SHORT COMMUNICATION

Preliminary investigations on the serine and aspartic protease inhibitors from *Nothopegia beddomei*

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Abstract: Aqueous stem bark extract of *Nothopegia beddomei* was assayed for serine and aspartic protease inhibitors by using trypsin and pepsin as the target enzymes, respectively. Crude bark extract was dialyzed and subjected to inhibition assays. The thermal stabilities of serine and aspartic protease inhibitors were studied by incubating the extract at 4 ºC, room temperature and 37 ºC for a period of one month and subjecting to higher temperatures (55 ºC, 75 ºC and 95 ºC) for 15 minutes. The crude bark extract of *N. beddomei* exhibited serine and aspartic protease inhibitory activities. However, the serine protease inhibitory activity was significantly higher. The approximate molecular mass of both serine and aspartic protease inhibitors were more than 8 kDa. Both types of inhibitors were identified as mixtures of thermally stable and labile compounds due to their variations in inhibitory activities with time at different temperatures. Attempts made to purify the protease inhibitors using ion exchange chromatography and ammonium sulphate precipitation were unsuccessful. In conclusion, the results indicate both serine and aspartic protease inhibition activities in *N. beddomei* bark extract are governed by mixtures of protenaceous and non-protenaceous molecules.

Keywords: Protenaceous, non-protenaceous, bark extracts, thermal stability.

INTRODUCTION

Proteases are one of the most important groups of enzymes which involve in plethora of physiological and biochemical processes in all organisms. The key roles governed by proteases in living organisms are regulation of protein catabolism, regulation of protein – protein interactions, processing of cellular information and in generation, transduction and amplification of molecular signals. Most interestingly proteases show notable functions in reproduction and toxin production by pathogens such as insects, nematodes, fungi, bacteria and other infectious agents including viruses which cause diseases to plants and specially humans (Motyan *et al*., 2013). Therefore, the function of proteases in living organisms is highly consequential. Regulation of protease activity is crucial in order to maintain the cellular homeostasis. Among several protease regulation methods found in biological systems, the use of Protease Inhibitors (PIs) is a promising approach. PIs prevent the association of proteases with their substrates. These inhibitors process either reversible inhibition or irreversible inhibition. PIs are either proteins or non-protein compounds such as phenolics, alkaloids and terpenes and are classified based on type of protease they inhibit and the mode of inhibition (Mahajan and Badgujar, 2010).

PIs have been identified as therapeutic agents in prevention and treating diseases. Among them, Serine Protease Inhibitors (SPIs) and Aspartic Protease Inhibitors (APIs) have been studied for many years due to their potential therapeutic values (Clynen *et al*., 2005, Tossi *et al*., 2010). SPIs and APIs are with potent functions against hypertension, inflammation, tumor metastasis, Alzheimer's disease, fungal or viral infections, malaria and Acquired Immune Deficiency Syndrome (AIDS) (Tossi *et al*., 2010, Mehandru and Markowitz, 2003). Furthermore, PIs exhibit great influence in plant defense system in order to protect against pathogens such as insects, nematodes, fungi, bacteria and viruses. These plant PIs are used to produce improved transgenic plants expressing a cocktail of PIs against many different pathogens (Sharma, 2015). Although PIs have been identified as potential therapeutic agents for certain diseases, only few of them are developed as recommended drugs whereas most of the rest is still under experimental level to date. Similarly, the applications of most plant PIs in agriculture is also still under investigation. Therefore, discovering more PIs from novel biological sources will significantly affect the acceleration of their use in the fields of medicine and agriculture.

Serine proteases and aspartic proteases represent the largest families of plant proteases. In order to keep their activity under control, plants synthesize their respective inhibitors, SPIs and APIs (Rustgi *et al*., 2018). In addition, synthesis of these plant PIs is triggered as a defense mechanism against pathogens (Ryan, 1990). Therefore, search for SPIs and APIs from plant sources has become an interesting area of research. From many plants studied for years, several plant families such as Family Anacardiaceae, Family Solanaceae, Family Poaceae and Family Fabaceae

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have been identified with species exhibiting high serine and aspartic protease inhibitory activities. Among them, PIs exhibiting important therapeutic values have been detected from several plant species belonging to Family Anacardiaceae. HIV protease 1 inhibitor from *Rhus javanica* (Filho *et al*., 2010) and inhibitor from *Semecarpus anacardium* nuts against tumor derived proteases of mammary carcinoma in rats (Mathivadhani *et al*., 2007) are such examples. Thus, there is a significant demand for Family Anacardiaceae in discovering novel PIs. *Nothopegia beddomei* is a native plant in Sri Lanka which belongs to Family Anacardiaceae which is currently being overlooked for PIs or other potential applications. Hence the present study was conducted to detect, partially purify and characterize SPIs and APIs from aqueous bark extract of *N. beddomei*.

