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PURIFICATION AND PARTIAL CHARACTERISATION OF A PROTEASE INHIBITOR FROM *Mimosa diplotricha*

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ABSTRACT

Search for inhibitors to insect proteases is one of many strategies to control pests. Previous work has demonstrated successful purification of effective inhibitors from plant origin. Thus, the current study attempted to purify protease inhibitors from locally available medicinal plants. The study demonstrated that the ethanolic extracts of *Mimosa diplotricha* leaves caused a significant 80% reduction in bovine trypsin activity. The inhibitory property of the proteinaceous nature of the extract was reconfirmed through qualitative analysis using the detection of trypsin inhibitors on the SDS-PAGE technique. The ammonium precipitated trypsin inhibitor was purified using Hi-Trap G25 and resolved into a single band with a molecular weight of approximately 20.8 kDa. By using the Dixon plot the competitive inhibitor has a K_i value of 2.16×10^{-4} mM. The purified protein inhibited the protease extract of *Chrysomya megacephala* at IC_{50} of 28 µg/mL. The results highlighted the presence of trypsin inhibitor in *Mimosa diplotricha* and its potential as a pest control agent.

Key words: *Chrysomya megacephala*, *Mimosa diplotricha,* pest control, protease inhibitor

INTRODUCTION

Natural defense-related proteins called protease inhibitors (PIs) are found in the seeds and vegetative parts of the majority of plant families (Ryan, 1990; Kanwar *et al*., 2021). Induced accumulation of PIs occurs in certain tissues and organs that are sites of attack by insects, phytopathogenic microorganisms, mechanical wounding, and UV radiation (Christeller, 2005). Due to these properties, PIs have been the subject of research to explore their bioinsecticides, antimicrobial activity, and anticarcinogenic properties. The research on plant proteases started when Kunitz (Kunitz 1946) and Bowman (Bowman 1946) first purified and characterized the protease inhibitors in soybeans. Numerous studies have been conducted to comprehend PI activity toward various plant pests, resulting in the development of a variety of PI genes expressing insect-resistant transgenic (Fan & Wu 2005). A lack of necessary amino acids in the guts of many insect species is caused by the inhibition of proteases by PIs, leading to a slowing of growth and eventually death by starvation. As a result, numerous studies have shown that transgenic plants can be used to reduce the growth and development of insect pests fed on transgenic plant diets. . Since PIs genes are primary gene products, they are excellent candidates for engineering pest resistance into plants and shows the most promising outcome (Boulter 1993). Among these is transgenic white poplar that expresses an *Arabidopsis thaliana* cysteine PI that is immune to attack by the chrysomelid beetle *Chrysomela populi* (Delledonne *et al*., 2001) and oilseed rape expressing mustard trypsin inhibitor causes diamondback moth (*Plutella xylostella*) mortality (De Leo *et al*., 2002). The first successful experiment of using the PIs gene was demonstrated by Hilder *et al*., (1987) where they transferred the trypsin inhibitor gene from *Vigna unguiculata* to tobacco, which conferred resistance to a wide range of insect pests including Lepidopterans, Coleopterans, and Orthoptera. For this investigation, local plants were tested for the presence of trypsin inhibitors.

Pest control has been a major pursuit in the area of agriculture due to its economic significance. Of the many strategies, inhibition of the proteases produced in the gut of the pest of interest. Thus, it was of great interest at the same time to explore the availability of the inhibitors to the enzyme in local plants with medicinal value. Hence, the work described the screening, purification, and partial characterization of the trypsin inhibitor and its potential as an inhibitor to proteases in pests such as *Chrysomya megacephala*.

MATERIALS AND METHODS

Chemicals

All chemicals used were of the best quality

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possible unless otherwise stated. Sodium-benzoyl-DL-tyrosine p-nitroanilide (BApNA), bovine trypsin (type XIII, 1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) treated) and dimethyl sulphoxide (DMSO) were from Sigma Chemical Co., St Louis, USA. Centrifugal concentrators were obtained from Vivascience (Gottingen, Germany). Acrylamide, bis-acrylamide, and concentrated Coomassie Brilliant Blue protein reagent were obtained from BioRad Laboratories, Hercules, CA, USA. Buffer components were purchased from Sigma Chemical Co., St Louis, USA.

