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RESEARCH ARTICLE

Purification of a Trypsin Inhibitor from *Cocculus hirsutus* and Identification of Its Biological Activity

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Abstract

Proteinase inhibitors play a significant role in plant defense against insect pests and phytopathogens by inhibiting their proteases. A thermotolerant monomeric trypsin inhibitor with molecular weight ~18kD was purified from *Cocculus hirsutus* (ChTI) using trypsin sepharose affinity column. Western blot analysis using ChTI IgY revealed its presence in vegetative parts and seeds. The second and third instar larvae of *H.armigera* fed with ChTI (5000TIU/ml) resulted in 84.59 and 58.71% reduction in mean larval weight respectively. An increase in the larval growth period was observed in ChTI fed larvae at all instars and inhibitor fed larvae could not complete their life cycle. ChTI caused 74 and 59.53% inhibition of bovine trypsin and *Helicoverpa* gut proteases respectively. ChTI exhibited strain specificity and inhibited growth and development of plant fungal pathogens. Bioassay studies on yeast strains indicated that ΔYNK and MNN1 are more sensitive to ChTI. The results suggest that phosphodiester linkage in cell wall components is likely to be the key determinants for binding of ChTI. Taken together, these studies indicate that ChTI is a potential candidate for development of transgenic plants against foliar diseases and insect pests.

Key words: *Cocculus hirsutus*, Menispermeaceae, serine proteinase inhibitor, Trypsin inhibitor, ChTI (*Cocculus hirsutus* trypsin inhibitor)

Introduction

Plants accumulate proteins during their growth and development to defend against the attacks of insects or other mechanical insults. These defensive proteins include thaumatin, osmotins, lectins, and protein proteinase inhibitors (PIs) (Chrispeels and Raikhel 1991; Monteiro et al. 2003; Moura and Ryan 2001) and some of these are inducible by insect damage, pathogen attack and physical injury (Bishop et al. 1984; Ryan et al. 1981). The defensive role of plant PIs relies on the inhibition of proteinases present in insect guts or secreted by microorganisms, thus causing a reduction in the availability of amino acids necessary for their growth and development (De Leo et al. 2002). Most PIs interact with their target proteases, resulting in the formation of stable protease-inhibitor complex (Norton 1991). Protease inhibitors have an enormous diversity of function by regulating the proteolytic activity of their target proteinases (Leung et al. 2000), especially serine proteinase inhibitors. Two major serine classes of proteinase inhibitors have been studied extensively in plants: PIs of high molecular weight with low cystein content are termed as Kunitz type (Odani and Ikeneka 1973) and Bowman-Birk inhibitors (BBIs) are cystein rich proteins of about 8 to 16kD with extensive disulphide bonds (Ryan 1990). Most of the plant serine PIs are characterized from Gramineae, Poaceae, Leguminosae, Fabaceae, Cruciferae, Liliaceae and Solanaceae families (Brzin and Kidric 1995; Deshimaru et al. 2003; Ferry et al. 2005; Johnson et al. 2006; Lin et al. 2006; Pompermayer et al. 2001). *Cocculus hirsutus* (Menispermeaceae) is a climber and its roots and leaves have unique medicinal properties (Badole et al. 2006; Nayak et al. 1993), mainly for its antidiabetic function (Raja et al. 2008; Sangameswaran and Jayakar 2007). It is relatively less prone to insect-pests and foliar diseases caused by fungal pathogens, making it an attractive candidate for the study of plant defense systems. The present study reports on the characterization of a serine proteinase inhibitor from *C.hirsutus* and its bioassay against insects and

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phytopathogens. This is the first Kunitz-type inhibitor identified from Menispemeaceae family.

Materials and Methods

Cocculus hirsutus leaves were collected from the Botanical Garden, University of Agricultural Sciences, Bengaluru, India. *Helicoverpa armigera* and *Spodoptera littoralis* larvae were obtained from Biological Control Laboratory, PCI, Bengaluru, India. Yeast (*Saccharomyces cerevisiae*) strains W303a, BWG7 and their mutants ΔYNK, MNN1, MNN4 and MNN6 were used in bioassay. The fungal isolates (*Fusarium oxysporum, Bipolaris maydis, Phonopsis convolvulus, Phoma pory, Alternaria helianthi, Metarhizium anisopliae, Verticilium dahlia, Drechelera turcium, Bipolaris oryzae, Alternaria alternata, Aspergillus flavus, Colletotrichum capsici, Fusarium solani, Rhizoctonia oryzae and Sclerotia* sp) were provided by the Department of Plant Pathology, University of Agricultural Sciences, GKVK, Bengaluru, India.

