J. Plant Biochemistry & Biotechnology Vol. **10**, 75 - 78, January 2001

Short Communication

Characterization of Serine Proteinase Inhibitor from Subabul (*Leucaena leucocephala* Lam) Seeds

P Nandeesha and D Theertha Prasad*
Department of Biotechnology, College of Basic Sciences and Humanities, University of Agricultural Sciences, GKVK, Bangalore 560 065, India

Partially purified subabul trypsin inhibitor (STI) showed high level of thermotolerance and pH stability with a molecular weight of ~15 kD. Bioassay results showed that STI is a strong inhibitor of *Helicoverpa armigera* larval gut proteinases. *In vitro* feeding experiments revealed 40% mortality in inhibitor fed larvae followed by 12 days extension in larval growth period and significant reduction in pupal weight. Differential activity staining for the larval gut proteolytic enzymes did not show any difference in the isoprotease pattern between the control and the larvae fed with STI.

Key words : proteinase inhibitor, legumes, *Leucaena leucocephala, Helicoverpa armigera.*

Proteinase inhibitors are among the defensive chemicals in plant tissues which are developmentally regulated and also induced in response to insect and pathogen attacks. The proteinase inhibitors found in plants are specific to each of four classes of proteolytic enzymes viz Serine-, Aspartic-, Cysteine- and Metalo- proteinases. The double-headed bifunctional inhibitors are known to inhibit both proteinases and amylases and are called α-amylase / proteinase inhibitors (1-3). Proteinase inhibitor and α-amylase inhibitor genes have provided novel systems for investigating the fundamental processes that underline the environmental and developmental regulation of natural defence system in plants. Attempts are now being made to engineer proteinase inhibitors and/or α-amylase inhibitor genes, either individually or in combination with other defensive genes for analysis of their effectiveness in enhancing defence against insects and pathogens (4-6). In the present study characterization of a serine proteinase inhibitor from subabul has been done.

Different cultivated legume seeds *viz* red gram (*Cajanus cajan*) *cv* GT1, TTB-7 and vegetable purpose, green gram (*Phaseolus aureus*) *cv* PDM90-1, 84-13, ICTP 8203, PRB-7 and ML-575, black gram (*Phaseolus mungo*), chickpea (*Cicer arietinum*), rice bean-KHRB-1 (*Vigna umbellata*), grain amaranth (*Amaranthus viridis*) and soybean (*Glycine max*), and wild species *viz. Cassia uniflora*, subabul (*Leucaena leucocephala*), *Crotolaria* sp, custard apple (*Anona squamosa*), *Erithrina* sp, *Mucuna pruriens*, neem (*Azadaricta indica*), copper pod (*Peltoforum* sp) camel foot tree (*Bauhinia* sp), rain tree (*Samania* sp) and *Teporosia* sp were collected from GKVK and western ghat area. The seeds were ground to fine powder and defatted with petroleum ether (40-60°C). The defatted flour was extracted with 0.1M potassium phosphate buffer, pH 7.6 (1:10 w/v) for one h at room temperature and centrifuged at 12,000 rpm for 30 min at 4°C. The supernatant, representing total soluble proteins (TSPs), was incubated at 70°C for 10 min, followed by further incubation on ice bath for 30 min. Centrifuged at 12,000 rpm for 30 min at 4°C. The supernatant having heat stable proteins (HSPs), was collected and assayed for protein content and trypsin, papain and pepsin inhibitory activity (7-10).

To partially purify subabul trypsin inhibitor (STI), pH of the TSPs was adjusted to 4.5 using 1M citric acid and incubated at 70°C for 10 min. The supernatant (subabul HSPs) after centrifugation at 12,000 rpm for 30 min at 4°C, dialyzed against distilled water overnight at 4°C and lyophilized. The HSPs dissolved in the elution buffer (0.1 M potassium phosphate buffer, pH 7.6 containing 0.02% sodium azide) were fractionated on Sephadex G-50 column (1.2 x 45 cm) pre-equilibrated with same buffer. 2.0 ml fractions were collected at 10 ml per h and the elution profile was monitored for protein by measuring the absorbance at 280 nm and for trypsin inhibitory activity. The active fractions were pooled, dialyzed against distilled water and lyophilized.

* Corresponding author. E-mail: dtprasad@132india.com

In thermal stability studies, the partially purified inhibitor was incubated at temperatures ranging from 40 to 100°C for 10 min and for pH stability, the inhibitor was incubated in 0.01 M buffer with pH ranging from 3.0 to 10.0 at room temperature as well as at 70°C for 10 min and the residual inhibitory activity was measured. Partially purified STI was subjected to SDS-PAGE (11) and for trypsin inhibitor activity staining on polyacrylamide gel (12). After native-PAGE, the gel was equilibrated with 0.1 M potassium phosphate buffer (pH 7.4), incubated in trypsin solution (100μg/ml) for 30 min followed by incubation in 10 ml substrate solution solution containing 2.5 mg of acetyl-DL-phenylalanine β-napthyl ester and 0.55 mg/ml fast blue RR in 0.1M potassium phosphate buffer, pH 7.4. The inhibitor bands were visualized as white bands in the pink background. Further, the gel was cut into 0.5 cm bits and the protein from the gel bits was extracted and assayed for the trypsin inhibitory activity using casein as substrate.

