PARTIAL PURIFICATION AND CHARACTERIZATION OF SERINE PROTEASE INHIBITORS FROM THE SEEDS OF ENDEMIC WILD *LEGUME* Dialium ovoideum

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Introduction

Protease inhibitors (PIs) are the compounds that inhibit or modulate the activities of proteases thus exerting dramatic biological effects. Plant originated protease inhibitors are widely used in research, therapeutic and biotechnological applications. Plant PIs (PPIs) are generally small molecules, ranged from 8 kDa – 25 kDa in size.

Serine proteases (EC 3.4.21) are one of the best-characterized family of enzymes, which possess a catalytic triad coordinated structure consisting three essential amino acids His^{57}, Ser^{195}, and Asp^{102} at the active site. They perform important functions in biological processes such as digestion, blood coagulation, fibrinolysis, complement activation, etc.

Seeds of plants belonging to family Fabaceae contain a number of proteinaceous inhibitors of serine proteases. These inhibitors are classified into at least three distinct inhibitor families, namely Bowman–Birk type inhibitors (BBIs), Kunitz type inhibitors, and Potato Inhibitor I Families (Habib *et al.*, 2007). Legume BBIs have been studied extensively for their ability to prevent carcinogenesis. BBI concentrate (BBIC) of soybean, achieved investigational new drug status from Food and Drug Administration in 1992 (Zhang *et al.*, 2008).

Dialium ovideum Thw. (Velvet tamarind (E), Gal siyambala (S), Kaddupuli (T) which belongs to family Fabaceae is endemic to Sri Lanka which thrives in semi-dry tropical evergreen and deciduous forests.

The objectives of the present study are to develop an assay procedure to detect serine protease inhibitory activity in the seed extracts of legumes and to partially purify and characterize the serine protease inhibitors from the seeds of *D. ovoideum*.

Materials and Methods

Mature pods of *D. ovoideum* were obtained from the Gal Oya National park, Ampara. Finely grounded seed powder was stored at -70°C until use. Other seeds were collected from various places of the country for screening. Trypsin activity was evaluated using casein as the

substrate at pH 7.6 in 0.05 M phosphate buffer. The optimized conditions (pH 7.6, 37°C and 1 hour incubation) were used to modify the assay by introducing the additional step to determine the serine protease inhibitory activity.

Five legume species (*Dialium ovoideum*, *Bauhinia racemosa*, *Cassia roxberghii* *Macropteluum lathyroides* and *Cassia auriculata*) were screened for potential trypsin inhibitory activities. *D. ovoideum* with significant inhibitory activity was selected for isolation, purification and characterization of inhibitors. Extraction of inhibitors was by shaking the crude extract for 22 hours at 4°C. Aliquots of immature and mature seed extracts were tested with the optimized assay. Ammonium sulphate precipitation was used to concentrate the proteins by successive addition of solid ammonium sulphate at 4°C. Two different pore sized dialysis membranes of 3500 MWCO and 14,000 MWCO were used in dialysis. Aliquots of crude and precipitated inhibitors were extensively dialyzed against 50 mM, pH 7.6, phosphate buffer at 4°C overnight. The percentage remaining inhibitory activity was calculated. The effect of inhibitors on chymotrypsin was also analyzed.

The extracted crude and precipitated samples obtained by 30% ammonium sulphate saturation were incubated at various temperatures of 37°C, 28°C and 4°C for 2 weeks. Aliquots were drawn in 2-day time intervals and inhibitory activity was determined. The precipitated inhibitor samples were incubated in different buffers (pH 2, 4, 6, 7, 6, 8 and 10) over 12 days at 4°C. Aliquots were drawn from each samples and pH was adjusted to pH 7.6 to detect the inhibitory activity. The agar plate assay for protease activity was modified to visualize trypsin inhibitory activity using 1.5% agar plates in 20 mM Tris-HCl buffer, pH 7.6 containing 2 mM $CaCl_2$ and 0.5% casein solution.

The anion exchange column (1000 mm x 10 mm) was prepared using DEAE-52 micro granular anion exchanger at pH 7.6. The crude extract, dialyzed in the same pH was loaded onto column. After washing out the unbound proteins with the buffer, the bound proteins were eluted with a linear gradient of 0 to 1.0 M NaCl in the above buffer. Fractions (2 ml) were collected and proteins were monitored at 280 nm. The trypsin inhibitory assay was performed for fractions collected. The fractions with inhibitory activity were pooled and concentrated using ultra filtration tubes (10,000 MWCO, Amicon) followed by extensive dialysis for further analysis.

Results and Discussion

Protease inhibitory activities of crude extracts of *Dialium ovoideum*, *Bauhinia racemosa*, *Cassia roxberghii*, *Macropteluum lathyroides* and *Cassia auriculata* were 70%, 59%, 68%, 71% and 28% respectively. The immature seed extracts of *D. ovoideum* exhibited only half of the activity of fully matured seeds, which explains the mobilization of seed reserves with maturity reflecting a physiological

fact related to biological rhythm of plants. Ammonium sulphate precipitation (30%) was successful in concentration of protease inhibitors. These precipitated samples were used in further characterization. The percentage remaining inhibitory activity in dialysis using 3500 MWCO dialysis membranes was about 80% where 14,000 MWCO membranes only retain 35% of existing activity.

Significant effect of the isolated inhibitors on chymotrypsin was not detected. Therefore, these inhibitors exhibit remarkable specificity against trypsin. About 40%, 60% and 80% inhibitory activity remained after 7 days at 37°C, 28°C and 4°C respectively. Results of the stability of inhibitors at different pHs clearly demonstrate that trypsin inhibitors from *D. ovoideum* are relatively stable in the pH range of 6.0 – 8.0 like most of the known serine protease inhibitors. Inhibitory activity was also confirmed by modified agar plate assay. The elution profile of anion exchange column demonstrates that the seed extract may contain two serine protease inhibitors, which is consistent with the previous results obtained by dialysis.

Conclusions

Seeds of *D. ovoideum* contain remarkable serine protease inhibitory activity. These inhibitors specifically inhibit trypsin activity against the substrate casein. Therefore, these inhibitors can be denoted as ***Dialium ovoideum* Trypsin Inhibitors (DOTI's)**. **DOTI's** may exhibit narrow range of thermal and pH stability. The seeds of *D. ovoideum* may contain two serine protease inhibitors according to elution profile of anion exchange chromatography.

References

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