MATERIALS AND METHODS

Preparation of aqueous bark extracts

Mature stem bark of *N. beddomei* was collected from Teldeniya, in Kandy district, Sri Lanka. Ten grams of the inner red-brown bark layers were weighed and ground while adding ice cold distilled water (50 ml). Finely ground sample was filtered and subjected to centrifugation at 4 ℃ at 15000 rpm for 10 minutes and the supernatant was taken. Finally, a 20% (w/v) aqueous bark extract was obtained and stored at -40 ℃.

Assaying serine protease inhibitory activity in the bark extract

Serine protease inhibitory activity of bark extract of *N. beddomei* was tested using a modified, previously developed trypsin inhibitory assay procedure by Kunitz in 1947. Each test and control procedures were conducted in replicates. Test samples were prepared by adding 40 μl of trypsin (5 mg ml⁻¹, with 0.01 min⁻¹ activity) and 50 μl of 20% (w/v) bark extract into 210 μl of phosphate buffer (0.1 M, pH 7.6). For the controls, only the above mentioned volumes of trypsin and phosphate buffer (0.1 M, pH 7.6) were added. Then, all the test and control samples were pre incubated for 15 min at 37 ℃. Then, 400 μl of pre incubated (for 5 min at 37 ℃) casein substrate (1% w/v) was added into each reaction mixture and they were further incubated for one hour at 37 ℃. After the incubation period, 800 μl of 5% TCA was added to each reaction mixture in order to terminate the reactions and precipitate undigested casein. Next, 50 μl of 20% (w/v) bark extract was added only to each control samples to adjust the final volume up to 1.5 ml. Then, all the samples were centrifuged at 14000 rpm for 15 min and resulting supernatants were transferred into separate eppendorf tubes. Subsequently, UV absorbance values of the supernatants were measured at 280 nm against the blank (0.1 M, pH 7.6 phosphate buffer) by using UV spectrophotometer (Shimadzu, Japan). Finally, the percentage inhibition values were calculated using the equation mentioned below.

$$\text{Percentage inhibition} = \frac{\Delta \text{ Optical Density (OD) 280}}{\overline{OD} \ 280 \ (\text{control})} \times 100 \ \%$$

where,

Δ OD 280 = (Average absorbance of control sample at 280 nm) – (Average absorbance of test sample at 280 nm)

and

OD 280 (control) = Average absorbance of control sample at 280 nm

Optimization of serine protease inhibitory assay

By using 20% (w/v) bark extract as the initial concentration, a dilution series of 10% (w/v), 5% (w/v) and 2.5% (w/v) bark extracts were prepared and each bark extract sample were subjected to the assay procedure. Then, the optimum bark extract concentration was determined. Next, the assay procedure was carried out for different volumes (10 μl, 20 μl, 30 μl, 40 μl and 50 μl) of bark extracts with optimal concentration selected above to determine the optimum bark extract volume. Then, the partially optimized assay procedure was carried out at different pre-incubation times (5 min, 10 min, 15 min, 20 min, 25 min and 30 min) to find the optimum pre-incubation time.

Assaying aspartic protease inhibitory activity in the bark extract and optimization of aspartic protease inhibitory assay

Aspartic protease inhibitory activity of bark extract of *N. beddomei* was tested using a modified, pepsin assay described by Anson in 1938. Briefly, the inhibitory assay procedure was carried out similar to the procedure conducted for assaying serine protease inhibitors but with different reagents. In that assay procedure, 20 μl of 0.2 mg ml⁻¹ pepsin was used as the target enzyme with 0.01 min⁻¹ activity where as 2.5% (w/v) hemoglobin and 1.0 M pH 2.0 phosphate buffers were used as the substrate and the buffer respectively. Furthermore, all the optimization procedures described in above section were conducted in order to develop an optimized assay.