Preparation of plant extract and protein purification

Plant samples used in the study were *Erythrina fusca, Cassia floribunda, Delonex, Acacia mangium, Caesalpinia pulcherrima, Cassia alata, Bauhinia blakeana, Andira inermis, Mimosa diplotricha, Pterocarpus indica, Adenanthera pavomina,* and *Acacia auriculiformis.* Fresh plant parts were oven-dried at a temperature of no more than 55 °C and then ground to a fine powder. Powdered plant material was soaked in dichloromethane overnight at room temperature. The solution was filtered and the obtained precipitate was dried overnight and later macerated in 50% (v/v) ethanol while shaking at room temperature. The plant debris was removed and the filtrate was repeatedly partitioned with dichloromethane. The organic soluble fraction was discarded, and the ethanol/water layer was concentrated on a rotary evaporator before 90% ammonium sulfate precipitation. The obtained proteinaceous material was then redissolved in distilled water and lyophilized. For further purification, the diluted sample was applied onto Hi-Trap G25 connected to AKTA Prime Plus equipped with a fraction collector. The collected fractions with protease inhibitory activities were then pooled and concentrated for further studies or separated to homogeneity on precast Novex® Tricine Gel (10-20%) (Invitrogen) for visualization.

Trypsin inhibitory assay

Trypsin inhibitory assay was essential as described by Heath *et al*., (1995). Reaction mixtures contained 50 mM Tris/HCl, pH 8.0, bovine trypsin, 1 mM BApNA (Nα-Benzoyl-DL-arginine p-nitroanilide hydrochloride) in 5% (w/v) dimethyl formamide (Sigma). The inhibitor was incubated with the enzymes for 30 min at 30 °C before the addition of substrate. The inhibitory property of the inhibitor was tested at different temperatures (15 °C, 30 °C, & 60 °C). The change in absorbance at 405 nm was recorded after 10 mins using JUSCO V6300 spectrophotometer. The catalytic hydrolysis of the substrate (BApNA) was measured using the molar extinction coefficient of 10000 $M^{-1}cm^{-1}$. The percentage difference of specific activity (μg/min/mg) was calculated. Protein content was estimated as described by Bradford (Bradford 1976) with bovine serum albumin as standard.

Detection of trypsin inhibitory activity in SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was run using the buffer system of *Lemmli* (1970). For detection of inhibitor on SDS-PAGE, a 17% separating gel containing 0.1% (w/v) gelatin was prepared according to Bushell *et al*., (1983). A sample of protease inhibitor was dissolved in an aqueous solution of 2.5% (w/v) SDS, 10% (v/v) glycerol, and phenol red. Electrophoresis was carried out at constant voltage and then the gel was washed in 100 mL of 2.5% (w/v) triton X-100 for 30 mins. The washing was repeated 3 times before the gel was washed for 1 hr in distilled water. The gel was then incubated in 150 mL of 0.1M glycine-NaOH buffer, pH 8.3, containing 6 mg trypsin. Incubation was conducted at 37 °C for 16 h. After incubation, the gel was washed as described and stained. Gels were stained with Colloidal Coomassie Blue according to Neuhoff *et al*., (1988). The stained gel was scanned using Image Scanner III (GE Healthcare) which is compatible with Image Master Software. The Image Master Software was used to visualize and analyze the gel.

Mode of inhibition (K_i), $IC_{50,}$ and thermostability determination

To calculate the inhibitory constant (Ki) using Dixon plots, kinetic studies were conducted utilizing BApNA as a substrate. A variety of inhibitor concentrations were added to a set quantity of trypsin at two distinct concentrations to conduct experiments (1.0 & 5.0 mM). For each of the two substrate concentrations ([S1] and [S2]), the reciprocal velocity (1/v) was plotted against inhibitor concentrations [I] (Dixon plot). Each concentration of substrate was represented by a single regression line, and the Ki was derived from the relationship between those two lines. The value of IC_{50} was estimated by plotting the percentage of residual activity and inhibition against the concentration of the inhibitor. The intersection of both curves was the concentration at which 50% of the activity was inhibited (IC_{50}). For the thermostability test, the sample was prepared in varying incubation periods ranging from 15 °C - 90 °C for 10 min before 30 µL of BapNA in DMSO was added to it.

