990) was done as described earlier under section 3.2.2.

3.3.2 Dialysis

The precipitate obtained after ammonium sulphate precipitation was further dialyzed against 0.01M-phosphate buffer (pH 7.0) as described previously under section 3.2.3.

3.3.3 Ion exchange chromatography

The active protease inhibitor fraction obtained after the dialysis of ammonium sulphate precipitation was further purified by Ion exchange chromatography, using DEAE cellulose as the anion exchanger (Rossomando, 1990). Proteins, due to surface charge, bind to ion-exchangers. These reversibly adsorbed proteins were eluted out either using a pH or a salt gradient.

3.3.3.1 Activation of DEAE Cellulose

The anion exchanger, DEAE Cellulose was activated as described below.
I. Ten gram of DEAE Cellulose (SRL, India) was soaked in citrate buffer (pH 3.0, 0.01M), allowed to settle, and the fine particles were removed by decanting.

II. It was then suspended in 1M NaCl for overnight.

III. Decanted the sodium chloride solution and washed several times with distilled water in sintered glass funnel using vacuum filtration, until the pH of the washings became neutral.

IV. Equilibrated the resin in appropriate buffer by repeated washing with the same buffer.

3.3.3.2 Standardization of binding pH of protease inhibitor to DEAE Cellulose

The pH at which the protease inhibitor binds at its maximum to the anion exchanger was determined by eluting the protease inhibitor solution after incubating with DEAE Cellulose equilibrated to different pH. DEAE Cellulose was activated as per the method described under section 3.3.3.1. The resin was resuspended in deionised water and equilibrated separately in 0.01 M glycine-HCl buffer (pH 2.0-2.5), citrate buffer (pH 3.0-5.5), phosphate buffer (pH 6.0-8.0), and carbonate-bicarbonate buffer (pH 9.0-10.5). One milliliter of diluted sample of ammonium sulphate precipitated fraction dialysed in the above buffers was mixed with 2 ml slurry of DEAE Cellulose equilibrated in each pH buffer. Incubated at 4°C for overnight, and the supernatant was collected by decanting without disturbing the DEAE particles. Washed with appropriate buffer, added 2 ml of 0.4 M NaCl, and incubated overnight to get eluted the bound proteins from the DEAE Cellulose. Supernatant collected was centrifuged at 10,000 rpm for
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15 minutes to remove fine particles and assayed for protease inhibitory activity and protein content as described under sections 3.1.3.1 and 3.1.3.3 respectively.

3.3.3.3 Purification using DEAE Cellulose column

DEAE Cellulose, activated as described under section 3.3.3.1, was carefully packed in XK16/26 column (30 cm height) (Amersham Pharmacia) without any air bubble and the column was equilibrated with citrate buffer (0.01 M) pH 3.0 overnight.

Fifteen milliliter of dialyzed sample, prepared as mentioned in section 3.3.2, with a protein content of 3.5 mg/ml was applied to the pre-equilibrated DEAE Cellulose column. After the complete entry of sample into the column, the column was connected to the reservoir containing the above said buffer (citrate buffer 0.01 M, pH 3.0) with a flow rate of 2 ml/ minute. The unbound proteins were washed out until the absorbance at 280 nm reached near to zero. Elution was done at a flow rate of 2 ml/minute using stepwise gradients of sodium chloride ranging from 0.1, 0.2, 0.3, 0.4 and 0.5 M, prepared in citrate buffer (0.01 M) pH 3.0. Five milliliter fractions were collected and the protein content of each fraction was estimated by measuring the absorbance at 280 nm. Peak fractions from the column were pooled and dialyzed against the phosphate buffer (0.01 M) pH 7.0 as described under section 3.2.3. The dialyzed fractions were assayed for protease inhibitory activity, protein content and specific activity as described under sections 3.1.3.1, 3.1.3.3 and 3.1.3.4 respectively. The yield and fold of purifications was calculated as described below.
Calculation of yield of protein, yield of protease inhibitory activity and fold of purification

Yield of protein and yield of protease inhibitory activity of each fraction during purification is the percent activity obtained by dividing the total protein content or activity of that fraction with the total protein content or activity of the crude extract as the case may be. Fold of purification in each step was calculated by dividing the specific activity of the respective fraction with that of the crude extract.

\[
\begin{align*}
\text{Yield of protein} &= \frac{\text{Total protein content of the purified fraction} \times 100}{\text{Total protein content of the crude extract}} \\
\text{Yield of activity} &= \frac{\text{Total activity of the purified fraction} \times 100}{\text{Total activity of the crude extract}} \\
\text{Fold of purification} &= \frac{\text{Specific activity of the purified fraction} \times 100}{\text{Specific activity of the crude extract}}
\end{align*}
\]

3.3.4 Preparative polyacrylamide gel electrophoresis

Active fractions pooled from ion exchange chromatography were lyophilized in 1ml aliquot and resuspended in 0.1ml of sample buffer (0.0625M Tris-HCl, 2% SDS, 10% sucrose, 0.01% bromophenol blue, pH 6.8). Aliquots of three tubes were loaded on to a polyacrylamide gel prepared as described under section 3.4.1.3.2 and subjected to electrophoresis (Tarsons Dual mini vertical electrophoretic unit). Low molecular weight markers of Amersham Pharmacia were used as standard. After electrophoresis, a portion of the gel with marker was stained, and
compared with the original gel, and the portion of the gel with protease inhibitor band was cut out and made into small pieces. The protein bands were eluted using an Electro elutor (FINE PCR Electro elutor) by applying current 25V for 10 minutes at 4°C into 200 µl of reservoir buffer for Native-PAGE (section 3.4.1.1). The eluted proteins in buffer was collected out and dialysed against phosphate buffer (0.01M) pH 7.0. The protein samples were lyophilized in aliquots, and stored.

