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 a pest control agent.

MATERIALS AND METHODS

Chemicals

All chemicals used were of the best quality...
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 (Göttingen, Germany). Acrylamide, bis-
acylamide, 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 diploptricha,
Pterocarpus caprus, Adenanthera pavonina, 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 redisolved
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
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.

Detection of trypsin inhibitory activity in SDS-
PAGE
Sodium dodecyl sulfate-polyacrylamide gel
electrophoresis (SDS-PAGE) was run using the buffer
system of Lemml (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 (K_i) using
Dixon plots, kinetic studies were conducted utilizing
BAPNA as a substrate. A variety of inhibitor
centrationes were added to a set quantity of trypsin
two distinct concentrations to conduct experiments
(1.0 & 5.0 mM). For each of the two substrate
centrations ([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 K_i
value 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 pavonina’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 finiysaniana, 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>
<thead>
<tr>
<th>Name of plants</th>
<th>plant parts</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrina fusca</td>
<td>leaves</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>flower</td>
<td>13</td>
</tr>
<tr>
<td>Cassia floribunda</td>
<td>leaves</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>fruits</td>
<td>8</td>
</tr>
<tr>
<td>Delonex regia</td>
<td>leaves</td>
<td>nil</td>
</tr>
<tr>
<td></td>
<td>fruits</td>
<td>2</td>
</tr>
<tr>
<td>Acacia mangium</td>
<td>leaves</td>
<td>6</td>
</tr>
<tr>
<td>Caesalpinia pulcherrima</td>
<td>leaves</td>
<td>12</td>
</tr>
<tr>
<td>Cassia alata</td>
<td>leaves</td>
<td>nil</td>
</tr>
<tr>
<td></td>
<td>fruits</td>
<td>nil</td>
</tr>
<tr>
<td>Bauhinia blakeana</td>
<td>leaves</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>fruits</td>
<td>nil</td>
</tr>
<tr>
<td>Andira inermis</td>
<td>leaves</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>fruits</td>
<td>nil</td>
</tr>
<tr>
<td>Mimosa diplotricha</td>
<td>leaves</td>
<td>nil</td>
</tr>
<tr>
<td>Pterocarpus indica</td>
<td>leaves</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>fruits</td>
<td>nil</td>
</tr>
<tr>
<td>Adenanthera pavonina</td>
<td>leaves</td>
<td>52</td>
</tr>
<tr>
<td>Acacia auriculiformis</td>
<td>leaves</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>fruits</td>
<td>nil</td>
</tr>
</tbody>
</table>
(lane 2) indicated the presence of a distinct band from *Mimosa diplotricha* leaves extract indicating the presence of protein protease inhibitors at MW lower than 31 kDa. 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* protease 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\textsubscript{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 K\textsubscript{i} value of 2.16 × 10\textsuperscript{-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 K\textsubscript{i} value of 1.4 × 10\textsuperscript{-3}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 I\textsubscript{C50} 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.
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.

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.
PROTEASE INHIBITOR FROM Mimosa diplotricha

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES


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