Preparation of protease extract from *Chrysomya megacephala*

Adult *Chrysomya megacephala* was homogenized in distilled water. Homogenates were then centrifuged at 12,000 × *g* for 10 min at 4 °C. Supernatants were used as crude enzyme extract for trypsin-like activity.

RESULTS AND DISCUSSION

Protease inhibitors (PIs) are widely distributed among bacteria, animals, and plants. We choose to focus on plants derived protease inhibitors because due to their insecticidal property that has emerged as an interesting strategy for insect pest control (Azzouz *et al*., 2005). Previous research discovered that legume plants especially from tropical rain forests contributed to a major repository of PIs diversity (Arindam & Cherukuri, 2009). We attempted to simplify the methods to screen and isolate protease inhibitors from the local plant family, namely *Leguminosae.* The study adopted a protocol for isolation of protein from plant material as described by Jennings *et al*., (2001) with slight modification. The extraction of the proteins using organic solvents successfully resulted in the isolation of proteins after ammonium sulfate precipitation. It was frequently observed that treatment with salt precipitation had caused precipitation of sticky and oily materials as well. At most times, the sticky material disappeared once the precipitated proteins were lyophilized.

Following extraction, the protein samples were screened for inhibitory activities against commercially available trypsin. As observed in Table 1, out of 12 samples screened, only three samples showed a reduction in specific activities at 50% and above. These included the extracts from *Mimosa diplotricha's* leaves, *Adenanthera pavonia's* leaves, and *Pterocarpus indicus's* leaves. Most serine protease inhibitors were isolated from this family (*Leguminosae*) (Chaudhary *et al*., 2008). Inhibitory activities were, however, absent in sample extracts originating from *Delonix regia's* leaves, *Ixora finiaysaniana,* and *Allamanda cathartica's* leaves even though there is a member of Leguminosae. Fruits of *Acacia auriculiformis, Pterocarpus indica, Andira inermis,* and *Bauhinia blakeana* were observed to have the non-detectable presence of PIs. Their leaves however indicated a considerably significant inhibition of trypsin. *The behavior is expected as* even though it was from the same plant, proteinase inhibitors are usually varied in their botanical origin and also in their effect and consequences on the storage organ, vegetative tissue, and reproductive organ within the plant (Arindam & Cherukuri, 2009).

To reconfirm their proteinaceous nature, the inhibitors were detected on SDS-PAGE as previously described. Detection of proteinaceous material with inhibitory activity was shown in Figure 1. The expectation was that the trypsin enzyme will degrade all the protein on the SDS-PAGE gels and if there is a presence of inhibitors in the selected plant's sample extract, the trypsin will not be able to hydrolyze that area, leave an intact band upon staining. Figure 1B

Table 1. Shows % inhibition of trypsin-specific activity by selected plant parts

Name of plants	plant parts	% inhibition
Erythrina fusca	leaves	44
	flower	13
Cassia floribunda	leaves	17
	fruits	8
Delonex regia	leaves	nil
	fruits	2
Acacia mangium	leaves	6
Caesalpinia pulcherrima	leaves	12
Cassia alata	leaves	nil
	fruits	nil
Bauhinia blakeana	leaves	21
	fruits	nil
Andira inermis	leaves	7
	fruits	nil
Mimosa diplotricha	leaves	nil
Pterocarpus indica	leaves	59
	fruits	nil
Adenanthera pavomina	leaves	52
Acacia auriculiformis	leaves	13
	fruits	nil

(lane 2) indicated the presence of a distinct band from *Mimosa diplotrica* leaves extract indicating the presence of protein protease inhibitors at MW lower than 31kDa. Upon ammonium sulfate precipitation and further purification using Hi-Trap G-25, the sample extract of *Mimosa diplotricha's* leaves resulted in single band resolution on SDS-PAGE at molecular weights estimated at 20.8 kD (Figure 2). *Acacia nilotica* proteinase inhibitor (AnPI) isolated by ammonium sulfate precipitation and DEAE-Sephadex A-25 was also of a similar molecular weight of approximately 18.6 kD (Babu *et al*., 2012).