3.4 CHARACTERIZATION OF PROTEASE INHIBITOR

Purified inhibitor was further subjected to characterization for their biophysical and physicochemical properties like molecular weight, isoelectric point, amino acid analysis, optimal temperature and pH for maximal activity, stability at different temperature and pH, and inhibition kinetics to determine the type of inhibition as described in the following sections.

3.4.1 Electrophoretic methods

The crude buffer extract of protease inhibitor prepared from fresh leaves, ammonium sulphate purified fraction and protease inhibitor obtained after ion exchange chromatography were all subjected to electrophoretic analysis by non denaturing Native–PAGE and denaturing SDS-PAGE in a vertical slab electrophoresis (Tarsons Dual mini vertical electophoretic unit) Electrophoresis was carried out in a 10% polyacrylamide gel according to the method described by Laemelli (1970). SDS–PAGE analysis of the purified inhibitor was carried out both under reducing and non-reducing conditions, i.e., with and without β-mercaptoethanol respectively.
3.4.1.1 Reagents for polyacrylamide gel electrophoresis

1) **Stock acrylamide solution**

   - Acrylamide (30%)  -  60.0 g
   - Bis-acrylamide (0.8%) -  1.6 g
   - Distilled water (DW) -  200.0 ml

   Stored at 4°C in amber coloured bottle

2) **Stacking gel buffer stock**

   - Tris buffer (0.5M) -  6 g in 40 ml DW
   - Titrated to pH 6.8 with 1 M HCl (~ 48 ml) and made up to 100 ml with DW. Filtered with Whatman No: 1 filter paper and stored at 4°C.

3) **Resolving gel buffer stock**

   - Tris buffer (3 M) -  36.3 g
   - Titrated to pH 8.8 with 1 M HCl (~ 48 ml) and made up to 100 ml with DW.
   - Filtered with Whatman No: 1 filter paper and stored at 4°C.

4) **Reservoir buffer for Native-PAGE (pH 8.3)**

   - Tris buffer -  3.0 g
   - Glycine -  14.4 g

   Dissolved and made up to 1L with DW.
   - Prepared in 10X concentration and stored at 4°C.

5) **Reservoir buffer for SDS-PAGE (pH 8.3)**

   - Tris buffer -  3.0 g
   - Glycine -  14.4 g
   - SDS -  1.0 g

   Dissolved and made up to 1L with DW.
   - Prepared in 10X concentration and stored at 4°C.
**Materials and Methods**

6) **Sample buffer for Native-PAGE**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl (pH 6.8)</td>
<td>0.0625 M</td>
</tr>
<tr>
<td>Glycerol (optional)</td>
<td>10% (v/v)</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>0.01%</td>
</tr>
</tbody>
</table>

Prepared in 2X concentrations and stored at 4°C.

7) **Sample buffer for Reductive SDS-PAGE**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl (pH 6.8)</td>
<td>0.0625 M</td>
</tr>
<tr>
<td>Glycerol (optional)</td>
<td>10% (v/v)</td>
</tr>
<tr>
<td>SDS</td>
<td>2% (w/v)</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>0.1 M</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>0.01%</td>
</tr>
</tbody>
</table>

Prepared in 2X concentrations and stored at 4°C.

8) **Sample buffer for Non-reductive SDS-PAGE**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl (pH 6.8)</td>
<td>0.0625 M</td>
</tr>
<tr>
<td>Glycerol (optional)</td>
<td>10% (v/v)</td>
</tr>
<tr>
<td>SDS</td>
<td>2% (w/v)</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>0.01%</td>
</tr>
</tbody>
</table>

Prepared in 2X concentrations and stored at 4°C.

9) **SDS (10%)**

10) **Sucrose (50%)**

11) **Protein staining solution**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coomassie brilliant blue (0.1%)</td>
<td>100 mg</td>
</tr>
<tr>
<td>Methanol (40%)</td>
<td>40 ml</td>
</tr>
<tr>
<td>Glacial acetic acid (10%)</td>
<td>10 ml</td>
</tr>
<tr>
<td>DW</td>
<td>50 ml</td>
</tr>
</tbody>
</table>
12) Destaining solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol (40%)</td>
<td>40 ml</td>
</tr>
<tr>
<td>Glacial acetic acid (10%)</td>
<td>10 ml</td>
</tr>
<tr>
<td>DW</td>
<td>50 ml</td>
</tr>
</tbody>
</table>

13) Protein markers for Native-PAGE

Separate markers from Sigma-Aldrich were used

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
<th>MW (M_r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine Serum Albumin</td>
<td>10 µl</td>
<td>66,000</td>
</tr>
<tr>
<td>Chickalbumin</td>
<td>10 µl</td>
<td>45,000</td>
</tr>
<tr>
<td>Carbonic anhydrase</td>
<td>5 µl</td>
<td>29,000</td>
</tr>
<tr>
<td>Lactalbumine</td>
<td>10 µl</td>
<td>14,200</td>
</tr>
</tbody>
</table>

Markers were prepared in Native 1X sample buffer, and 30 µl of marker mix was loaded to the gel.