Estimation of approximate molecular weights of the serine and aspartic protease inhibitors

The crude bark extract (20% w/v) of 10 ml was placed in a cellulose dialysis bag with a cut off pore size of 8 kDa and subjected to an overnight dialysis against 500 ml of deionized distilled water. Then, the dialyzed bark extract was subjected to the optimized serine and aspartic protease inhibitory assays and the percentage inhibitory activities were determined.

Thermal stability at 4 ℃, room temperature and 37 ℃

The bark extracts with optimum concentrations for serine and aspartic protease inhibitory assays were separately kept at 4 ℃, room temperature and 37 ℃ initially for one week and the same samples were further tested for thermal stability for another three weeks. During this time period, bark extracts were subjected to the optimized serine and aspartic protease inhibitory assay procedures (daily for the first week and in 6 days time interval for the rest of

the period). Finally, the percentage remaining inhibitory activities of trypsin and pepsin inhibitors were calculated using the equation mentioned below.

Remaining percentage inhibition =

$$\frac{\text{Percentage inhibition by current sample}}{\text{Percentage inhibition by initial sample}} \times 100 \ \%$$

Temperature effect on the inhibitors at 55 ºC, 75 ºC and 95 ºC

The bark extracts with optimum concentrations for serine and aspartic protease inhibitory assays were separately heated at 55 ºC, 75 ºC and 95 ºC for 15 minutes and subjected to the optimized assay procedures. Finally, the percentage inhibitory activities were calculated.

Ion exchange chromatography

Anion exchange chromatography was conducted using 1.5 ml Diethyl-aminoethyl (DEAE) cellulose columns at pH 8.5 and pH 7.0. Dialyzed crude bark extracts (against buffers at respective pHs) were injected to the columns and fifteen fractions (1.5 ml) were eluted from each column using elution buffers (1 M NaCl in 0.05 M, pH 8.5 and pH 7.0). Finally, the unbound fraction, washed and eluted fractions were subjected to the optimized serine and aspartic protease inhibitory assays and the percentage inhibitory activities were calculated. The same procedure was conducted for cation exchange chromatography using Carboxymethyl (CM) cellulose columns at pH 5.5 and pH 7.0.

Ammonium sulphate precipitation

Crude bark extract (20% w/v, 10 ml) was mixed with solid ammonium sulphate for the 20% saturation according to the method described by Green and Hughes in 1955. Then, the mixture was kept at 4 ºC for one hour with constant stirring. After that, the mixture was centrifuged at 4 ºC at 10000×g for 15 minutes. The resulting pellet was dissolved in 1.5 ml of phosphate buffer (0.05 M, pH 7.6) and the supernatant was transferred to a beaker containing the required weight of ammonium sulphate for the 40% saturation. Subsequently the same procedure was repeated for 60%, 80% and 100% saturations. Then, the optimized serine and aspartic protease inhibitory assays were conducted for all the dissolved pellet samples and the final supernatant. Finally, the percentage inhibitory activities were calculated.

RESULTS AND DISCUSSION

Inhibitory activities in crude bark extract

The aqueous bark extract exhibited a significant potential to inhibit both serine and aspartic proteases and the highest inhibition was displayed by serine protease inhibitors. The inhibitory activities were studied using target enzymes trypsin and pepsin as they are well characterized and readily available model enzymes of serine and aspartic proteases respectively. The optimized bark extract concentration, volume and pre incubation time for serine protease inhibitory assay procedure were 2.5%, 30 µl and 15 min

respectively. Similarly, the optimized aspartic protease inhibitory assay procedure was obtained by 40 µl of 10% bark extract with 15 min of pre incubation period. The optimized serine protease inhibitory assay was able to show 75.78% trypsin inhibition which was greater than that of the inhibitory percentage shown by the bark extract before the optimization (67.36%). Similarly, pepsin inhibition percentage also was increased from 40.21% to 46.62% after the optimization of aspartic protease inhibitory assay.

Approximate molecular weights of the serine and aspartic protease inhibitors

After dialysis of bark extract, the trypsin inhibition percentage was increased from 75.78% to 81.24% whereas the pepsin inhibition percentage was increased from 46.62% to 59.75%. Therefore, it can be concluded that the size of the SPIs and APIs must be greater than the pore size of the dialysis tube which was 8 kDa. The removal of the small interfering molecules during the dialysis might be the reason for increased activity in SPIs and APIs. In general, plant PIs varies from 4 to 85 kDa, with the majority in the range of 8 to 20 kDa (Fan and Wu, 2005). Kunitz trypsin inhibitors (20 kDa), barley trypsin inhibitors (13 kDa), cathepsin D inhibitor found in potato tubers (27 kDa) and pepsin inhibitor (63 kDa) from aqueous extract of the roots of *Anchusa strigosa* are some of the examples for molecular weights of PIs (Major and Constabel, 2008, Odani *et al*., 1983, Lawrence and Koundal, 2002, Abuereish, 1998).