Extraction and purification of ChTI

Cocculus hirsutus leaf acetone powder was stirred with extraction buffer (50mM Tris buffer pH7.6 containing 50mM EDTA, 25mM ascorbic acid, 10mM β-mercaptoethanol and 100mg polyvinylpyrrolidone (PVPP)/g of powder) in 1:20 (W/V) for 10min at 4℃. The slurry was centrifuged at 12,000rpm for 20min at 4℃. Supernatant containing total soluble proteins (TSPs) was subjected to thermal denaturation at 70 ℃ for 10 min; snap chilled for 30min on dry ice, centrifuged at 12,000rpm for 20min at 4℃ to remove the precipitated proteins. The clear supernatant having heat stable proteins (HSPs) was assayed for trypsin inhibitory activity (TIA).

Trypsin-Sepharose affinity column was saturated with repeated of HSP fraction in wash buffer (40mM Tris, 10mM $CaCl_2$, pH 7.6). The column was washed thoroughly to remove unbound proteins. The inhibitor was decoupled with 0.2N HCl (pH 3.0). The pH of the eluent was adjusted to 7.6 with 2N NaOH immediately, assayed for TIA and analyzed on SDS-PAGE (Laemmli 1970).

Trypsin Inhibition Assay (TIA)

TIA was carried out using 1% casein as substrate (Kakade et al.1969) and absorbance of TCA soluble products was measured at 280nm. The inhibitory activity was calculated as the difference between the proteolytic activity with and without inhibitor. In gel TIA staining was carried out after separating the protein samples on 10% PAGE. The gel was incubated with PPB (0.1M potassium phosphate buffer pH7.4) for 10min followed by incubation with trypsin solution (100μg trypsin/ml PPB) for 30min at 37℃. After sequentially washing with distilled water and PPB, the gel was incubated with 10ml of substrate solution containing 2.5mg of acetyl-DL-phenylalanine-β-napthylester and 0.55mg/ml fast blue RR in PPB, till color development (Filho and Moreira 1978).

Thermal and pH stability

For thermal stability, ChTI was incubated at 50-100℃ for 10min and snap chilled. For pH stability, the inhibitor was incubated in 0.01M buffer (Glycine HCl, pH3.0; sodium citrate, pH5.0; potassium phosphate, pH7.0 and Tris HCl pH9.0) for 30min at room temperature, diluted with PPB. Residual TIA activity was measured in all samples.

Western Blot Analysis

Laying hens were injected subcutaneously with 300µg affinity purified ChTI in Freund's complete adjuvant followed by three booster doses in Freund's incomplete adjuvant at two week intervals and IgY was purified from egg yellow (Song et al. 1985). HSPs from leaf, shoot, seed and root were separated on 10% SDS-PAGE and electroblotted onto PVDF membrane. The membrane was blocked with 1% casein in phosphate-buffered saline (PBS), pH7.2 for 1h at 37℃. ChTI specific band was detected with ChTI-IgY (1:500v/v) and anti-IgY rabbit antibody coupled with alkaline phosphatase (1:1000v/v). The bands were visualized by incubating with NBT/BCIP substrate solution (Sambrook et al. 1989). Serological cross reactivity of ChTI IgY was also carried out against Subabul trypsin inhibitors, Rain tree trypsin and chymotrypsin inhibitors I and II.

Bioassay with insect larvae

Helicoverpa armigera (2^{nd} and 3^{rd} instars) and *Spodoptera littoralis* (2^{nd} instar) larvae were reared on artificial diet with affinity purified inhibitor (ChTI -5000TIU/ml). The concentration was chosen after preliminary experiments and based on early studies in the lab. Control larvae were maintained on identical feed but without inhibitor (Bhavani et al. 2007; Nandeesha and Prasad 2001). One hundred grams of whole kabuli gram was washed thoroughly and soaked in sterilized water for 24h. Seeds were blended in a grinder into a fine pulp. Other ingredients of the diet (Brewer's Yeast-15.0g, Weasson Salt-7.0g, Ascorbic Acid-1.0g, Vitamin E tablet-1, Methyl parabene-2.0g, Streptomycin-0.5g, Ethyl alcohol-2.0ml, Water (distilled) -800.0ml, Agar Agar-14.0g) were added, mixed and cooked for 10min. A total of 3ml of hot media was poured into each glass vial and allowed to solidify. Larval weight was taken at regular intervals until pupation and the diet was changed once in 3day intervals. Midgut from 15-day-old larvae was homogenized in 0.2M Glycine -NaOH buffer pH10.0 (1:5w/v), and centrifuged at 4℃ for 15min at 12,000rpm. Supernatant was used as the source of gut proteinase for assay.