14) Protein Markers for SDS-PAGE

Low molecular weight marker mix of Amersham Pharmacia was used. Lyophilized marker mix was reconstituted in 1X sample buffer for reductive SDS-PAGE, boiled for 5 minutes, and 5µl of marker was loaded on to the gel. The composition of the marker mix is as given below.

<table>
<thead>
<tr>
<th>Components</th>
<th>MW (M_r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphorylase b</td>
<td>97,000</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>66,000</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>45,000</td>
</tr>
<tr>
<td>Carbonic anhydrase</td>
<td>29,000</td>
</tr>
<tr>
<td>Trypsin inhibitor</td>
<td>20,100</td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td>14,400</td>
</tr>
</tbody>
</table>
3.4.1.2 Native polyacrylamide gel electrophoresis

3.4.1.2.1 Gel preparation

**Resolving gel (10%)**

- Acrylamide: bis-acrylamide (30:0.8) - 10.00 ml
- Resolving gel buffer stock - 3.75 ml
- Ammonium persulphate (APS) - a pinch
- Water - 16.25 ml
- TEMED - 15.00 µl

**Stacking Gel (2.5%)**

- Acrylamide: bis-acrylamide (30:0.8) - 2.5 ml
- Stacking gel buffer stock - 5.0 ml
- Ammonium persulphate (APS) - a pinch
- Water - 12.5 ml
- TEMED - 15.0 µl

**Sample buffer (1X)**

- Native-PAGE sample buffer (2X) - 1.0 ml
- 50% Sucrose - 0.4 ml
- DW - 0.6 ml

**Sample preparation**

Added 100 µl of 1X sample buffer to lyophilized sample, mixed well and 25 µl sample and 5 µl marker mix was loaded to the gel.

**Procedure**

(a) Cleaned and assembled the gel plates.

(b) **Resolving gel** – Added all the components except APS in to a beaker, mixed gently and finally added APS. Immediately poured the mixture into the cast and poured a layer of butanol over the gel and allowed to polymerize at least for one hour.
(c) **Stacking gel** - Added the components of stacking gel except APS into a beaker, mixed gently and finally added APS. Pour the contents into the cast above the resolving gel and immediately inserted the comb between the glass plates. Allowed to polymerize at least for 1 hour.

(d) Gel was placed in the electrophoresis apparatus, and upper and lower reservoir was filled with reservoir buffer for Native-PAGE.

(e) The gel was pre run for 1 hr at 80 V.

(f) Loaded the gel with the protein sample.

(g) The gel was run at 80V till the sample entered the resolving gel.

(h) When the dye front entered the resolving gel, increased the current to 100V.

(i) The run was stopped when the dye front reached 1 cm above the lower end of the glass plate.

(j) Removed the gel from cast and stained for at least one hr in the staining solution.

(k) Destained the gel till the bands became clear and observed the protein bands under a transilluminator.

### 3.4.1.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The purified inhibitor protein was subjected to reductive and non reductive SDS-PAGE for evaluating the nature of polypeptide. Low
molecular weight marker of Amersham Pharmacia was used as standard and molecular weight of protease inhibitor was determined using Quantity One Software from Biorad.

3.4.1.3.1 Reductive SDS–PAGE

Gel preparation

Resolving gel (10%)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock Acrylamide solution</td>
<td>10.00 ml</td>
</tr>
<tr>
<td>Resolving gel buffer stock</td>
<td>3.75 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>Ammonium persulphate (APS)</td>
<td>a pinch</td>
</tr>
<tr>
<td>Water</td>
<td>15.95 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>15.0 µl</td>
</tr>
</tbody>
</table>

Stacking Gel (2.5%)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock Acrylamide solution</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Stacking gel buffer stock</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>Ammonium persulphate (APS)</td>
<td>a pinch</td>
</tr>
<tr>
<td>Water</td>
<td>12.3 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>15.0 µl</td>
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</tbody>
</table>

Sample buffer (1X)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS-PAGE sample buffer (2X)</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>50% Sucrose</td>
<td>0.4 ml</td>
</tr>
<tr>
<td>DW</td>
<td>0.6 ml</td>
</tr>
</tbody>
</table>

Sample preparation

Added 100 µl of 1X sample buffer to pure lyophilized sample, mixed well, boiled for 5 minutes in a water bath, cooled
to room temperature, and 25 μl sample and 5 μl low molecular weight marker mix was loaded to the gel.

**Procedure**

Procedure followed for SDS polyacrylamide gel electrophoresis was essentially the same as that of Native-PAGE which was described under section 3.4.1.2 with the exception that the reservoir buffer used was that of SDS-PAGE.

### 3.4.1.3.2 Non-reductive SDS–PAGE

**Gel preparation**

Resolving and stacking gel was prepared as described under section 3.4.1.3.1.

**Sample buffer (1X)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample buffer for Non-reductive SDS-PAGE (2X)</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>50% Sucrose</td>
<td>0.4 ml</td>
</tr>
<tr>
<td>DW</td>
<td>0.6 ml</td>
</tr>
</tbody>
</table>

**Sample preparation**

Added 100 μl of 1X sample buffer to pure lyophilized sample, mixed well, and 25 μl sample and 5μl low molecular weight marker mix was loaded to the gel.

**Procedure**

Procedure followed for SDS-polyacrylamide gel electrophoresis was essentially same the as that of Native-PAGE
which was described under section 3.4.1.2 with the exception that the reservoir buffer used was that of SDS-PAGE.

