Original Research Article

Characterization of partially purified trypsin and chymotrypsin inhibitor of *Vigna pilosa*

Abstract

Vigna pilosa is a crop plant belonging to the family of Leguminosae. A proteinaceous inhibitor of trypsin (VPTI) and chymotrypsin (VPCI) was isolated from *Vigna pilosa* seeds. Protein was extracted in 0.1 M Tris–Cl buffer (pH-8.0) and subjected to precipitation using 60% ammonium sulphate, dialyzed against 0.1 M phosphate buffer and eluted on DEAE cellulose column chromatography. Fold purification obtained was 16.79 for VPTI and 12.99 for VPCI. A purified sample was analyzed on gelatin embedded native PAGE for isoform detection. Two isoforms of VPTI were observed. SDS-PAGE analysis of *Vigna pilosa* protease inhibitor (VPPI) showed closely related two polypeptide bands of ~20 and ~19.5 kDa. Solution assay with BApNa and BTpNa at 38 µg and 55 µg of inhibitor concentration showed 50% inhibition of VPTI and VPCI respectively. Both the inhibitors showed activity between pH ranges 6.5 to 7.5. It lost its complete activity when heated at 100°C for 50 minutes.

Keywords plant seed inhibitor, protease inhibitor, trypsin inhibitor, chymotrypsin inhibitor *Vigna pilosa.*

1. Introduction

Trypsin inhibitors are small proteins found in a variety of seeds, especially in food legumes. They interact with their target enzyme by contact with the active site of the enzyme resulting in the enzyme inhibitor complex that is incapable of enzymatic activity (Norton, 1991) Legumes mostly contain Bowman-Birk and Kunitz type of inhibitors (Shewry, 1999; Francisco, 2004). Bowman-Birk inhibitors are serine proteinase inhibitors. They are single polypeptides that interact independently but simultaneously with two proteases, which may be the same or different (Birk 1985, Raj *et al*., 2002). The plant Kunitz inhibitors are one or two polypeptide chains and low cysteine content. The members of this family are mostly active against trypsin, chymotrypsin, and subtilisin (Laing and McManus, 2002; Park, 2005). Formally trypsin inhibitors are often associated with its negative effect as an antinutritional factor but now it is known that they are helpful for human health due to its anticarcinogenic, anti-inflammatory activity, antiulcer activity (Kumar & Gowda, 2013).

Vigna pilosa is a crop plant belonging to the family Leguminosae. It is a perennial climbing herb that belongs to subgenus *Dolicho vigna*. In India it has restricted distribution in Western ghat, where it is locally known as *alsunda*. Trypsin and chymotrypsin inhibitors were found in many species of the Vigna.

2. Materials and Methods

Plant material Seeds of ripened fruit of *Vigna pilosa,* were procured from local stores.

Chemicals The chemicals used in the present investigation were procured from the following sources. Bovine serum albumin (BSA), acrylamide, N-N'-methylene bis-acrylamide, Coomassie brilliant blue (CBB) , N-N, N'-N'-tetramethyl ethylenediamine, 1, 2-diaminoethane (TEMED), ammonium per sulfate (APS), ammonium sulfate, sodium chloride, sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate, sodium hydroxide, ethylene diamine tetra acetic acid (EDTA), Glycine, Sodium dodecyl sulfate (SDS), were purchased from Himedia laboratories Pvt. Ltd. Mumbai India. Bovine pancreatic trypsin (2 × crystallized, type III, EC 3.4.21.4), ∝-N-benzoyl-DL-arginine-*p*-nitroanilide (BApNA), ∝-N-benzoyl-DL-tyrosine-*p*-nitroanilide (BTpNA),

purification material DEAE cellulose were purchased from Sigma Chemical Co., St. Louis. MO, USA. SDS-PAGE medium and low range molecular weight markers were procured from Merck Bioscience, Bangalore, India.

Protein extraction and partial purification

The seeds were cleaned, dehusked, crushed, and the powder was defatted with hexane. The defatted powder was stored at 4°C for further use. A 10% (w/v) suspension in 0.1 M Tris–Cl Buffer (pH-8.0) was extracted overnight at 4°C with continuous stirring. Next day, it was centrifuged at 14,000 rpm for 30 min. Crude extract was saturated for 40 to 60 % $(NH_4)_2SO_4$ precipitation by addition of finely ground solid $(NH_4)_2SO_4$ at 4 °C. The solution was allowed to stand overnight in cold for complete precipitation of the proteins. The precipitate was collected by centrifugation at 10, 000 · g for 30 min at 4 °C. The precipitate obtained was dissolved in minimal quantity of water and dialyzed extensively against 0.1 M phosphate buffer (2 · 1000 mL). The protein content was determined using BSA (250µg/mL) as a standard protein (Lowry et al., 1951).