Thermal stabilities of the inhibitors

As illustrated in Figure 1, with the time and rise in incubating temperature, the remaining percentage inhibitory activities of both trypsin and pepsin inhibitors have been reduced. After the first 7 days of incubation, the remaining percentage inhibitory activity of trypsin inhibitors at 4 ºC, room temperature and 37 ºC were 93.80%, 88.02% and 77.11% respectively. Similarly, pepsin inhibitors exhibited remaining inhibitory activities of 90.00% (4 ºC) 74.91% (room temperature) and 71.53 % (37 ºC) at the end of the one week of incubation period. However, both trypsin and pepsin inhibitors were able to maintain considerable remaining activities even after exposure to above temperatures for a period of one week. But when the samples were further kept at respective temperatures for another three weeks, remaining activities of both trypsin and pepsin inhibitors were gradually decreased. At the end of one month of incubation period, bark extract kept at 37 ºC was capable of maintaining remaining percentage activities of trypsin inhibitors by 45.00% and pepsin inhibitors only by 18.35% implying the significantly higher thermal stability of trypsin inhibitors than pepsin inhibitors.

Furthermore, heating of bark extract at higher temperatures was resulted a significant decrease in trypsin and pepsin inhibitory activities. The bark extract heated at 55 ºC was capable of showing 65.96% of trypsin inhibition percentage and 33.26% of pepsin inhibition percentage indicating the relatively high thermal stability of trypsin inhibitors than pepsin inhibitors. However, bark extracts exposed to 75 ºC and 95 ºC exhibited only 39.52% and 13.14% of trypsin inhibition percentages respectively. The
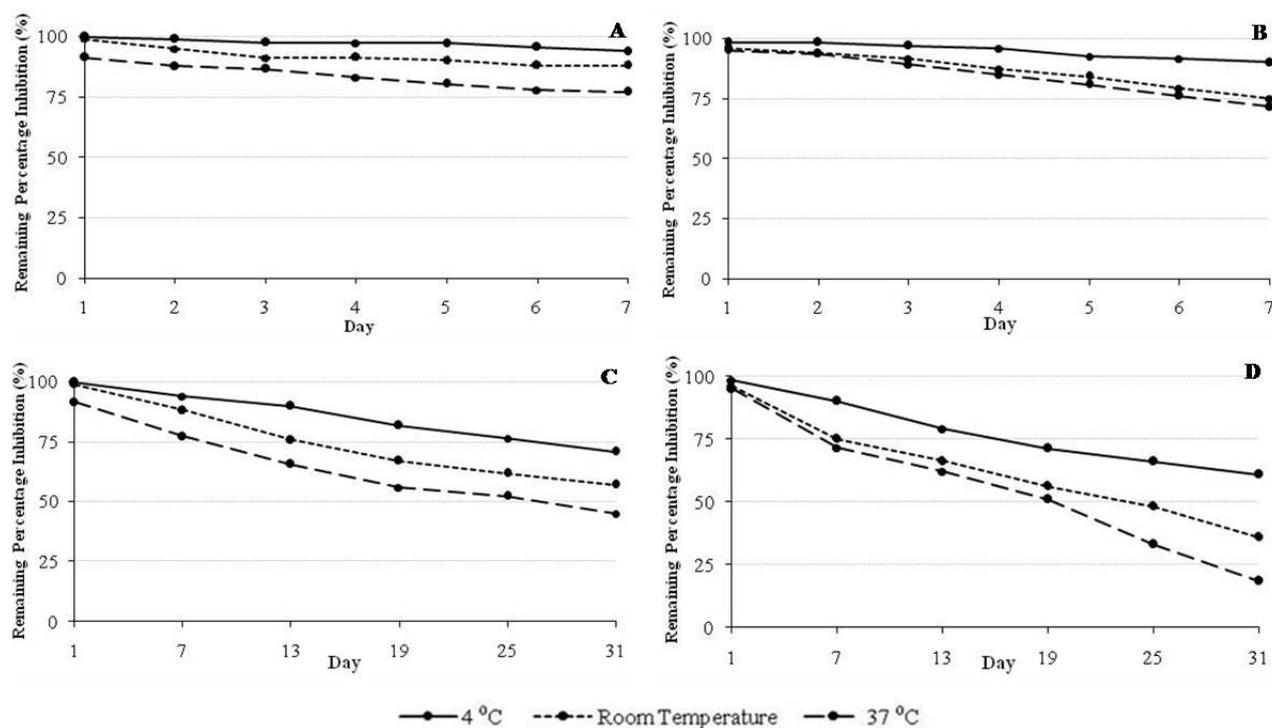
Figure 1: The graphs showing remaining percentage inhibitory activities of trypsin and pepsin inhibitors in crude bark extracts incubated at 3 different temperatures (4 ºC, room temperature and 37 ºC). Graph A and Graph B indicate the temperature effect on trypsin inhibitors and pepsin inhibitors over a period of one week respectively. Graph C and Graph D indicate the temperature effect on trypsin inhibitors and pepsin inhibitors over a period of one month respectively. In all graphs, Y axes represent the remaining percentage inhibition and X axes represent the time duration in days.