3.4.1.4 Analysis of protease inhibitor by Dot - Blot method

The purified fraction collected from ion exchange chromatography, was analyzed for its protease inhibitory activity according to the method of Veerappa et al., (2002) as described below.

I. 3µl of protease inhibitor was mixed with 3µl trypsin (0.5mg/ml) and spotted on to a strip of X-ray film.

II. 3µl of trypsin was mixed with 3µl phosphate buffer 0.1M (pH 7.0) as the control and spotted on to the X-ray film.

III. Incubated the X-ray film at 37°C for 10 minutes.

IV. Washed the film under tap water till the zone of gelatin hydrolysis by trypsin was visualized.

V. Where the inhibitor is present, the trypsin does not degrade the gelatin on the x-ray film. If the inhibitor is absent, a clear zone is formed at the site of sample application on the X-ray film.

3.4.1.5 Reverse zymography

Protease inhibitory activity of the purified protein was further confirmed by Reverse Zymogram on Gelatin-PAGE, performed by adding gelatin (0.1% final concentration) to the poly acrylamide prepared according to the method of Felicioli et al. (1997).
3.4.1.5.1 Gel preparation

**Resolving gel (10%)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide: bis-acrylamide (30:0.8)</td>
<td>10.0 ml</td>
</tr>
<tr>
<td>Resolving gel buffer stock</td>
<td>3.75 ml</td>
</tr>
<tr>
<td>Gelatin (1%)</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>SDS (10%)</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>Ammonium persulphate (APS)</td>
<td>a pinch</td>
</tr>
<tr>
<td>Water</td>
<td>12.95 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>15.0 µl</td>
</tr>
</tbody>
</table>

Stacking gel and sample buffer was prepared as described under section 3.4.1.3.2.

**Sample preparation**

Added 100 µl of 1X sample buffer to pure lyophilized sample, mixed well, and 25 µl sample was loaded to the gel.

**Procedure**

Procedure followed for gelatin-polyacrylamide gel electrophoresis was essentially the same as that of SDS-PAGE, which was described under section 3.4.1.3.2.

After the electrophoretic run, the gel was washed with 2.5 % (v/v) Triton X 100 for 30 minutes followed by rinsing the gel in phosphate buffer pH 7.0 (0.1M) and incubated at 37°C for 15 minutes in a bath containing 0.5mg/ml trypsin in phosphate buffer pH 7.0 (0.1M).
Materials and Methods

After the gelatin hydrolysis, the gel was washed with distilled water, stained with the Coomassie Brilliant Blue (Sigma Aldrich) for 1 hr and destained.

3.4.2 Molecular weight determination of protease inhibitor by gel filtration chromatography on Sephadex G75

Gel filtration chromatography was performed for the ammonium sulphate precipitated fraction of crude protease inhibitor using Sephadex G75 (Sigma Aldrich) in order to determine the molecular weight of protease inhibitor.

3.4.2.1 Preparation of column

a) 23g of Sephadex G75 (Sigma Aldrich) was suspended in distilled water and allowed to hydrate for 3 hrs at 100°C in a water bath, and fine particles were removed by decantation.

b) Hydrated gel suspension was degassed under vacuum to remove the air bubbles.

c) Filled the column with distilled water or eluent without air bubble. Gel suspension was carefully poured into the column (Amersham Pharmacia XK 26/70 column) without air bubbles and allowed to settle under gravity while maintaining a slow flow rate through the column.

d) Column was stabilized by allowing two times the bed volumes of eluent (0.1 M phosphate buffer, pH 7.0) to pass through the column bed in a descending eluent flow.
e) The position of the flow adapters are re-adjusted as required to maintain contact of the plungers with the gel bed.

3.4.2.2 Sample preparation and application

Two milliliter of dialyzed sample, prepared as described under section 3.3.2, with a protein content of 18.6 mg/ml was applied to the column. Care was taken to make sure that the sample was completely free of undissolved substances. After the complete entry of sample to the column, the proteins were eluted using 0.1M phosphate buffer pH 7.0, with a flow rate of 1ml/minute. One milliliter fractions were collected and the protein content was estimated by measuring the absorbance at 280nm in a UV-visible spectrophotometer (Shimadzu, Japan). Peak fractions from the column were pooled and assayed for protease inhibitory activity and protein content as described under sections 3.1.3.1 and 3.1.3.3 respectively.

3.4.2.3 Calculation of molecular weight

The molecular weights of the eluted proteins were calculated by calibrating the column with low molecular weight gel filtration protein markers from Amersham Pharmacia.

3.4.2.3.1 Column calibration

The gel filtration column prepared by Sephadex G 75 was calibrated with low molecular weight gel filtration protein markers from Amersham Pharmacia.
**Materials and Methods**

**Protocol**

a) Prepared a fresh solution of Blue Dextran 2000 (0.1mg/ml) (Sigma Aldrich) in the eluent buffer (0.1M phosphate buffer, pH 7.0).

b) Applied the Blue Dextran (1-2% of the total gel bed volume) to the column to determine the void volume ($V_0$).

c) Dissolved the proper combination of the calibration kit proteins in the eluent buffer. The concentration of each protein was between 5-20 mg/ml.

