Identification of plant extracts expressing trypsin inhibitor

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ABSTRACT

Plant protease inhibitors (PPIs) are compounds which play a potent defensive role against pest and pathogens, thereby protecting the plants. In this work we tested different plant extracts to identify extracts containing inhibitor against trypsin activity. Plant extracts were made by homogenizing soaked leaves/seeds/root modification of plants in bicarbonate buffer, pH 9.0.The homogenates were centrifuged at 10,000 rpm at 4°C for 10 minutes. The supernatant containing the soluble proteins were used for protease inhibition assay. Protease inhibition assay was carried out in a total volume of 1 ml containing 67 µl trypsin (Bovine), 25 µl plant extract, 511 µl Tris buffer, 67µl NaCl and 330 µg Nα-Benzoyl-DL-Arginine-P-Nitro Anilide (BAPNA) as substrate. On screening 19 extracts from different plants, we found 9 of them inhibited trypsin activity greater than 40%. The percentage inhibition of these plants are *Garcinia xanthochymus* (96.7± 0.4 %)*, Datura stramonium* (86.1±1.6%), *Ricinus communis* (74.05±0.45%), *Phyllanthus amarus* (72.05±2.15%)*, Santalum album* (64.4±1.7 %), *Plectranthus ambonicus* (62.15±2.45%), *Croton hirtus* (55.85±0.9%), *Zea mays* (48±2.1%) and *Prunus cerasifera* (45.38±0.125%). Of these, to our knowledge no trypsin inhibitor was reported from *Garcinia xanthochymus, Datura stramonium, Plectranthus ambonicus, Prunus cerasifera*, *Phyllanthus amarus*, *Santalum album*, and *Croton hirtus.* As trypsin is a serine protease, a predominant class of protease in the gut of lepidopteran larvae, this screening will be useful to identify extract containing protease inhibitor against the gut protease of lepidopteran pests. Protease inhibitors from these extracts can be exploited for control of lepidopteran pests.

Keywords: trypsin, proteases, plant protease inhibitors, *Garcinia xanthochymus, Datura stramonium*

INTRODUCTION

Protease inhibitors (PIs) are molecules that inhibit the function of proteases. Many naturally occurring PIs are proteins. In seeds and tubercles about 10% of their total protein is represented by protease inhibitors [1-3]. These plant protease inhibitors (PPIs) play essential roles in biological systems such as regulating proteolytic processes, and participate in defence mechanisms against attack by insects, fungi, and other pathogenic microorganisms.

Plant protease inhibitors were first reported more than 65 years ago. Soybean trypsin inhibiter was crystallised by Kunitz in 1947 [4]. This inhibiter was the main tool for studies that helped to understand the mechanism of PI interaction for the majority of serine proteases. The PIs binds to the active site on the enzyme to form a complex with a very low dissociation constant. A binding loop on the inhibitor, usually "locked" into conformation by a disulphide bond, projects from the surface of the molecule and contains a peptide bond which can be cleaved by the enzyme [5,6]. This peptide

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bond may be cleaved in the enzyme inhibitor complex but the inhibitor remains with the enzyme. The inhibitor thus directly mimics a normal substrate of the enzyme [6]. The specificity of the inhibitor enzyme interaction is primarily determined by the specificity of proteolysis determined by the enzyme [7].