The partially purified STI was mixed with the basic *Helicoverpa* diet (13) at three levels, 5,000, 10,000 and 20,000 TIU/ml of diet. 5-day-old larvae of mean weight 0.86 mg (Ten each) were fed on control diet and diet with STI. The growth rate in terms of body weight at every three days intervals; number of days required for pupation, pupal weight and % mortality rate of the larvae were recorded. Crude extract from the *Helicoverpa* larval gut was prepared by homogenizing the gut tissue (1:5 w/v) in 0.1M potassium phosphate buffer, pH 7.6 at 4°C, and centrifuged at 12,000 rpm for 15 min at 4°C. 100 μg of protein in the supernatant was used for determination of proteolytic activity and to determine inhibitory activity of STI at pH 3.0, 7.6, 8.5 and 10 using BSA and casein as substrates respectively. Further the gut extract was separated on native PAGE and stained for the proteinase activity using acetyl-DL-phenylalanine β-napthyl ester and fast blue RR (12).

The highest protein content in both TSPs and HSPs was recorded in *Crotalaria* sp (295 and 278 mg/g flour) and lowest in red gram-GT7 (15.75mg/g flour) for HSPs and grain amaranth (39.6 mg/g flour) for TSPs respectively. The highest trypsin inhibitor activity (TIA) was observed in *Samania* sps (4880 and 4130 TIU) followed by *Peltoforum* sp (4570 and 3190 TIU), *Erithrina* sp (4470 and 4810 TIU), *Mucuna pruriens* (4570 and 3190 TIU), *Teporosia* sp (4630 and 3790 TIU), Subabul (4290 and 4150 TIU) and custard apple (3250 and 3520 TIU) and the lowest activity was observed in grain amaranthus (410 and 640 TIU) for TSP and HSP fractions respectively. The highest papain inhibitory activity (PIA) was observed in green gram (PRB-7 4900 and 3900 PIU), followed by *Crotolaria* (3900 and 5400 PIU), neem (2400 and 2900 PIU), grain amaranth (5300 and 3000 PIU) and lowest activity was found in red gram (170 and 300 PIU) for TSPs and HSPs respectively.

The highest pepsin inhibitory activity (PelA) was found in red gram (3100 and 2400 PelU) followed by *Crotolaria* sp, (2200 and 2100 PelU), grain amaranthus (2000 and 1000 PelU), *Cassia uniflora* (200 and 1700 PelU) and lowest activity was found in green gram (70 and 110 PelU) for TSPs and HSPs respectively. There was no pepsin inhibitory activity observed in *Samania* sp, *Peltoforum*, *Teporosia and Bauhinia* sp. There was negative correlation observed with trypsin and pepsin inhibitory activity and TSPs, whereas papain inhibitory activity showed positive correlation. In case of HSPs there was negative correlation with protein content and trypsin inhibitory activity but positive correlation with papain and pepsin inhibitory activity.

The partially purified STI using thermal denaturation at pH 4.5 followed by gel filtration chromatography showed 6.6 fold purification. SDS-PAGE analysis of STI showed two major bands with molecular weight~13 and ~15 kD. The inhibitor was also visualized by specific stain for trypsin inhibitor, using acetyl-DL-phenylalanine β-naphthyl ester/fast blue RR substrate mixture, as unstained band against the pink background (data not shown). HSP proteins showed large smear with two fast moving inhibitor bands and the quantitative data revealed that 15 kD band had the highest specific inhibitory activity against trypsin.

STI was found to be highly stable up to 80°C, and possesses only 50% and 20% of activity at 90°C and 100°C respectively, and also stable over wide range of pH from 3.0 to 12.0 both at room temperature and at 70°C for 10 min. These results are on par with proteinase inhibitors reported from jackfruit seeds (JSTI)-stable up to 100°C and pH from 3.0 to 12.0 (14), and rice bean proteinase inhibitor-stable up to 100°C pH range 6.0 to 10.0 but not at acidic pH (15). STI molecular weight as determined by SDS-PAGE analysis was ~ 15 kD. STI seems to be a Bowman-Birk type inhibitor, stable at wide range of pH and temperature, although the molecular weight is slightly higher (8-10 kD), which is not unusual to this class of inhibitors (16).