DEAE cellulose column chromatography

The active protease inhibitor fraction obtained after dialysis was further purified by ion-exchange chromatography. The DEAE cellulose column was prepared according to protocol designed by manufacturer (Sigma Chemical Co., St. Louis. MO, USA) with minor changes. The column was carefully packed without any air bubble and was equilibrated with phosphate buffer (0.02M) pH 7.8. A dialyzed protein sample was applied to the pre-equilibrated column. After the complete entry of sample into the column, the column was eluted with phosphate buffer (0.02M) pH 7.8 with a flow rate of 1ml/min. using step wise gradient of sodium chloride. The protein content of each fraction was estimated by measuring the absorbance at 280 nm. Then the fractions were assayed for trypsin and chymotrypsin inhibitory activity. Peak fractions from the column were pooled and dialyzed against the phosphate buffer (0.02M) pH 7.8. The yield and fold of purification was calculated.

Assay method

The trypsin and chymotrypsin inhibitory activity were spectrophotometrically measured by assaying amidolytic activity for trypsin and chymotrypsin in the absence and presence of a known quantity of inhibitor using the chromogenic substrates BApNA and BTpNa, respectively (Kakade et al., 1969; Kumar & Gowda, 2013). All the spectrophotometric measurements were performed on a UV-visible spectrophotometer (Shimadzu, Model -UV-1800).

Assay of trypsin and trypsin inhibitor activity

Trypsin was assayed using the substrate BApNA. 40 mg of BApNA was dissolved in 2 mL dimethyl sulfoxide (DMSO) and then diluted (1:100) in 50 mM Tris-HCl buffer, pH 8.2 containing 20 mM $CaCl_2$, prior to enzyme assay. The assay reaction consisted of 0.5 mL of trypsin solution (50 g of trypsin in 1 mM HCl), 0.5 mL of water, and 1.25 mL of the substrate. The reaction was carried out at 37 °C for 10 min and the reaction arrested by adding 0.25 mL of 30 % acetic acid. The absorbance of *p*-nitroanilide liberated was measured at 410 nm against an appropriate blank.

Trypsin and trypsin inhibitory unit

One trypsin (TU) unit is arbitrarily defined as an increase in absorbance of 0.01 at 410 nm under conditions of assay. The trypsin inhibitory unit (TIU) is defined as the number of trypsin units inhibited under the same conditions (Kakade *et al.*, 1969).

Assay of chymotrypsin and chymotrypsin inhibitory activity

The stock of BTpNA (20 mM) was prepared by dissolving 16.2 mg in 2 mL of DMSO and then made up to 100 mL with 80 mM Tris-HCl buffer, pH 7.8 containing 100 mM $CaCl_2$ and 20 % DMSO (v/v). 0.5 mL chymotrypsin solution was added to 0.5 mL of distilled water and incubated with 1.25 mL of substrate at 37 °C for 10 min. The reaction was stopped by adding 0.25 mL of 30 % acetic acid and the liberated product, *p*-nitroanilide measured at 410 nm.

Chymotrypsin and chymotrypsin inhibitory unit

One chymotrypsin (CU) unit is arbitrarily defined as an increase in absorbance of 0.01 at 410 nm under assay conditions. The chymotrypsin inhibitory unit (CIU) is defined as the number of chymotrypsin units inhibited under the same conditions.

Polyacrylamide gel electrophoresis

Vertical slab gel electrophoresis was carried out in a GENEI mini model electrophoresis unit, at 25 ± 2 °C. The gel was scanned in BioRads Gel Doc™ EZ imager and analyzed in image lab software.

Gelatin embedded PAGE for trypsin and chymotrypsin inhibitory activity staining

Gelatin–PAGE (Felicioli *et al*., 1997) was performed by adding gelatin (1 %, w/v final concentration) to the acrylamide gel. Following electrophoresis, the gel was washed with distilled water for three times and then incubated at 37°C in 0.1 M Tris-HCl buffer (pH 8.0) containing trypsin (40g/mL) for 1 h. After gelatin hydrolysis, the gel was washed with distilled water and stained with CBB and destained. The presence of the trypsin inhibitor was detected as a dark blue band in a clear background due to the complex of the unhydrolyzed gelatin with the stain.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE (10%T, 2.7%C) at alkaline pH (8.3) was carried out by running the gel at constant current (50 volts) until the tracking dye reached the anode tank buffer (Laemmli, 1970).

