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CHARECTERIZATION OF PARTIALLY PURIFIED TRYPSIN INHIBITOR FROM LABLAB PURPUREUS

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ABSTRACT

Lablab purpureus is a protein rich crop plant belonging to the family Leguuminosae. A proteinacious inhibitor of trypsin *(*LPTI*)* was isolated from *Lablab purpureus* seeds. Protein was extracted in 0.1 M tris–Cl buffer (pH-8.0) and subjected to precipitation using 20% ammonium sulphate, dialyzed against 0.1M phosphate buffer and further purified by using DEAE cellulose column. Fold purification obtained was 12.0 of LPTI. Three isoforms of LPTI were observed in gelatin embedded native PAGE. SDS-PAGE analysis of *Lablab purpureus* seed extract eluted from DEAE cellulose column showed three polypeptide bands of~30.1 kDa, ~20.6 kDa and ~17.9 kDa. Solution assay with casein showed 36 µg of inhibitor concentration causes 50% inhibition of trypsin. Thermo stability study showed that the LPTI are quit thermostable, it lost its complete activity when heated at 100°C for 90 minutes. Inhibitors are stable between pH 6.to 8.

KEYWORDS: plant seed inhibitor, protease inhibitor, trypsin inhibitor, *Lablab purpureus.*

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INTRODUCTION

The proteinacious inhibitors are small protein found in all life forms. Plant trypsin inhibitors are involved in plant defence found in a variety of seeds especially in legumes. These inhibitors interact with trypsin and forms enzyme inhibitor complex that is incapable of enzymatic activity.[1] In trypsin inhibitors, reactive sites are formed by arginine or lysine residues linked to another amino acid.[2] Several inhibitors of legumes are double-headed proteins while some inhibitors have a single peptide bond.[3] Bowman-Birk and Kunitz type of inhibitors are common in pulses[4]. Bowman-Birk inhibitors are serine proteinase inhibitors. They are single polypeptide interact independently but simultaneously with two proteases, which may be same or different.[5,6] The plant Kunitz inhibitors are one or two polypeptide chains and low cysteine content. Kunitz are mostly active against trypsin, chymotrypsin and substilisin.[7] Now day's trypsin inhibitors are helpful for human health due to its anticarcinogenic, anti-inflammatory activity, antiulcer activity.[8] The lablab bean originated in India and grows in the wild in Bengal and Assam. It was cultivated throughout the tropic for food[9]. Plant is annual, short lived, perennial vines. The flowers are white and others may blue or purplish. Fruit is legume pod variable in shape, size and colour. It contains two to four seeds. The seeds are white, brown, red or black depending on the cultivar. In India, the lablab bean, like black gram, was sown in standing rice in small holes made between the rice plants, two seeds in a hole. The green pods were consumed as a vegetable and the stalks as fodder.[10] Nowadays, the dry seed is soaked, sprouted, dehulled, and then cooked as an alternative to dhal in Western India.

MATERIALS AND METHODS

PLANT MATERIAL

Seeds of *Lablab purpureus* were collected from local store Maharashtra (India).

CHEMICALS

The chemicals used for extraction, inhibitory assay and electrophoresis, were purchased from Himedia laboratories Pvt. Ltd. Mumbai India. Purification material DEAE cellulose was 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.

(i)Protein Extraction And Partial Purification

The seeds were cleaned, dehusked and crushed to obtain fine powder. The powder was defatted with hexane and suspended 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 with 0 to 20 % $(NH_4)_2SO_4$ for precipitation of protein by the addition of finely ground solid $(NH_4)_2SO_4$ at 4°C. The solution was allowed to stand overnight at 4°C 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. The protein content was determined using BSA (250µg/mL) as a standard protein.[11]

(ii)DEAE- Cellulose Column Chromatography

The dialyzed sample 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. Dialyzed protein sample was applied to the pre-equilibrated column. After the complete entry of sample in to 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 inhibitory activity. Peak fraction from the column were pooled and dialyzed against the phosphate buffer (0.02M) pH 7.8. The yield and fold of purification was calculated.

(iii)Assay Method

The trypsin inhibitory activity was spectrophotometrically measured by assaying amidolytic activity for trypsin in the absence and presence of a known quantity of inhibitor using the substrates casein. All the spectrophotometric measurements were performed on a UV-visible spectrophotometer (Shimadzu, Model -UV-1800).

