Journal of Science-FAS-SEUSL (2026) 07(01) 36-43
ISSN: 2738-2184

In vitro assessment of trypsin inhibitors in *Phaseolus lunatus*

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Abstract

Proteases, particularly serine proteases like trypsin, play vital roles in maintaining physiological functions, while their dysregulation contributes to pathological conditions like cancer, and neurodegenerative diseases. Therefore, protease inhibitors are recognized as potential therapeutic agents for these diseases. Plants constitute an abundant natural source of protease inhibitors, especially legumes. Therefore, this study aims to assess the trypsin-inhibitory activity of *Phaseolus lunatus* (Sinhala: Pathuru Bonchi, Tamil: Mochai). Trypsin inhibitory activity of the seed extract at different concentrations was determined by the Kunitz method (1947), and the approximate molecular weight of the inhibitor was determined by dialysis. The active trypsin inhibitor/s were purified through ammonium sulfate precipitation and DEAE-cellulose anion exchange chromatography. Also, the effect of different metal ions, non-ionic detergents, reducing agents, temperature, and pH on its inhibitory activity was tested. Among the tested concentrations, the 2.5% seed extract exhibited the highest inhibitory activity, and dialysis results suggest that the inhibitor(s) is larger than 14 kDa. The purified inhibitor was optimally active at a temperature of -20℃ and a pH of 7.6 and showed considerable instability over temperatures ranging from 25℃ to 55℃ and pH ranging from 4 to 8.8. The inhibitor was substantially active and stable in the presence of some metal ions, reducing agents, and non-ionic detergents. This study demonstrates that *Phaseolus lunatus* is a promising source of trypsin inhibitors. The findings provide a foundation for molecular characterization of trypsin inhibitors.

Keywords: In vitro assessment, *Phaseolus lunatus*, seed water extracts, Trypsin inhibitors, Serine proteases

Introduction

Proteases are essential enzymes that cleave polypeptide bonds in proteins into smaller peptides and amino acids in organisms, to regulate key physiological processes. Among these proteases, serine proteases are critical because they are involved in major physiological functions, including cell signaling, protein digestion, immune responses, fibrinolysis, and blood coagulation [1]. However, their dysregulation causes serious consequences that are strongly related to diseases like cancers, neurodegenerative diseases, and autoimmune diseases, etc. Therefore, the function of serine proteases has to be properly regulated [2].

Protease inhibitors (PIs) are molecules that bind to proteases and regulate their activity. These PIs are either protein or non-protein compounds such as alkaloids, phenolics, or terpenes, etc. Hence, PIs can be used as therapeutic agents for diseases like cancer [3]. Plants, especially members of the family Fabaceae, are rich natural sources of trypsin inhibitors, which mainly function in defense against pathogens and regulation of endogenous proteins [4]. Among several PIs, serine PIs (SPI) have been studied widely due to their potential therapeutic values. Trypsin is one of the well-characterized serine proteases that play key roles in many essential

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physiological processes in organisms, and trypsin inhibitors are abundant in plants [5]. Despite the identification of PIs as potential therapeutic agents for certain diseases, only a few have progressed into clinically approved drugs, whereas most remain at the experimental stage. Therefore, the identification of novel PIs from unexplored plant sources could significantly play a role in medicine in the future [2].

The family Fabaceae has broad diversity, thus, it contains structurally and functionally diverse varieties of PIs [4]. Although PIs of many cultivated legumes have been studied, there are many underutilized legume plants in Sri Lanka that remain unexplored with respect to protease inhibitory studies. *Phaseolus lunatus*, a member of the family Fabaceae, is classified under the subfamily Faboideae. *Phaseolus lunatus* is a medicinal legume native to the neotropics and now widely distributed in tropical regions, characterized by its broad pods and high protein content [6]. It is also known as lima beans and in Sinhala, it is known as 'pathuru bonchi,' and in Tamil it is called 'Mochai'. *P. lunatus* is currently recognized globally as a versatile legume crop; however, it remains a largely underutilized species in Sri Lanka, often constrained to home gardens or found in the wild. Despite this, *P. lunatus* seeds are known to contain a high protein content, making them a valuable source of dietary proteins [7]. Although the research was conducted to highlight the general presence of bioactive compounds, the specific trypsin inhibitors have not been investigated within the local lima bean varieties of Sri Lanka. Hence, this study aims to assess trypsin inhibitory activity and characterize the trypsin inhibitor/s present in the seed extract of local *Phaseolus lunatus*.