3. Results and Discussion

Partial purification of inhibitor

The inhibitory activity of the crude extract indicates that the *Vigna* seeds contain proteinaceous trypsin inhibitors. Therefore, to identify the inhibitory protein, partial purification was carried out by using a DEAE cellulose column. The bound protein was eluted as a single peak with phosphate buffer pH 7.8. The advantage of using this step was the exclusive binding of only the inhibitor forms and removal of other contaminants. Increased NaCl concentration induced dissociation results in the release of inhibitors from DEAE cellulose. Specific inhibitory activity of purified VPTI was 1074 TIU/mL and VPCI was 534.8 CIU/mL. The purification is summarized in Table 1. The inhibitory protein bonded to the DEAE column at pH 7.8 showed that the net charge on protein is anionic.

Isoform detection

The purified inhibitor was electrophoresed on native PAGE as described earlier and detected by staining with CBB. Native PAGE followed by incubation independently with bovine trypsin and chymotrypsin showed that *V. pilosa* inhibitor inhibits trypsin and chymotrypsin enzyme (Fig. 3). It also showed that *V. pilosa* seeds contain multiple forms of VPTI inhibitors.

Molecular weight determination

The purified VPPI showed closely related two indistinct bands having the same staining intensity under reducing condition in SDS PAGE (Fig. 5). It shows that the inhibitor present in doublet form indicating presence of two polypeptide chains that share the same amino acid, but they are differing in certain key positions. It also showed

that out of total loaded protein in well, 52% retained in upper band while 48% in lower band. The gel analyzed in the image lab showed that the VPPI was separated between the R_f values of 0.4-0.6. The apparent molecular weights of the two isoinhibitors were ~20kDa and ~19.5kDa. (Fig. 4)

Stoichiometry

The stoichiometry of inhibition for the partially purified inhibitor against bovine pancreatic trypsin and chymotrypsin was assessed using BApNA and BTpNA respectively. Increasing concentrations of inhibitors were incubated with a fixed concentration of the enzyme. The plot of [I] Vs % residual activity was used to evaluate the stoichiometry. For 50% inhibition of trypsin, 38µg of VPTI and for chymotrypsin 55 µg of VPCI were needed.

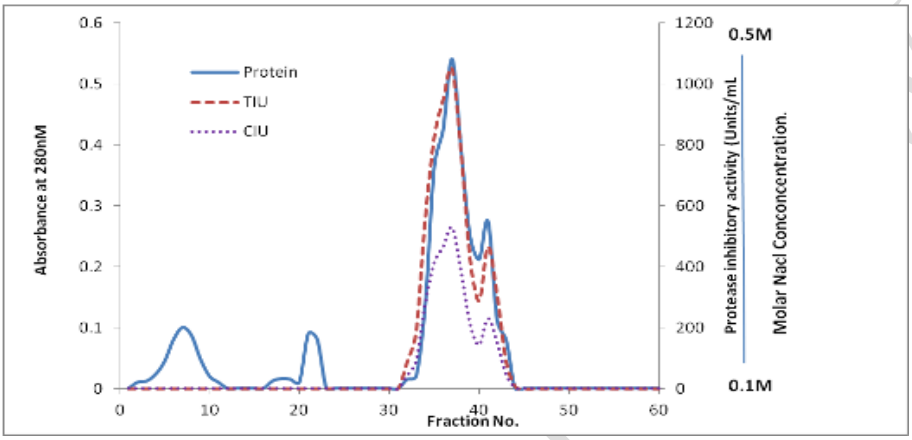


Fig. 1 DEAE cellulose chromatographic profile of VPPI

S/N	Sample Name	Protein mg/mL	Inhibitory Activity (IU/mL)	Specific Activity (TIU /mg)	TIU/ CIU	Yield of protein (%)	Fold Purification
01	Crude extract	3.4	TIU 218 CIU 140	TIU 64.11 CIU 41.17	1.55	100	1 1
02	Dialysis	1.8	TIU 640 CIU 380	TIU 355.5 CIU 211.1	1.68	52.94	5.54 5.12
03	DEAE Cellulose chromatography Pulled extract	1.234	TIU1326 CIU 660	TIU 1074 CIU 534.8	2.00	36.29	16.76 12.99