The calibration markers used included the following:

<table>
<thead>
<tr>
<th>Components</th>
<th>MW ($M_r$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribonuclease A</td>
<td>13,700</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>67,000</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>45,000</td>
</tr>
<tr>
<td>Chymotrypsinogen A</td>
<td>25,000</td>
</tr>
</tbody>
</table>

d) Applied the calibration kit proteins to the column. The volume of calibration solution was 1% of the total gel bed volume.

e) Determined the elution volume ($V_e$) for each kit proteins by measuring the volume of the eluent from the point of application to the center of the elution peak.

f) Calculated the $K_{av}$ value (Partition coefficient) for each protein and prepared a calibration curve of $K_{av}$ versus log molecular weight.

$$K_{av} = \frac{V_e}{V_0}$$
where $V_e$ is the elution volume of each protein and $V_0$ is void volume of the column, which was calculated by running the column with Blue Dextran 2000.

Molecular weight of the protease inhibitor was calculated from the calibration curve prepared using the calibration kit proteins.

### 3.4.3 Amino acid analysis

Amino acid analysis of the purified inhibitor protein sample was done using Shimadzu High-Performance liquid chromatography (LC - 4A) “Amino Acid Analysis System” (Ammu et al., 2001) at Central Institute of Fisheries Technology (CIFT), Kochi.

### 3.4.4 Optimal pH for protease inhibitor activity

Optimum pH for the maximal activity of the protease inhibitor was determined by performing protease inhibitor assay at different pH ranging from 2.0-12.0 as described under section 3.1.3.1 with minor modifications. The substrate 1% casein was prepared in the respective buffer for each pH. The buffer systems used were, glycine-HCl Buffer (pH 2-3.5), citrate buffer (pH 4-6), phosphate buffer (pH 6-8), Tris-HCl buffer (pH 8-9), carbonate-bicarbonate buffer (pH 9.5-10.5), boric acid/potassium chloride/ sodium hydroxide (pH 11.0), disodium hydrogen phosphate/sodium hydroxide (pH 12.0). Protease inhibitory activity was calculated as described under section 3.1.3.1.
3.4.5 Stability of protease inhibitor at different pH

The stability of protease inhibitor over a range of pH was determined by evaluating the inhibitor activity at pH 7.0, after incubating the purified protease inhibitor in different buffers of pH ranging from 2.0-12.0 for 24 hrs, at 4°C. 1.2 ml of purified inhibitor was incubated with 10.8 ml of different buffer systems, which included, glycine-HCl buffer (pH 2-3.5), citrate buffer (pH 4-6), phosphate buffer (pH 6-8), Tris-HCl buffer (pH 8-9), carbonate-bicarbonate buffer (pH 9.5-10.5), boric acid/potassium chloride/sodium hydroxide (pH 11.0) and disodium hydrogen phosphate/sodium hydroxide (pH 12.0). After incubation, 1 ml of sample was assayed for protease inhibitory activity as described under section 3.1.3.1 and was expressed as percent inhibition of protease activity.

3.4.6 Optimal temperature for protease inhibitor activity

Optimum temperature for the maximal activity of protease inhibitor was determined by assaying the inhibitor activity at different temperatures ranging from 10°C-80°C. The assay method followed was essentially the same as that described under section 3.1.3.1.

3.4.7 Stability of protease inhibitor at different temperatures

Temperature stability of purified inhibitor at different temperatures was evaluated by incubating 1.2 ml of purified protease inhibitor at different temperatures ranging from 30°C-70°C. The sample was drawn at different time intervals: 30 minutes, 1hr, 2hrs, 4hrs, 8hrs and 12hrs, and was further incubated at 4°C for 15 minutes. The protease inhibitory activity of each
sample was assessed by conducting the assay as described under section 3.1.3.1.

3.4.8 Effect of stabilizers on thermal stability of protease inhibitor

Effect of stabilizers on protease inhibitor against thermo inactivation was determined at 50° and 60°C, respectively, at which they were found to lose activity. The effect of various additives on the thermal stability was determined by incubating the inhibitor in the presence of an additive at the desired temperature for a stipulated period of time. Sample was drawn at different time intervals of 1 hr, 2 hrs, 4 hrs and 8 hrs. At the end of incubation, the inhibitor was further incubated on ice for 15 min and the residual activity was determined. Stabilizers added included glycine (1M), cysteine hydrochloride (10mM), PEG 8000 (10mM), glycerol (10%), sorbitol (10%), casein (1%), CaCl₂ (10mM), urea (10mM), sucrose, BSA and starch (at 1% level). Protease inhibitor assay was carried out as described under section 3.1.3.1.

3.4.9 Effect of various metal ions on protease inhibitor activity

Effect of various metal ions on activity of protease inhibitor was evaluated by incubating the protease inhibitor along with different concentrations of various metals ions in the inhibitor solution for 30 minutes followed by measuring the protease inhibitory activity as described under section 3.1.3.2. The metals studied included sodium chloride, calcium chloride, magnesium sulphate, cupric sulphate, sodium molybdate, zinc sulphate, ferric chloride, manganese chloride, nickel chloride, mercury chloride, barium chloride, cadmium sulphate, and aluminum sulphate which
Materials and Methods

The metal ions, Na$^+$, Ca$^{2+}$, Mg$^{2+}$, Zn$^{2+}$, Cu$^{2+}$, Fe$^{3+}$, Mn$^{2+}$, Ni$^{2+}$, Hg$^{2+}$, Ba$^{2+}$, Cd$^{2+}$, Mo$^{6+}$ and Al$^{3+}$ each at 1 and 10 mM final concentrations respectively.