Materials and Method

Sample collection

Fresh mature seeds of *Phaseolus lunatus* were collected from a home garden in Kandy district, Sri Lanka. Aqueous crude seed extracts were prepared by homogenizing the seeds with ice-cold distilled water, followed by centrifugation at 10,000 rpm for 10 min to remove insoluble materials and cellular debris. The resulting clear supernatant was collected and used as the crude extract. A series of extract concentrations (1, 2.5, 5, and 10% w/v) was prepared using distilled water for subsequent analyses [8].

Trypsin inhibitory activity

Trypsin inhibitory activity of the seed extract at each concentration was determined by the Kunitz method (1947). The crude extract was incubated with trypsin and buffer at 37 ℃ for 15 minutes. Then, the mixture was incubated with 0.5% pre-incubated casein at 37 ℃ for 60 minutes. Thereafter, 5% trichloroacetic acid was added, and the mixture was centrifuged. The absorbance of the supernatant was measured at 280 nm. The percent trypsin inhibitory activity (TIA) was calculated as follows [9],

$$\text{Protease Inhibitory Activity} = \frac{(\text{Average absorbance of control samples} - \text{Average absorbance of test samples})}{\text{Average absorbance of control samples}}$$

Dialysis and effect of metal ions, non-ionic detergents and reducing agents on inhibitory activity

Then, the 2.5% seed extract was dialyzed against 0.1 M phosphate buffer at pH 7.6 using 8 kDa and 14 kDa dialysis tubes, and trypsin inhibitory assay was conducted after dialysis to determine the approximate molecular weight of the inhibitor [10]. After that, the effect of various agents on inhibitory activity was evaluated by incubating the seed extract with metal ions, Calcium Chloride, Magnesium Sulphate, Zinc Chloride, Sodium chloride, with 1 mM and 10 mM concentrations, selected based on concentrations commonly employed in previous studies investigating the influence of metal ions on protease inhibitors [9], 1% Triton X-100 and Tween-20 non-ionic detergents and β-mercaptoethanol and dithiothreitol (DTT) reducing agents, with 30 µM and 60 µM concentrations for 30 minutes. After incubating with non-ionic detergents, the crude extract was dialyzed against 0.1 M phosphate buffer at pH 7.6 using an 8 kDa dialysis tube. Followed by, trypsin inhibitory activity was assessed [9].

Ammonium Sulphate precipitation and DEAE cellulose ion exchange chromatography

After that, the 2.5% seed extract was fractionated using the ammonium sulphate salting-out method. First, the crude extract was added to 0-30% saturation and centrifuged to precipitate proteins. The resulting supernatant was subjected to further precipitation by 30-45%, 45-60% and 60-75% ammonium sulphate saturations, respectively. Following, the precipitates were dissolved in PBS buffer and dialyzed against 0.1 M phosphate buffer (pH 7.6) using a 14 kDa dialysis tube [10].

Thereafter, the inhibitor was partially purified using DEAE cellulose anion exchange chromatography. First, the DEAE cellulose column was equilibrated to pH 7.6 with 0.05 M phosphate buffer and dialyzed seed extract was applied to the column, which allowed binding of inhibitor to the column. Then, the column was washed with 0.05 M phosphate buffer (pH 7.6) and bound proteins were eluted using Sodium Chloride. When eluting, five 0.5ml fractions were collected, and their inhibitory activity was assessed [11].

Temperature and pH stability

Finally, the temperature and pH stability of the inhibitor was tested by incubating the partially purified inhibitor at 4, 25, 37, 40 and 55 ℃ temperatures for three days and at pH 4.0, 5.5, 7.6, and 8.8 for 30 minutes before the trypsin inhibitory assay [10].

Statistical analysis

All experiments were carried out in triplicate to check the reproducibility of results. Data were analyzed using ANOVA (Analysis of Variance) and a P-value of less than 0.05 was considered significant. The data are presented here as mean ± SD (Standard Deviation).