Table 1 Summary of the purification of VPPI

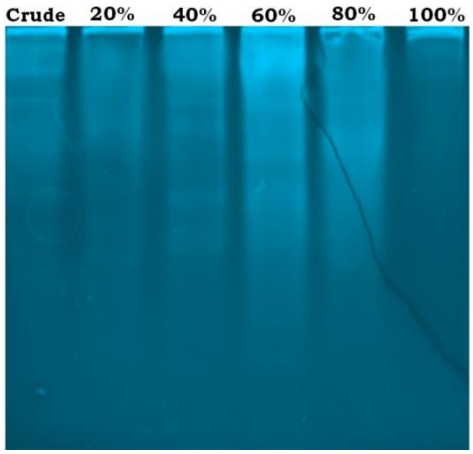


Fig. 2 Native-PAGE (10% T, 2.7% C) profile of Ammonium sulphate precipitate fractions of VPPI inhibitor showing trypsin inhibitory activity.

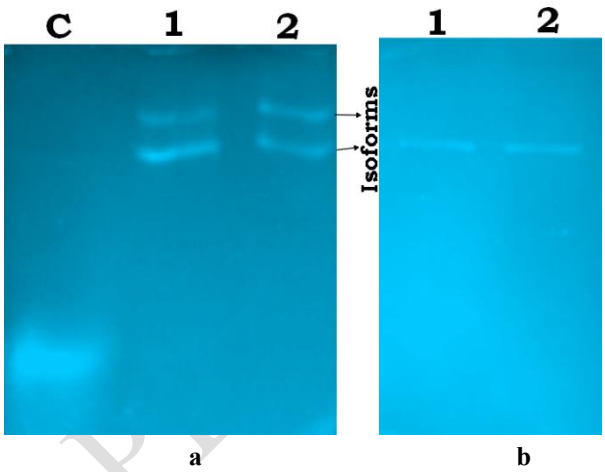


Fig. 3 Native-PAGE (10% T, 2.7% C) profile of VPPI showing trypsin inhibitory activity **and** chymotrypsin inhibitory activity **b**.

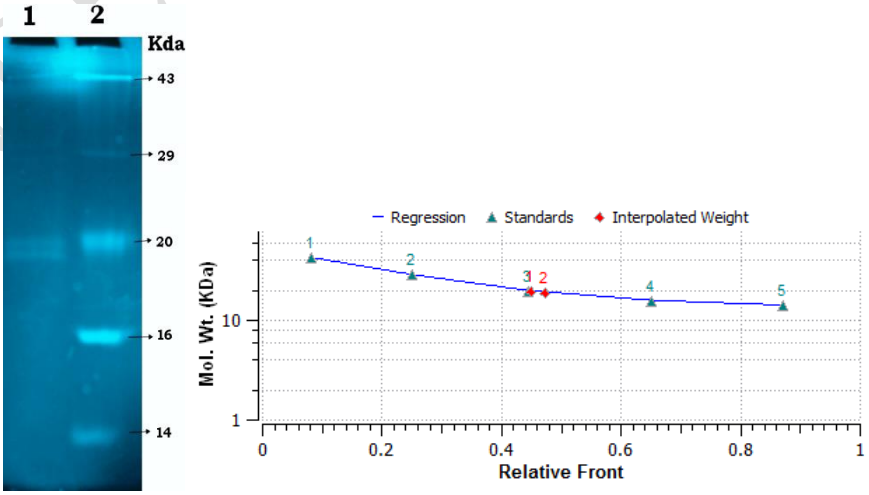
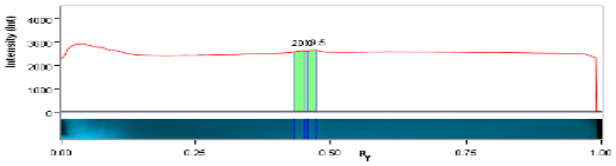
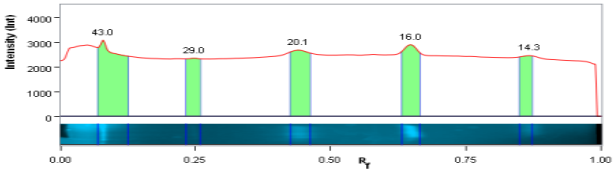