The digestive proteolytic enzymes in the different orders of commercially important insect pests belong to one of the major classes of proteinases predominantly. Coleopteran and hemipteran species utilizes cysteine proteinases [8] where as lepidopteran, hymenopteran, orthopteran and dipteran species mainly make use of serine proteinases [1,9]. There are many examples to show that both of these classes of proteinases are inhibited by plant proteinase inhibitors [10]. But inhibition of insect gut proteinase by PPIs is often circumvented by insects through developing resistance to the PPIs. In many cases there are two populations of digestive enzymes in target pests, one susceptible to inhibition and the other resistant [11,12] . Some insects respond to ingestion of PPIs like soybean trypsin inhibitor [13] and oryzacystatin [11] by hyper-producing inhibitor-resistant enzymes. In plants over-expression of both endogenous and exogenous inhibitors against insect pest have been done. Although significant protection against insect pests has been routinely achieved, these genetically modified plants do not show levels of resistance considered commercially viable. A variety of strategies have been tried to improve effectiveness of proteinase inhibitors towards pests, including mutagenesis for novel inhibitory activity, and engineering inhibitors with multiple functions. As proteinase inhibitor alone is insufficient to offer enough protection, they are being used in transgenic crops in combination with other insecticidal genes. For example in genetically engineered cotton varieties which express Bt toxins as an insecticidal protein against lepidopteran larvae, the CpTI (cowpea trypsin inhibitor) gene has been employed as a second transgene to improve protection [14]. Thus for better insect control using PPIs, there is a need for developing better PPIs which are less prone to resistance. In this article we screened several plants to identify extracts containing inhibitor against trypsin. As the predominant proteinase in the gut of lepidopteran pests is trypsin-like serine proteases, this screening will be helpful to identify PPIs which are useful in the control of lepidopteran insect pests.

MATERIALS AND METHODS

Nα-Benzoyl-DLArginine-P-NitroAnilide (BAPNA) was purchased from Sigma-Aldrich Corporation, USA. Trypsin was obtained from HIMEDIA, India. Other reagents used were of analytical grade.

Collection of plant parts and preparation of extracts

Plant parts (seeds, fruit, leaves, root modification) were collected from Kottakkal, Malappuram, Calicut University Park, Calicut and Bangalore. The plant parts were soaked in bicarbonate buffer, pH 9.0 (1ml/g tissue) overnight and then ground using mortar and pestle. The homogenates were centrifuged at 10,000 rpm in a Rota 4R-V/FA, Plasto Crafts centrifuge for 10 minutes at 4°C.The soluble proteins recovered from the supernatant (inhibitor) were used for protease inhibition assay.

Protease assay

The protease assay was carried out in a total volume of 1 ml, by mixing 67 µl bovine trypsin (0.125 mg /ml; dissolved in 1mM HCl), 67 µl of 0.9% NaCl, 536 µl Tris buffer (200 mM Tris; 20 mM Calcium chloride, pH 7.8) and 330µl Nα-Benzoyl-DLArginine-P-NitroAnilide (BAPNA)

(1mg/1ml) as substrate. A blank was also done along with it by adding 67 µl 1mM HCl, instead of trypsin.

Protease inhibition assay

In the protease inhibition assay, 25 µl of plant extract was pre-incubated with 67 µl of trypsin (0.125 mg /ml) for 10 minutes, followed by addition of 67 µl NaCl (0.9%) and 511 µl Tris buffer. The reaction was started by adding 330µl BAPNA (1mg/1ml). Proteolytic activity was measured by continuous spectrophotometric rate determination method using UV Spectrophotometer, by recording the increase in absorbance at 405nm for 5minutes. All assays were done in duplicate and percentage of inhibition calculated by taking the activity in presence of the enzyme alone as 100%.

RESULTS AND DISCUSSION

Extracts from parts (seeds/fruit/leaves/root modification) of 19 different plants were used to test their capacity to inhibit trypsin activity. Out of these, 9 plant extracts showed inhibition greater than 40 % (Table 1). Of the different extracts tested, the seeds of *Garcinia xanthochymus* showed the highest inhibitory activity (96.7±0.4%). It has been shown that the leaf of *G. xanthochymus* has potent antioxidant activity which was similar to that of the standard antioxidant BHT [15]. To our knowledge no trypsin inhibitor is reported from the seeds of *G. xanthochymus*. Seeds of *Datura stramonium* exhibited 86.1±1.6 % inhibition followed by extract from *Ricinus communis* seeds (74.05±0.45%). Crude protease inhibitor has been isolated from *Ricinus communis* [16].

Table 1. List of plants showing greater than 40% inhibition.