3.4.10 Metal chelation of protease inhibitor using EDTA

The metal ion concentration of purified protease inhibitor and its effect on inhibitory properties in its native state was determined by metal chelation using 30mM EDTA (SRL, India) according to the method described by Jack et al. (2004). Purified protease inhibitor (2.7mg/ml) was dialyzed extensively against 30mM EDTA for over night at 4°C for chelation of metal ions. The EDTA was removed further by dialyzing over night with frequent changes of deionised water. The inhibitory activity of the demetallized protease inhibitor was determined by conducting protease inhibitor assay as described in section 3.1.3.2.

3.4.11 Metal ion concentration of protease inhibitor

Metal ion (Ca$^{2+}$, Mg$^{2+}$ and Zn$^{2+}$) concentrations of the protease inhibitor was determined as follows:

I. An aliquot of protease inhibitor was dialyzed extensively against distilled water and was used for mineral analysis by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES).

II. Another aliquot of protease inhibitor (2.7mg/ml) was dialyzed extensively against deionised water and was used for mineral analysis by ICP-AES.

III. A third aliquot was dialyzed extensively against 30mM EDTA for over night at 4°C for chelation of metal ions and then the EDTA
was removed by dialyzing against deionised water over night with frequent changes in deionised water. The mineral concentration of dialyzed sample was also determined by ICP-AES.

3.4.12 Effect of additional supplementation of Zn$^{2+}$, Ca$^{2+}$ & Mg$^{2+}$ to demetallized protease inhibitor

Impact of additional supplementation of different concentrations of metals to the demetallized protease inhibitor, in order to contribute Zn$^{2+}$, Ca$^{2+}$ & Mg$^{2+}$ metal ions towards regaining its inhibitory property was evaluated. One and 10mM final concentrations of calcium chloride, magnesium sulphate and zinc sulphate was incubated along with protease inhibitor for 30 minutes, followed by measuring the residual protease inhibitory activity as described under section 3.1.3.2.

3.4.13 Effect of various detergents on protease inhibitory activity

Effect of various non-ionic and ionic detergents such as Triton X-100, SDS, Tween-80, Tween-20, and Brij-35 (1% each w/v) on protease inhibitory activity was determined by incubating the protease inhibitor in each detergents for 30 minutes, dialyzed against 0.01M phosphate buffer pH 7.0 and estimated the residual inhibitory activity as described under section 3.1.3.2.
3.4.14 Effect of oxidizing agents on protease inhibitory activity

Impact of oxidizing agents on the activity of protease inhibitor was studied by incubating the protease inhibitor with 1, 2, 3, 4 & 5 % (v/v) of hydrogen peroxide and dimethyl sulfoxide for 30 minutes and measuring the residual inhibitory activity as described under section 3.1.3.2.

3.4.15 Effect of reducing agents

The effect of reducing agents on the activity of protease inhibitor was studied by incubating the protease inhibitor with 0.2, 0.4, 0.6, 0.8 and 1% (v/v) of dithiothreitol, β-mercaptoethanol and sodium thioglycolate for 30 minutes and measuring the residual inhibitory activity as described under section 3.1.3.2.

3.4.16 Chemical modifications of amino acids in protease inhibitor

To determine the impact of chemical structure of the amino acids at the reactive sites of inhibitor molecule on its inhibitory activity, selected amino acids of the inhibitor molecules were chemically modified. Thus five different amino acids were individually modified using specific chemical modifiers and the effect of modifiers on the anti proteolytic activity of the inhibitor molecule was determined. Chemical modifications of amino acids of purified inhibitor was carried out using different chemical modifiers under their respective reaction conditions. 2 ml of purified inhibitor (2.7mg/ml) was used for this study. After the incubation with different concentrations ranging from 5, 10, 15, 20 and 25 mM of each modifier, the
sample was dialyzed against phosphate buffer and the residual protease inhibitory activity was estimated as described under section 3.1.3.2.

<table>
<thead>
<tr>
<th>Chemical modifier</th>
<th>Amino acid Modified</th>
<th>Reaction conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Ethylmaleimide</td>
<td>Cysteine</td>
<td>30°C 0.1M Tris/HCl buffer (pH-7.0) for 60 min (Colman &amp; Chu, 1970)</td>
</tr>
<tr>
<td>Succinic anhydride</td>
<td>Lysine</td>
<td>30°C 0.1M Sodium carbonate buffer (pH-8.0) for 120 min (Habeeb et al., 1958)</td>
</tr>
<tr>
<td>Diethyl pyrocarbonate</td>
<td>Histidine</td>
<td>30°C 0.1M Tris/HCl buffer (pH-7.0) for 30 min (Ovaldi et al., 1967)</td>
</tr>
<tr>
<td>PMSF</td>
<td>Serine</td>
<td>25°C 0.05M Tris/HCl buffer (pH-7.8) for 120 min (Gold &amp; Farney, 1964)</td>
</tr>
<tr>
<td>N- Bromosuccinamide</td>
<td>Tryptophan</td>
<td>30°C 0.01M Tris/HCl buffer (pH-7.0) for 30 min (Spande &amp; Witkop, 1967)</td>
</tr>
</tbody>
</table>

3.4.17 Effect of acid treatment on protease inhibitor

Sensitivity of protease inhibitor in an acidic environment was evaluated by incubating purified protease inhibitor with different
concentrations of HCl ranging from 0.02, 0.04, 0.06, 0.08 & 1M (pH 2.0) for 30 minutes. After the incubation, the pH was neutralized with 1 ml of 0.1M Tris-HCl buffer pH 9.0. The residual protease inhibitory activity was estimated as described under section 3.1.3.2.