The Dixon plot has been chosen as the method to determine the K_i value of the PI. Dixon plot was generated by plotting the reciprocal velocity (1/v) versus [I] (concentration of inhibitors) for both substrate concentrations (Figure 3). From the Dixon plot, it was revealed that the inhibitor exhibits a competitive inhibition characteristic when incubated with the trypsin with a calculated Ki value of 2.16×10^{-4} mM. The calculated value was far less significant in terms of its effectiveness as an inhibitor as compared to that of PI isolated from *Putranjiva roxburghi* seeds with a Ki value of 1.4×10^{-8}mM. (Chaudry *et al*., 2008).

The PI inhibitory activity was shown to increase as the reaction temperature increased from 15 °C to 60 °C. At 15 °C and 30 °C, the inhibitory activity was 70%, and 82% respectively. The optimal temperature was 60°C which gave 99% of inhibition of the trypsin. The inhibitory activity started to decrease as the reaction temperature increased higher than 60°C (data not shown). The PI isolated from *Putranjiva roxburghi* seeds has also shown that the inhibitory property was completely retained up to 70 °C and started to slightly decreased when the temperature reached above 70 °C (Chaudry *et al*., 2008). PI isolated from *Schistocerca gregaria* also showed no significant loss of activity when this inhibitor was incubated at a temperature ranging from 37 °C to 90 °C (Brillard-Bourdet *et al*., 2006). This paper proved that some serine protease inhibitors can retain their properties in a wide range of temperatures which further supports the thermostability findings of *Mimosa diplotricha* leaves that were able to retain their inhibitory activity at temperatures ranging from 15 °C-60 °C.

Chrysomya megacephala that are commonly known as blow flies are well known for being mechanical carriers of several pathogens such as viruses, bacteria, protozoa, and helminth eggs that are recorded to cause illness and disease in humans and are an annoyance to humans and agronomic livestock. Our study has found that the PI was able to inhibit 52% of trypsin-specific activity in the insect's gut. This of absolutely lower compared to the inhibitory activity that was obtained using trypsin treated from the bovine pancreas which resulted in 82%. Understandably that the protease source was not prepared for homogeneity, the finding somehow suggests the occurrence of inhibition of the protease by the isolated PI. The calculated IC_{50} value obtained in this study was 28 µg/mL (data not shown). This showed a promising future for the use of the isolated PI as a strategy for insect pest control, especially against flies such as *Chrysomya megacephala*.

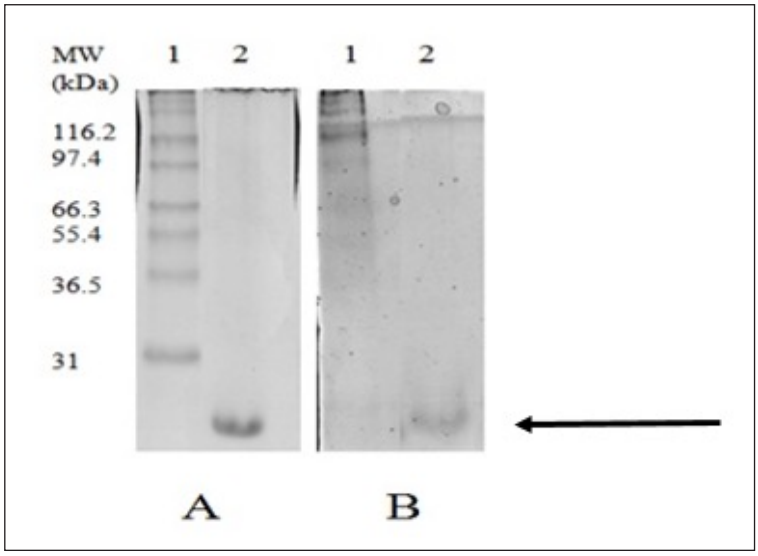

Fig. 1. SDS-PAGE indicates the detection of trypsin inhibitory activity of the *Mimosa diplotricha* extract. Gel A was untreated with protease while gel B was with treated protease. Lane 1 of gel A and B are markers and bands in lane 2 in gel B (after treatment with protease) indicate the presence of inhibitory material at MW below 31 kDa. The gel was stained with coomassie blue.