Fig. 4 SDS-PAGE (10%T, 2.7%C) profile of purified inhibitor lane1: VPPI lane 2: molecular weight markers. (Small size)



a



b

Fig. 5 SDS –PAGE lane profile of VPPI (a-lane 1, b-lane 2)

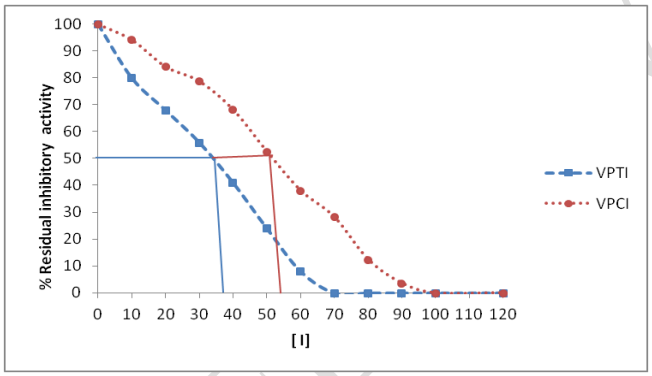


Fig. 6 Determination of IC_{50} value for trypsin and chymotrypsin inhibition.

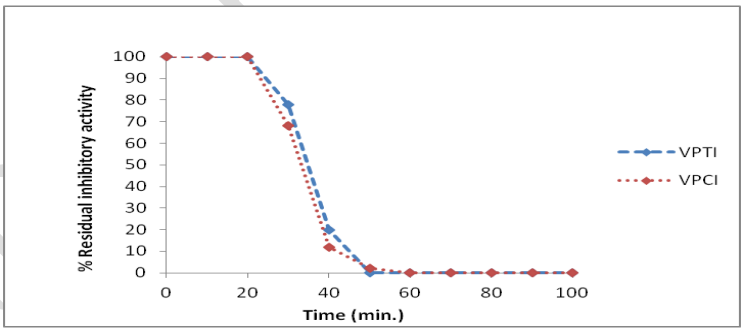


Fig. 7 Determination of thermo stability for trypsin and chymotrypsin inhibition.

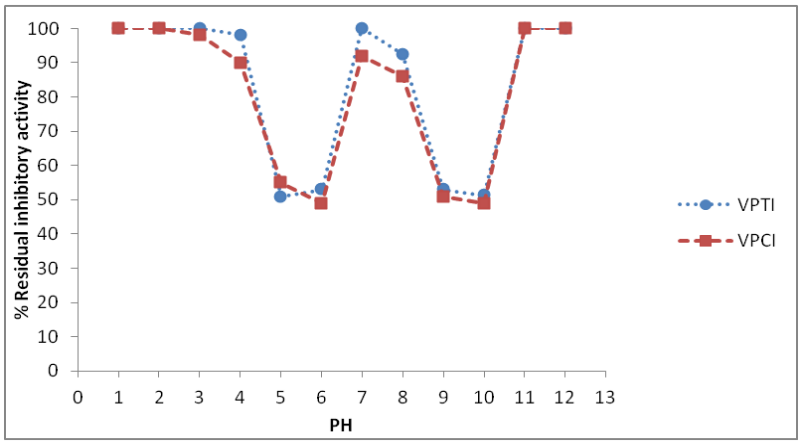


Fig. 8 Effect of pH on trypsin and chymotrypsin inhibition.

Conclusion

The result indicates that the *Vigna pilosa* seeds contain proteinaceous-protease inhibitors. The protein precipitated at 60% ammonium sulphate concentration has the highest protease inhibitory activity (Fig. 2). Gelatin embedded native PAGE of purified VPPI incubated independently with either trypsin or chymotrypsin showed a single protein species with similar mobility (fig. 3) indicating that VPPI inhibiting both the enzymes. It also shows that the seeds contain multiple forms of VPTI. The apparent molecular weight of VPPI was evaluated by SDS-PAGE. The molecular weights of two isoforms of VPTI as determined by SDS-PAGE were 20 and 19.5 kDa (Fig. 4). 70 µg of partially purified protein (VPTI) causes 100% of trypsin inhibition while 100 µg (VPCI) causes 100% chymotrypsin inhibition. Thermal stability of *V. pilosa* inhibitor was assessed to check whether it can withstand high temperatures. Preliminary results suggest that the trypsin inhibitor was not stable to heat.

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