Bioassay for antifungal activity:

i. Fungal isolates

Mycelia / conidia of fungal isolates were grown in Potato Dextrose Broth with vigorous shaking at 30℃ for 48h. Aliquots of different fungal strains were uniformly spread on Potato Dextrose Agar plate. Sterilized filter paper discs containing 5-20µg of purified ChTI were placed on the plates. Sterile water was used as control. Inhibition pattern was recorded after 24-48h of incubation at 30℃.

ii. Yeast strains

To understand the basis of specificity of ChTI, yeast strains as a model system was used. A total of 100µl of freshly grown yeast strains were (0.5 OD at 600nm) uniformly spread on Yeast Extract Potato Dextrose (YEPD) agar plate. Sterile filter paper discs containing 5-20µg of protein was placed. Sterile water served as control. Ten µg of purified ChTI was incubated with yeast strains (100ul, 0.5 OD at 600nm) at 30℃ for 2h. 5µl aliquots from serially diluted (1:10, 1:100, 1:1000, 1:10000v/v) samples were spotted on the YEPD agar plates. Inhibition pattern was recorded after 24-48h of incubation at 30℃

Fluorescein isothiocyanate conjugated *Cocculus hirsutus* trypsin inhibitor (FITC-ChTI) was prepared for *in situ* binding studies (Kim et al. 2003). Yeast and fungal cultures were incubated with Florescein-ChTI. The cell suspension was washed with distilled water several times before the observations were recorded using UV fluorescent microscope (Leica-DM-LB2).

Results and Discussion

Trypsin inhibitors are known to confer protection against pests and diseases. They also play an important role in regulation of endogenous proteinases in plants. Protease inhibitors (PIs) are found effective in controlling insect-pests by inhibiting their gut proteases (Bhavani et al. 2007; Franco et al. 2003;Giri et al. 1998, 2003;Rodrigues et al. 2003).

Characterization of ChTI

Trypsin sepharose affinity column, commonly used in purification of serine proteinase inhibitors (Nandi et al. 1999; Rayas-Duarte et al. 1992), was employed to purify ChTI from leaves. ChTI, purified to 33.70 fold, showed inhibitory activity against bovine trypsin, but not with chymotrypsin and amylase (data not shown). SDS electrophoresis and specific activity staining of ChTI revealed that it is a monomeric protein with a molecular mass of ~18kD (Fig.1A). Affinity purified ChTI exhibited high TIA (6274.0TIU/mg) when compared to HSP (2770.0TIU/mg).

PIs are known to be stable under extremes of pH and temperature. Trypsin Inhibitors from onion and legumes were reported to possess substantial inhibitory activity even after exposure to high temperature and pH (Bhattacharyya et al. 2006; Deshimaru et al. 2003; Nandeesha and Prasad 2001; Rayas-Duarte et al. 1992). ChTI was found to be stable at pH range 7.0-9.0, beyond which activity decreased drastically. ChTI was stable up to 70℃ and lost considerable activity when incubated over 80℃. These findings suggest that ChTI can retain compact structure at temperatures around 70℃ without significant changes in activity.

Western blot analysis revealed that ChTI is distributed in leaves, seeds, shoots and roots (Fig.1B). Expression of proteinase inhibitors in vegetative parts of plants indicates their possible role in defense against insects and pathogens and possibly a significant role in regulating endogenous proteolytic activities during tissue development (Franco et al. 2003; Habu et al. 1996). Cross reactivity of ChTI IgY with rain tree trypsin and chymotrypsin inhibitors, high and low molecular weight Subabul Trypsin Inhibitors (Fig.1C) suggests that ChTI shares significant serological homology with other inhibitors as well, similar to onion trypsin inhibitor - 3 (Deshimaru et al. 2003).