3.4.18 Effect of protease treatment on protease inhibitor

Sensitivity of protease inhibitor to gastric enzymes like trypsin was assessed by incubating the purified protease inhibitor with different concentrations of trypsin (from Bovine pancreas, SRL, India) ranging from 0.2, 0.4, 0.6, 0.8 and 1% for 30 minutes at 37°C. The residual protease inhibitory activity was estimated as described under section 3.1.3.2.

3.4.19 Stoichiometry of protease-protease inhibitor interaction

The molar concentration of the purified protease inhibitor for the complete inactivation of the trypsin was determined by preincubating 1 nM trypsin (based on $M_r$ 23,800) in 100µl of 0.1M phosphate buffer pH 7.0 with different amounts of chromatographically purified protease inhibitor (0.25 - 2.0 nM, based on $M_r$ 23,600) at 37°C for 60 minutes. The long incubation time was necessary to ensure that the reaction was complete. The remaining activity of the trypsin was determined by the addition of 1% casein, followed by incubation and spectroscopic examination according to Kunitz method as described under section 3.1.3.1.
3.4.20 IC$_{50}$ value of protease inhibition

The amount of protease inhibitor needed for 50% inhibition of protease activity was determined by conducting the assay as described under section 3.1.3.1.

3.4.21 Kinetic studies of inhibition of trypsin by protease inhibitor

Using the enzyme rate data a double reciprocal-plot was prepared and analyzed to determine whether the nature of protease inhibition is competitive, uncompetitive or non competitive. Protease inhibition kinetics was studied using various concentrations of substrate ranging from 0.001mM - 2.0mM. The assay was carried out (Erlanger et al., 1961), as detailed below:

a) 500µl of one nM trypsin was preincubated in 0.1M phosphate buffer alone for 20 minutes at 37°C.

b) 500µl of different concentrations of purified protease inhibitor (4, 6 & 8 nM) was preincubated with 100µl of one nM trypsin for 20 minutes at 37°C.

c) The pre-incubated mixtures were then added separately to the substrate solution 0.001mM-2.0 mM Nα-Benzoyl-Arginine-4-nitroanilide (BAPNA) (Sigma Aldrich) at 37°C for 10 minutes.

d) The reaction was arrested by adding 200µl of 30% (v/v) acetic acid.

e) The liberated p-nitro aniline was measured at 410 nm in a UV-visible spectrophotometer (Schimadzu, Japan).

f) One protease unit was defined as the amount of enzyme that increased absorbance by 1 OD/min and one protease inhibitory unit
is defined as the amount of protease inhibitor that inhibited one unit of protease activity and was expressed as percent inhibition as described under section 3.1.3.1.

The velocity of the enzymatic reaction (v) based on the rate of change in absorbance ($A_{410}$) of the reaction mixture was determined for each concentrations of BAPNA used.

The initial velocity data was plotted as the function of the concentration of substrate by the linear transformation of the Michaelis-Menten Equation and usual non-linear curve fitting of the Michaelis-Menten equation for the calculation of $K_m$ and $V_{max}$ of the reaction.

A Lineweaver-Burk curve, $1/v$ versus $1/[s]$ was plotted and the dissociation constant ($K_i$) and maximum Velocity ($V_{max}$) were calculated (Dixon, 1953).

3.5 APPLICATION STUDIES

3.5.1 Specificity with different pharmaceutically important proteases

The affinity of purified inhibitor with different classes of proteases having roles in much pharmaceutical and agricultural industry was tested. The proteases tested were, Cathepsin-B (Sigma Aldrich), Thrombin (Sigma Aldrich), Elastase (Sigma Aldrich), Chymotrypsin (Sigma Aldrich), Collagenase (Sigma Aldrich) and Papain (Sisco Research Laboratories Pvt. Ltd, India).
3.5.1.1 Assay of Cathepsin-B inhibitory activity

Cathepsin-B inhibitory activity was assayed using a solution containing 1.25-2.5 units/ml of Cathepsin B (Sigma Aldrich) in cold deionised water described by Barrett (1981a). 25μl of Cathepsin B was preincubated with 25μl of purified inhibitor solution (0.27mg/ml) at 30°C for 10 minutes. After the incubation, added 200 μl of 20 mM N-Succinyl-Ala-Ala-Pro-Phe-P-Nitroanilide in dimethyl sulphoxide and incubated at 30°C for 5 minutes. One unit of enzyme will release one micromole of p-nitro aniline per minute from N-Succinyl-Ala-Ala-Pro-Phe-P-Nitroanilide at pH 7.0 at 30°C and one cathepsin inhibitory unit is defined as the amount of protease inhibitor that inhibited one unit of cathepsin activity and was expressed in percent inhibition as described under section 3.1.3.1.

3.5.1.2 Assay of thrombin inhibitory activity

The thrombin inhibitory activity of protease inhibitor was evaluated. Prepared a stock solution containing 1mg/ml thrombin (Sigma Aldrich) in 0.1M Tris-HCl buffer pH 7.5, and 100 μl of 10μg/ml solution was preincubated with 100μl of purified inhibitor solution (0.27mg/ml) at 37°C for 10 minutes. Added 200μl of 1% casein to the mixture and incubated at 37°C for 30 minutes. The thrombin inhibitory activity of purified protease inhibitor was measured as described under section 3.1.3.1.