Assay Of Trypsin And Trypsin Inhibitory Activity

Trypsin was assayed according to the photometric method of using the substrate casein. 1% of casein was dissolved in 0.1M of Phosphate Buffer (pH 7.8) The assay reaction consisted of 1 mL of trypsin solution, 1 mL of Phosphate Buffer (pH 7.8) and 2.5 ml of 0.4M TCA . The reaction was carried out at 40 °C for 30 min and the reaction arrested by adding 0.4M TCA. The absorbance was measured at 280 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[12]

(iv)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 Native Page

Gelatin–PAGE 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 (40 μg/mL) for 1 h. After gelatin hydrolysis, the gel was washed with distilled water and stained with coomassie brilliant blue 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.[13]

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.[14, 15]

RESULTS AND DISCUSSION

(i)Partial Purification of Inhibitor

The inhibitory activity of the crude extract indicates that the seeds of *lablab purpureus* contain proteinacious trypsin inhibitors. Therefore to study the inhibitory protein, partial purification was carried out by using DEAE cellulose column. The bound protein 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 induce dissociation results in the release of inhibitor from DEAE cellulose. Specific inhibitory activity of purified LPTI was 1628 TIU/mL The purification is summarised in (Table 3.1). The inhibitory protein bonded to DEAE coloumn at pH 7.8 showed that the net charge on protein is anionic.

Figure 3.1
DEAE cellulose chromatographic profile of LPTI

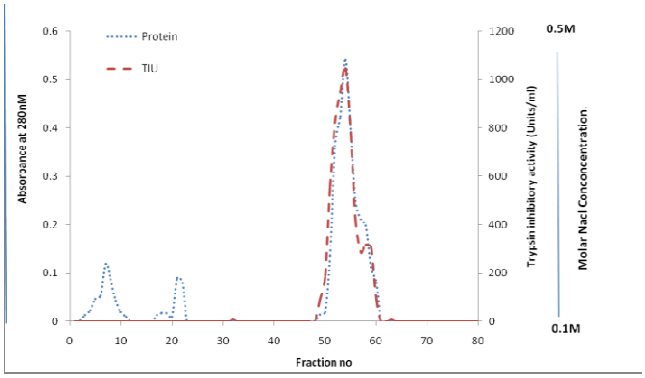


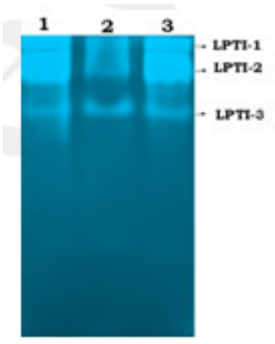
Table 3.1
Summary of the purification of LPTI

Sr.No.	Sample Name	Protein mg/mL	Inhibitory Activity (TIU/mL)	Specific Activity (TIU /mg)	Yield of protein (%)	Fold Purification
01	Crude extract	10.29	633	61.51	100	1
02	Dialysis	3.8	1122	295.26	36.92	4.8
03	DEAE Cellulose column Pulled extract	2.2	1628	740	21.37	12.0

(ii)Isoform Detection

The partially purified inhibitor sample on gelatin embedded native PAGE showed that *L. purpureus* inhibitor inhibit trypsin enzyme. It also showed that *L. purpureus seeds* contain multiple forms of LPTI inhibitors.

Figure 3.2
Native-PAGE profile of LPTI showing trypsin inhibitory activity. (All three lane contains same sample)



(iii)Molecular Weight Determination

The purified LPTI showed closely related three indistinct bands under reducing condition in SDS PAGE. The gel analysed in image lab showed that the LPTI were separated in between the R_f value 0.1-0.8. The apparent molecular weights of the three isoinhibitors were ~30.1 kDa, ~20.6 kDa. and ~17.9kDa.(Fig. 3.3).

Figure 3.3
SDS-PAGE profile of purified inhibitor lane1: LPTI lane 2: molecular weight markers.

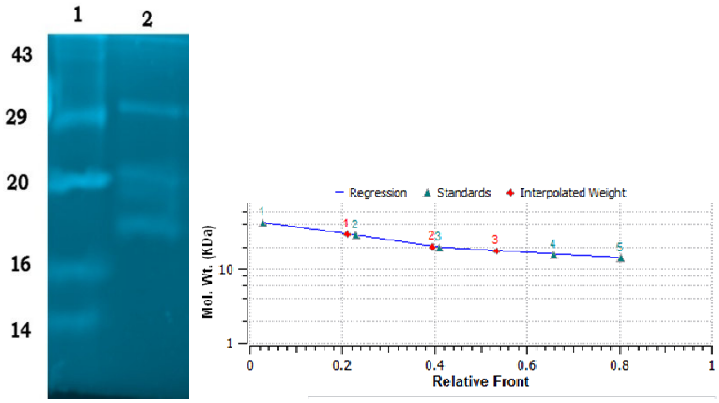
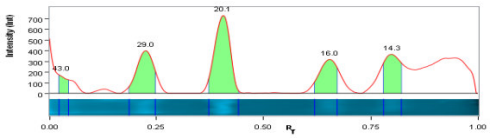
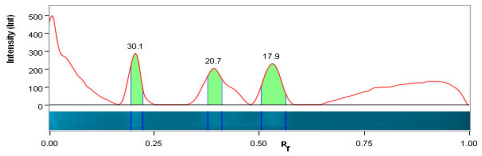