Name of plant	Common name	Plant part Used	Mean % inhibition± SE
Garcinia xanthochymus	Sour mangosteen	Seed	96.70± 0.40
Datura stramonium	Thorn apple	Seed	86.10±1.60
Ricinus communis	Castor oil seed	Seed	74.05±0.45
Phyllanthus amarus	Carry me seed	Fruit	72.05±2.15
Santalum album	Sandal	Leaf	64.40±1.70
Plectranthus ambonicus	Indian borage	Leaf	62.15±2.45
Croton hirtus	Hairy croton	Seed	55.85±0.90
Zea mays	Maize	Seed	48.00±2.10
Prunus cerasifera	Red plum fruit	Seed	45.38±0.13

Extract from the fruits of *Phyllanthus amarus* inhibited the trypsin activity by 72.05±2.15 %. The extracts and the compounds isolated from *P. amarus* show a wide spectrum of pharmacological activities including antiviral, antibacterial, antiplasmodial, , antimalarial, antimicrobial, antioxidant properties [17]. To our knowledge no trypsin inhibitor is reported from this fruit. The leaf extracts of *Santalum album* and *Plectranthus ambonicus* also inhibited trypsin activity to the extent of 64.4±1.7 % and 62.15±2.45 % respectively. Extract from the seeds of *Croton hirtus* showed 55.85±0.9 % inhibition of trypsin activity. To our knowledge no trypsin inhibitor is reported from these plants.

The extent of inhibition was similar for *Zea mays* and *Prunus cerasifera*, 48±2.1 % and 45.38±0.125 % respectively. No trypsin inhibitor from the seeds of *Prunus cerasifera,* was reported. Extract from *Zea mays* was reported to contain trypsin/factor XII A inhibitors. It is a potent inhibitor

of mammalian trypsin and a specific inhibitor of factor XII A. It belongs to the Cereal trypsin/Alpha-amylase inhibitor family [18].

Trypsin inhibitor has been reported from different plants. Soybean trypsin inhibitor was the first PI isolated and characterized [1]. This trypsin inhibitor was shown to be toxic to the larvae of the flour beetle (*Tribolium confusum*) [19]. A trypsin inhibitor has been reported from sunflower (*Helianthus annuus*) called sunflower trypsin inhibitor 1 (SFTI-1) [20,21]. In studies using *in vitro* assays against insect gut proteases, Chickpea varieties showed inhibitory activity against the gut protease of *Helicoverpa armigera* [22]. The expression of some of the PIs are induced by pest or pathogen attack. For example the expression of rice Bowman Brick Inhibitor (BBI) from *Oryza sativa* is up regulated and induced by pathogens or insects during germination of rice seeds [23]. Feeding experiments proved that diet incorporated with proteinase inhibitor retards larval growth in insect pests. Soybean trypsin inhibitor and potato proteinase inhibitor II when incorporated into an artificial diet; both proteinase inhibitors significantly reduced the growth and development of the *Heliothis zea* and *Spodoptera exiqua* larvae [13].

As plant proteinase inhibitors have been shown to act as defensive compounds, their genes encoding for proteinase inhibitor were isolated and incorporated in transgenic crop plants. Growth of *Manduca sexta* larvae (tobacco hornworms), feeding on leaves of transgenic plants expressing tobacco inhibitor II significantly retarded the growth of the larva [24]. Genetically modified crops (GM crops) expressing plant proteinase inhibitors are more advantages than GM crops expressing toxins like Bt toxin, because proteinase inhibitors are naturally present in plants and their toxic effect on humans is less likely.

But GM crops expressing proteinase inhibitors are not very successful as the insects feeding on it gets adapted to it by secreting new proteases insensitive to plant proteinase inhibitors or digest the proteinase inhibitors itself. For circumventing this problem it is essential to identify more potent and novel protease inhibitors to which insects will not develop resistance very easily. Another approach is to modify the existing PPIs to make them less vulnerable.

Hence, plant extract showing high inhibition against trypsin activity reported in this study will be useful in insect control as the major protease in lepidopteran pests is trypsin-like serine proteases. Thus the reported trypsin-inhibitor containing extracts may be used to test on lepidopteron gut extracts or pests so as to establish their potential for insect pest control. Studies in this line are ongoing in our laboratory.

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