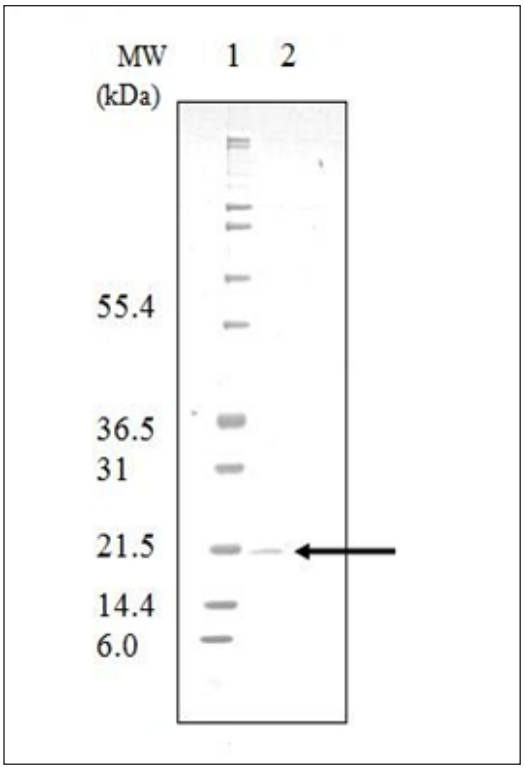

Fig. 2. Tricine gel (10-20%, Invitrogen) electrophoresis indicates the presence of protease inhibitor purified from *Mimosa diplotricha* leaves. Lane 2 indicates the pure PI migrated at MW 20.8 kDa (indicated by arrow). Lane 1 is Mark12 Marker (Invitrogen).

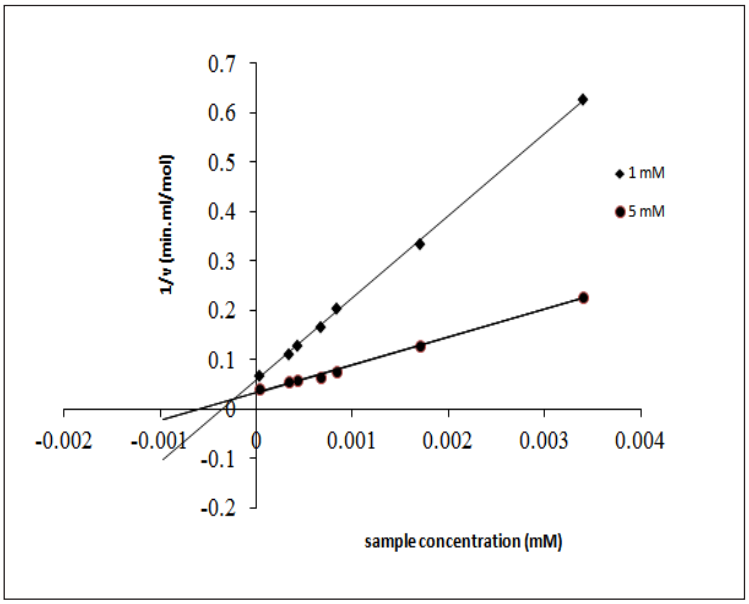

Fig. 3. The Dixon plot shows the intersection of the linear plot generated from two substrate concentrations.

CONCLUSION

Our work has continued into looking at the detailed biochemical and biophysical aspects of the inhibitor. Early attempt to identify the identity of the protein has not resulted in reliable outcome using MALDI-tof mass spectrometry. Thus, further, work to characterize needs to be done especially in detail inhibitory properties of the inhibitor against the enzyme. The work, however, has shown simplicity in screening and isolating a PI from a local plant, to suggest the potential of the plants as a source of protease inhibitors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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