Figure 3.4
SDS –PAGE lane profile of LPTI



Lane 1



Lane 2

Figure 3.5
Determination of thermo stability for trypsin inhibition

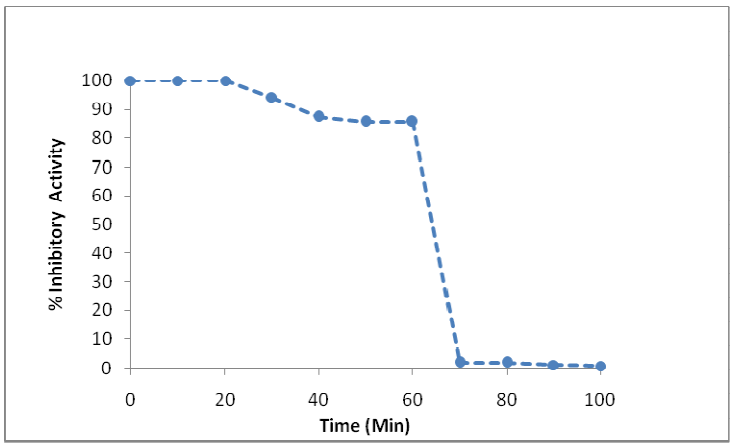
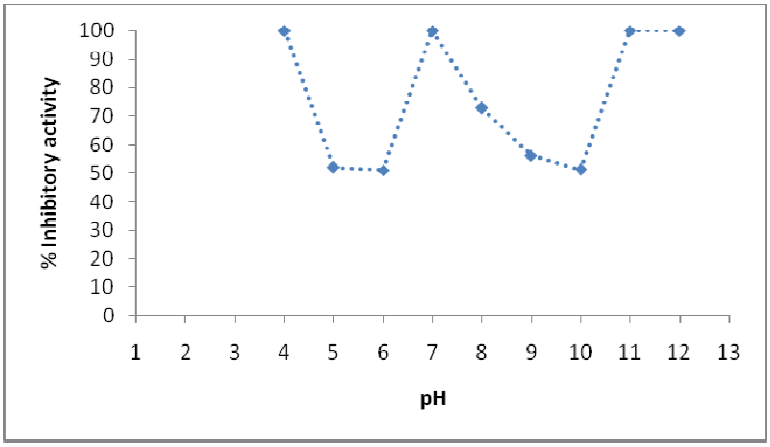


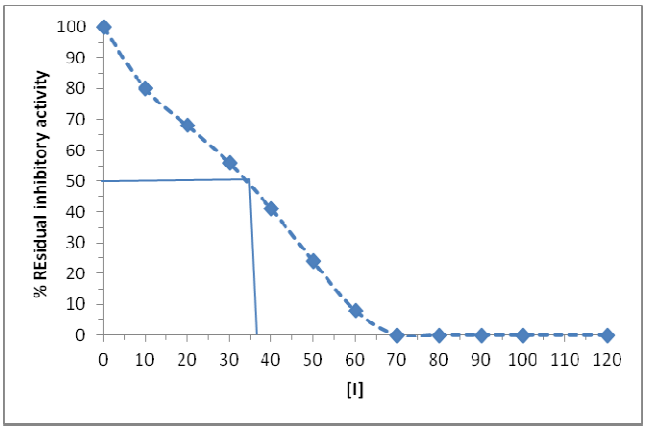
Figure 3.6
Effect of pH on trypsin inhibition



(iv)Stoichiometry

The stoichiometry of inhibition for the partially purified inhibitor against bovine trypsin was assessed using casein. Increasing concentrations of inhibitor 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 36 µg of LPTI were needed.

Figure 3.7
Effect of Inhibitor concentration on trypsin inhibition



CONCLUSION

The result indicates that the *Lablab purpureus* seeds contain proteinacious-protease inhibitors. The protein precipitated at 20% ammonium sulphate concentration has highest trypsin inhibitory activity. Gelatin embedded native PAGE of purified LPTI incubated independently with trypsin showed that the seeds contain multiple forms of LPTI. The apparent molecular weight of LPTI was evaluated by SDS-PAGE. The molecular weights of three isoforms of *L. purpureus inhibitor as* determined by SDS-PAGE were ~30.1 kDa of LPTI-1, ~20.6 kDa. of LPTI-2 and ~17.9kDa. of LPTI-3 respectively. 70µg of partially purified protein (LPTI) causes 100% of trypsin inhibition. Thermal stability of *L. purpureus* inhibitor was assessed to check whether it can with stand high temperatures. Preliminary results suggest that the trypsin inhibitor was quite stable to heat for 30 minutes. The optimum pH for the inhibitory activity is pH 7.5

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CONFLICT OF INTEREST

The authors whose names are listed for a manuscript do not have any conflict of interest.

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