3.5.1.3 Assay of elastase inhibitory activity

The elastase inhibitory activity was tested with 10μg/ml elastase (Sigma Aldrich) solution with 0.27mg/ml purified protease inhibitor
according to Kunitz caseinolytic (Kunitz, 1947) method as described under section 3.1.3.1.

3.5.1.4 Assay of collagenase inhibitory activity

The collagenase inhibitory activity of purified protease inhibitor was checked by using 1% gelatin as substrate according to Ian (2001). 1mg/ml collagenase (Sigma Aldrich) was prepared in cold deionised water prior to assay. Preincubated one millilitre of collagenase with 0.27mg/ml purified inhibitor at 37°C for 10 minutes. Added 2ml of 1% gelatin to the reaction mixture and further incubated for 30 minutes at 37°C. The reaction was terminated using 0.44M TCA and the released amount of peptides were measured as described under section 3.1.3.1.

3.5.1.5 Assay of papain inhibitory activity

Papain inhibitory activity was evaluated by Caseinolytic method according to Murachi (1970). Prepared 6mg/ml solution papain, and preincubated 0.1 ml of papain with 0.1 ml purified inhibitor solution at 37°C for 10 minutes. After incubation, 0.5 ml of casein solution (1%) was added to the solution and the reaction mixture was incubated for 10 minutes at 37°C. The reaction was interrupted by adding 1.5 milliliter of trichloroacetic acid (5%) (w/v). The precipitate was removed by centrifugation at 10,000 rpm for 15 minutes and the released amount of tyrosine was estimated as described under section 3.1.3.1.
3.5.1.6 Assay of chymotrypsin inhibitory activity

Chymotrypsin inhibitory activity was assayed according to the modified method of Fritz et al (1966). Chymotrypsin from Bovine pancreas (Sigma Aldrich) was prepared by dissolving freeze dried material in 0.001 M HCl at a concentration of 1 mg/ml. Standard assay mixture contained 0.05 M Tris-HCl buffer, pH 7.6, 20 mM peptide substrate, N-Suc-Ala-Ala-Pro-Phe-p-nitroanilide, 0.27 mg/ml inhibitor solution and chymotrypsin (10 μg/ml). One unit of enzyme is defined as the amount of enzyme that induces the conversion of 1 μmol substrate/min. One chymotrypsin inhibitory unit is defined as the amount of protease inhibitor that inhibited one unit of chymotrypsin activity and was expressed in percent inhibition as described under section 3.1.3.1.

3.5.2 Activity spectrum of purified protease inhibitor towards commercially important proteases

The activity spectrum of purified protease inhibitor with different commercially available proteases from different sources were tested. All the enzymes were purchased from Sigma Aldrich. One milliliter of proper dilutions of each enzyme was incubated with one milliliter of protease inhibitor (0.27 mg/ml) to check the affinity of the inhibitor with the proteases and the assay for protease inhibition was carried out as described under section 3.1.3.1.
<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Enzyme</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Protease</td>
<td>Bacillus amyloliquifaciens</td>
</tr>
<tr>
<td>2</td>
<td>Protease</td>
<td>Bacillus licheniformis</td>
</tr>
<tr>
<td>3</td>
<td>Protease</td>
<td>Bacillus sp.</td>
</tr>
<tr>
<td>4</td>
<td>Protease</td>
<td>Aspergillus oryzae</td>
</tr>
<tr>
<td>5</td>
<td>Subtilisin</td>
<td>Subtilisin calves</td>
</tr>
<tr>
<td>6</td>
<td>Esperase</td>
<td>Bacillus lentis</td>
</tr>
<tr>
<td>7</td>
<td>Proteinase K</td>
<td>Tritirachium album</td>
</tr>
<tr>
<td>8</td>
<td>Pronase E</td>
<td>Streptomyces griseus</td>
</tr>
<tr>
<td>9</td>
<td>Protease</td>
<td>Engyodontium album</td>
</tr>
</tbody>
</table>

### 3.5.3 Role of protease inhibitor in seafood preservation

The activity of *Moringa oleifera* protease inhibitor towards the seafood spoiling microorganism and its effect on protein degradation of *Peneaus monodon* during preservation under different storage conditions like room temperature, 4°C and -20°C was evaluated.

For this, the sample was peeled and head removed. 10g sample (*Peneaus monodon*) was weighed and taken in a sterile plastic bag, sealed and kept at each storage condition as control. As the test experiment, the same weight of samples were taken in the same conditions as that of the control and incubated in a sterile polyethylene bag containing 10ml of (0.2mg/ml) purified protease inhibitor prepared as mentioned under section 3.3.3 at each temperature for 8hrs, 24hrs and 168hrs respectively.
After incubation, the sample was drawn and extract was prepared by homogenizing the samples in sterile distilled water using a mortar and pestle under sterile conditions and kept in a rotary shaker for 30 minutes at 150rpm. One milliliter of extract was taken under sterile condition, and serially diluted the sample in physiological saline and the total microbial population of each sample was analyzed by pour plating the samples on Casein agar plates prepared by incorporating 1% casein in Nutrient agar medium.

The complete protein of the samples were extracted using 5% NaCl in 0.02M sodium bicarbonate according to Chandrasekaran (1985) and the cell pellet were removed by centrifugation at 10,000 rpm for 15 minutes at 4°C. The clear supernatant was assayed for total protein content as described in section 3.1.3.3.