Results and Discussion

Trypsin inhibitory activity of crude extract

The percentage trypsin inhibitory activity of the crude extract of *P. lunatus* seeds at different concentrations is shown in the following table 01. Among the tested concentrations, the maximum trypsin inhibitory activity (71.19%) was exhibited by the 2.5% crude extract.

Table 01: The percentage trypsin inhibitory activity of crude extract of *P. lunatus* seed samples

Concentration of the crude extract (%)	The percentage trypsin inhibitory activity (%)
1.0	69.71 ± 0.45
2.5	**71.19 ± 0.35**
5.0	67.34 ±0.20
10.0	62.21 ± 0.45

The results of this study demonstrated that *P. lunatus* has a higher trypsin inhibitory activity, as all tested concentrations of crude extract exhibited an inhibitory activity higher than 60%. Similar to this, many other edible legumes like soybeans, chickpeas, mung beans, common beans, velvet beans and cowpea have also been reported to contain high trypsin inhibitory activity [12]. Although, trypsin inhibitors serve defensive roles, certain inhibitors can limit protein digestibility if unprocessed. Therefore, mature seeds like velvet beans and sword beans are not suitable for direct consumption. However, pods and leaves of lima beans are suitable for direct consumption [13].

The trypsin inhibitory activity after dialysis as follows.

Table 02: The percentage trypsin inhibitory activity of 2.5% crude extract of *P. lunatus* seed samples after dialysis

Pore Size (kDa)	The percentage trypsin inhibitory activity (%)
8	67.70
14	69.36

In dialysis, molecules larger than the pore size are retained within the tube, and smaller molecules diffuse into the external solution. According to the results, since the inhibitory activity was fully retained after dialysis using both 8 kDa and 14 kDa membranes, this indicates that the molecular weight of inhibitor/s is greater than 14 kDa. Therefore, it could probably be a protein because proteins are generally larger than 10 kDa. This is consistent with the molecular weight range reported for Kunitz-type trypsin inhibitors (KTI) across legume species. The trypsin inhibitor from soybean is approximately > 20 kDa, winged bean is nearly 20 kDa, and cowpea is >15 kDa. Reported molecular weights for KTIs across legume species generally cluster around 18-24 kDa, with some variations [14]. This suggests that P. *lunatus* trypsin inhibitor's size places within the expected Kunitz type range. Also, some legumes such as common bean (*Phaseolus vulgaris*) and lentil (*Lens culinaris*) contain smaller Bowman-Birk family of inhibitors that typically range from 7-10 kDa [12]. However, dialysis results provide only an approximate estimate of the molecular weight and SDS-PAGE or mass spectrometry would be needed to confirm the precise molecular weight.

In this study, the effect of selected metal ions, non-ionic detergents and reducing agents on trypsin inhibitory activity was tested to determine whether these agents were capable of enhancing trypsin inhibitory activity. However, all metal ions tested (Ca^{2+}, Mg^{2+}, Zn^{2+} and Na^{+}), non-ionic detergent Tween-20 and reducing agents DTT and β-mercaptoethanol did not

significantly alter inhibitory activity. This stability suggests that the active site of P. *lunatus* trypsin inhibitor is not disrupted by the tested agents. For legume seeds like soybean, chick pea, and winged bean, trypsin inhibitory activity is generally reduced by many metal ions, non-ionic detergents, and reducing agents. Conversely, there are specific cases where the trypsin inhibitory activity of legumes like cowpea can even increase after incubation with metal ions and non-ionic detergents [10].