Table 1. The effect of subabul trypsin inhibitor on growth parameters of *Helicoverpa armigera* larvae

Age of larvae (days)	Mean of the survived larval weight in mg				F Value	CD at 5%
	Control	T_1 (5000 TIU/ml diet)	T_2 (10000 TIU/ml diet)	T_3 (20000 TIU/ml diet)		
9	58.80 (10)	43.40 (10)	39.80 (10)	35.00 (10)	NS	
12	242.20 (10)	140.20 (8)	136.20 (6)	111.80 (8)	*	36.09
15	290.80 (10)	224.00 (8)	160.00 (6)	126.00 (8)	*	74.85
18	480.40 (10)	314.60 (8)	198.00 (6)	171.20 (8)	*	83.91
21$	–	385.60 (8)	255.00 (6)	206.00 (6)		–
24$	–	321.40 (8)	207.40 (6)	156.80 (6)		–
327$	–	–	189.50 (6)	156.00 (6)		–
Pupal weight	296.4(5)	249.20 (8)	195.80 (6)	82.00 (6)	*	19.91
Mortality	0.0	1/5 (20%)	2/5 (40%)	2/5 (40%)		
Extended larval growth by#		6 days	10 days	12 days		

Note : The values given in the table are mean of 10 observations; the value in the parentheses indicates the no. of larvae survived at that particular day
$ - Statistical analysis is not done.
# - Larval growth extended in comparison with the control.
NS - non significant,
* - Significant at 5%.

The results on bioassay of STI on *Helicoverpa armigera* larval development showed significant reduction in mean larval weight with mortality to an extent of 40% (Table 1). The larval duration was extended by 5, 10 and 12 days in T1-T3. The lowest pupal weight of 82.0 mg was observed in the T3 as against 296.4 mg in the control. Prolongatin in larval duration, growth retardation and mortality of *Helicoverpa armigera* and *Lacanobia oleracea* has been reported with the soybean Kunitz trypsin inhibitor and soybean Bowman-Birk trypsin-chymotrypsin inhibitor (17, 18). Our studies indicated that *Helicoverpa armigera* midgut has appreciable amount of trypsin like activity. The highest gut proteolytic activity was observed at pH 10, whereas the highest inhibitory activity was observed at pH 7.6 (69% of gut proteolytic activity). Earlier reports indicate *de novo* synthesis of the midgut proteinases in the insects (*H. zea, H. armigera, Plutella xyloslella, Lymenthria dispar, Spodoptera litura*) fed with varying levels of proteinase inhibitors in the artificial feed (19, 20). However, the feeding experiments on *H. armigera* using STI showed no detectable variation in the isoprotease pattern among the control and STI fed samples (data not shown). Hence, the reduction in growth of the larva fed with STI may be attributed to the inhibiton of gut proteolytic enzymes, limiting supply of the essential amino acids required for its growth. In conclusion, based on our results we opine that the STI is a typical member of serine proteinase inhibitors, which shows very good inhibitory activity against *H. armigera* mid gut proteolytic enzymes. Therefore, efforts are being made to purity STI to homogeneity and to isolate the gene encoding for STI for its exploitation to develop transgenic plants against devastating polyphagous pest, *H. armigera.*

Received 28 September, 2000; revised 26 December, 2000.

References

1 **Garcia-Olmedo F,** *Oxf Surv Plant Mol Cell Biol,* **4** (1987) 275.

2 **Geugen J & Cerletti P,** In *New and developing sources of food proteins* (BSF Hudson, Editor), Champan & Hal, London (1994) p 146.

3 **Richordson M**, *Methods Plant Biochem,* **5** (1991) 259.

4 **Laskowski M Jr & Kato I,** *Ann Rev Biochem,* **49** (1980) 593.

5 **Ryan CA,** *Ann Rev Phytopathol*, **28** (1990) 425.
6 **Gatehouse LN, Shanon AL & Burgess EPJ,** *Insect Biochem Mol Biol*, **27** (1997) 929.
7 **Kunitz M,** *J Gen physiol*, **30** (1947) 593.
8 **Arnon R,** *Methods Enzymol*, **19** (1970) 226.
9 **Herriot RM,** *Methods Enzymol*, **4** (1955) 3.
10 **Kakade ML, Simons N & Liener IE,** *Cereal Chem*, **46** (1969) 518.
11 **Laemmli UK,** *Nature*, **227** (1970) 680
12 **Filho JX & Moreira RDA,** *Anal Biochem*, **84** (1978) 296.
13 **Veerareddy CG & Bhattacharya AK,** *J Inset Sci*, **3** (1990) 23.
14 **Annapurna SS, Ramadoss CS & Prasad DS,** *J Sci Food Agric*, **54** (1991) 605.
15 **Maggo S, Malhotra SP, Dhawan K & Singh R,** *J Plant Biochem Biotech*, **8** (1999) 61.
16 **Hajela N, Pande AH, Sharma S, Rao DN & Hajela K,** *J Plant Biochem Biotech*, **8** (1999) 57
17 **Johnston KA, Gatehouse JA & Anstee JH,** *J Insect Physiol*, **39** (1993) 657.
18 **Gatehouse AMR, Norton E, Davidson GM, Babbe SM, NeWell CA & Gatehouse JA,** *J Insect Physiol*, **45** (1999) 545.
19 **Bown DP, Wilkinson HS & Gatehouse JA,** *Insect Biochem Mol Biol*, **27** (1997) 625.
20 **Jongsma MA & Bolter C,** *J Insect Physiol*, **43** (1997) 885.