pepsin inhibition percentage shown by the bark extract heated at 75 ºC was 20.93% and at 95 ºC, it was only 4.68%. A previous study by Mikola and Mikkonen in 1999 showed that trypsin inhibitors extracted from oat possess a thermal stability in a wide range of temperatures and it described that the partial inactivation of oat trypsin inhibitors at different temperatures was apparently due to the presence of both heat stable and labile inhibitors. Moreover, another study has described a proteinaceous trypsin inhibitor named CpaTI from *Crotalaria pallida* seeds which was stable at 50 ºC and lost 40% of activity at 100 ºC and even though it has a protenaceous origin, that relative stability was possible due to the presence of intra-molecular disulfide bridges (Gomes *et al*., 2005). Similarly, *N. beddomei* crude bark extract can contain a mixture of thermal stable and labile components including small proteins, oligo peptides, phenolic compounds, alkaloids and terpenes responsible for both trypsin and pepsin inhibitory activities. This might be the reason for reduced but not completely inactivated nature of the inhibitors due to the effect of temperature.

Partial purification of the inhibitors

Only a few bound fractions eluted from both anion exchange columns and cation exchange columns were capable of showing trypsin inhibition and pepsin inhibition activities in very low levels (<10%). However, both trypsin inhibitors and aspartic inhibitors were not effectively bound to either positively charged DEAE columns at pH 7 and pH 8.5 or negatively charged CM cellulose columns at pH 7 and pH 5.5. Similarly, partially purified fractions obtained by the ammonium sulphate precipitation procedure also

exhibited a low serine and aspartic protease inhibitory activities which were almost less than 10%. According to these results, it can be assumed that the most of the trypsin and pepsin inhibitors in *N. beddomei* bark extract are the most probable non-proenaceous molecules. There are several records on non-protenaceous plant SPIs and APIs such as amidiol and faradiol terpene trypsin inhibitors from *Chrysanthemum mortifolium* and a non-protein macro molecular pepsin inhibitor isolated from *Anchusa* sp. (Polya, 2003). Interestingly one of the previous studies explained that *Rhus typhina* (Family Anacardiaceae) showed a broad spectrum of inhibitory activity to serine proteases and suggested that polyphenols, tannins and the flavonoids present in higher amounts in *Rhus typhina* plant extracts could be responsible for the observed anti-protease activity (Jedinak *et al*., 2010). Therefore, trypsin inhibitors and pepsin inhibitors in *N. beddomei* also have a high potential to be such non protein compounds.

CONCLUSIONS

N. beddomei can be considered as one of the important species belonging to the Family Anacardiaceae containing potent serine and aspartic protease inhibitors. However, SPIs are the major inhibitors found in bark extract of *N. beddomei* and both of these SPIs and APIs are water soluble compounds with molecular weights greater than 8 kDa. Furthermore, it is most likely that both SPIs and APIs responsible for anti-protease activities of bark extract are mixtures of protenaceous and non-protenaceous inhibitors exhibiting different thermal stabilities. The optimized conditions for the serine protease inhibitory assay for bark

extract of *N. beddomei* are 30 µl from 2.5% (w/v) bark extract and a pre-incubation period of 15 minutes whereas for aspartic protease inhibition assay, it is 40 µl from 10% (w/v) bark extract and 15 minutes of pre-incubation period.

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