Fractionation with ammonium sulphate precipitation

Proteins present in the 2.5% crude extract were separated using the ammonium sulphate precipitation method to eliminate unwanted constituents and partially purify the trypsin inhibitor. As ammonium ion concentration increases, the solubility of the proteins gradually decreases, allowing differential protein fractionation. This principle was used to fractionate proteins from the crude extract and to purify the trypsin inhibitor [15]. Among the 0-30, 30-45, 45-60, and 60-75% ammonium sulphate saturations, the highest trypsin inhibitory activity (76.35%) was shown by the 30-45% saturation fraction. The percentage trypsin inhibitory activity shown by 0-30, 45-60, and 60-75% ammonium sulphate saturations was 11.85%, 69.36%, and 15.56%, respectively. Higher inhibitory activity in two consecutive fractions sometimes suggests that the seed extract may contain more than one trypsin inhibitor or multiple isoforms of a single inhibitor. However, proteins precipitated from the 30-45% fraction were selected for further purification of trypsin inhibitor by ion exchange chromatography. This precipitation pattern is closely related to other legume trypsin inhibitors, where the maximum inhibitory activity concentrates in mid-range saturation fractions. For example, trypsin inhibitor from *Capsicum frutescens* was similarly discovered on 30-60% saturation fraction [10]. Trypsin inhibitor from *Canavalia ensiformis* (Wonder bean) was concentrated in 30-65% fraction [16]. By contrast, some studies have reported peak inhibitory activity at higher saturations, for instance 60-80% in *Phaseolus vulgaris* (White beans) suggesting that optimal precipitation range is species-dependent [17].

Further purification of the inhibitor was done by using DEAE cellulose anion exchange chromatography, where negatively charged proteins bind to the positively charged DEAE cellulose column. Bound proteins were subsequently eluted using a gradient increase in ionic strength, collecting five separate elutions [17]. From the five fractions collected, the maximum trypsin inhibitory activity was exhibited by the second fraction, suggesting successful partial purification of the inhibitor from other non-inhibitory compounds. This indicates that the isolated trypsin inhibitor is negatively charged. Trypsin inhibitors of *Phaseolus vulgaris* [17] and *Canavalia ensiformis* were also purified through DEAE cellulose anion exchange chromatography [16]. However, some inhibitors, such as trypsin inhibitors from soybeans, were collected through CM cellulose ion exchange chromatography, and those inhibitors are positively charged [18].

Temperature and pH stability

The inhibitory activity was expressed as 100% after incubation at -20℃, and the corresponding inhibitory activities are shown in table 04.

Table 03: The percentage trypsin inhibitory activity after incubating at different temperatures

Temperature (℃)	Inhibitory activity (%)
-20	100.00 ± 0.00
4	97.28 ± 0.66
25	56.95 ± 0.64
37	46.74 ± 0.52
40	43.80 ± 0.49
55	41.72 ± 0.83

These results indicate that the inhibitor is relatively stable at 4 and -20 ℃ but progressively loses activity as temperature increases. This reflects the inhibitor's heat sensitivity and, hence, its protein nature. The high retention of activity at 4 and -20 ℃ is consistent with cold storage being the standard method for preserving protease inhibitory activity. However, the steep loss of activity within the 25-55 ℃ temperature range reports thermal sensitivity of some legume trypsin inhibitors [10]. This thermal inactivation of trypsin inhibitors could be due to folding and unfolding of the protein due to changes in temperature. This comparatively lower thermal tolerance may indicate a Kunitz-type rather than a Bowman-Birk type inhibitor. Also, the classical soybean Kunitz-type inhibitors are sensitive to heat and lose their inhibitory activity [18].

The inhibitory activity after incubating the partially purified inhibitor at varying pH is shown in the following table 05.

Table 04: The percentage trypsin inhibitory activity after incubating at different pH

pH	The percentage inhibitory activity (%)
4.0	55.18 ± 0.33
5.5	60.80 ± 0.46
7.6	68.95 ± 0.40
8.8	57.93 ± 0.96

The inhibitor exhibited the highest inhibitory activity at pH 7.6, with reduced activity at pH 5.5, 8.8, and 4.0, indicating optimal stability near neutral pH conditions and progressive decline toward both acidic and basic extremes. This pH dependent behavior reflects the protein nature of the inhibitor. Similar thermal and pH stability patterns have been reported for trypsin inhibitors derived from legumes such as soybean, cowpea, and mung bean, suggesting that this stability is characteristic of many legume-derived trypsin inhibitors [10].

Conclusion

The results of this study indicate that seeds of *Phaseolus lunatus* have a significant trypsin inhibitory activity. This finding highlights P. *lunatus* as a promising source of trypsin inhibitors and provides a basis for further structural and molecular characterization and evaluation of their potential therapeutic applications.

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