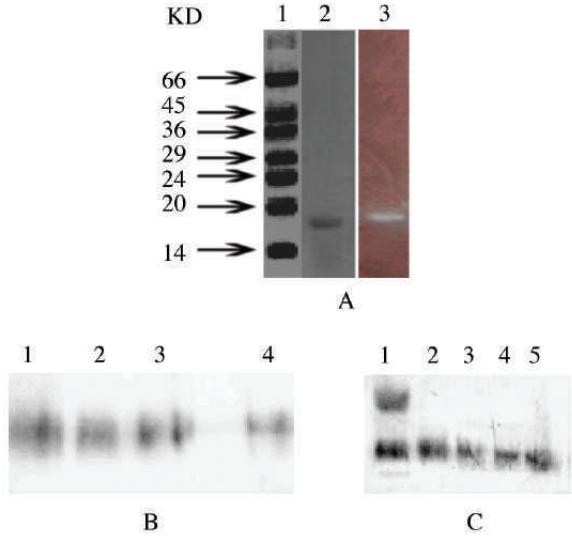

Fig. 1. Characterization of ChTI: (A) SDS-PAGE analysis of ChTI- lane1. Molecular marker, lane 2. Affinity purified ChTI, lane 3. Activity staining of ChTI. (B) Western analysis with ChTI IgY: HSPs from *Cocculus hirsutus*- lane1.leaves, lane 2. seeds, lane 3.shoots, lane 4. roots; (C) lanes1and 2. Subabul HMW and LMW trypsin inhibitors, lanes 3 and 4. Rain tree Chymotrypsin inhibitor I and II and lane 5. Rain tree trypsin inhibitor

Bioassay with Lepidopteron larvae

Larvae of two Lepidopteron insects were tested for the effect of the ChTI in feed. Egyptian cotton worm (*Spodoptera littoralis*) larvae fed on ChTI (5000TIU/ml) supplemented diet were very sensitive and could not survive. The control larvae completed each instar of their life cycle (data not shown). But in the case of the larvae of *Helicoverpa armigera* the second instar larvae fed with ChTI showed a significant decrease in growth within a span of three days compared to controls, whereas third instar larvae showed growth reduction only after nine days (Fig. 2). At the end of the 16th day, the second instar larvae fed with ChTI showed minimal growth (20.02mg) compared to its respective control (322.92mg), (Table1, Fig. 3A). The third instar larvae also showed a similar trend in the growth pattern with ChTI containing media (Tables 1 and 2). The control larvae pupated from the 17th day onwards whereas ChTI treated larvae could not complete their life cycle and mortality was 100%. The ChTI-fed second and third instar *H.armigera* larvae resulted in a significant reduction in larval weight (84.59 and 58.79% respectively) compared to the control. These findings suggest that ingestion of inhibitor in early stages of life cycle would drastically suppress the growth and development of Lepidopteron insects. Reduction in larval weight, growth retardation, enhancement in the duration of larval growth and mortality of *H. armigera* has been reported with Subabul Trypsin Inhibitor (Bhavani et al. 2007; Nandeesha and Prasad 2001).

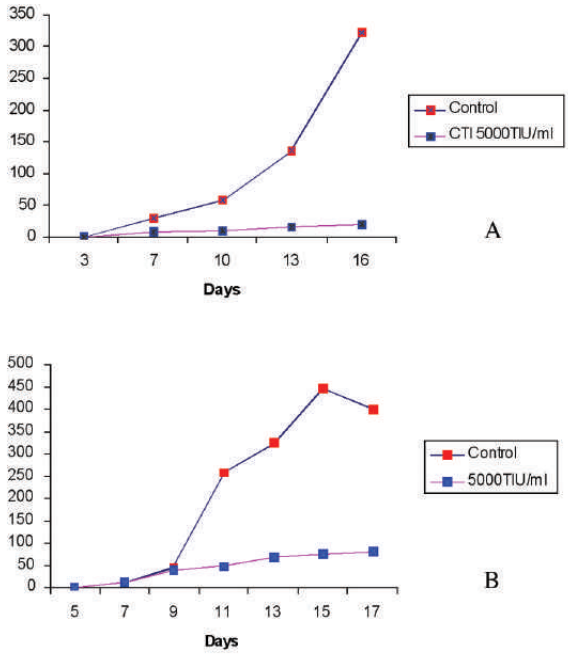

Fig. 2. Larval growth curve of (A) second and (B) third instar *Helicoverpa armigera* larvae fed on artificial diet containing ChTI (5000TIU/ml) and control on same diet without ChTI. Each value represents the mean weight of 20 larvae from five independent replicates ± S.E.

Table 1. Effect of ChTI on growth of 2nd instar *Helicoverpa armigera* larvae.

Age of larvae in Days	Mean of the survived larval weight (mg) Control	Mean of the survived larval weight (mg) ChTI	SE	CD
3Φ	-	-	-	-
7	29.35** (20)	7.27** (20)	0.62	3.55
10	58.54** (20)	10.80** (16)	2.00	11.77
13	135.08** (20)	16.44** (10)	4.55	29.57
16	322.92** (20)	20.02** (5)	12.25	112.77
Day 19 Pupal weightΦ	307.20	No Pupation	-	-
Mortality (%)	0	100	-	-

ChT1: Artificial diet treated with 5000 TIU/ml of ChTI,
** - significance at 1%. Φ - Data for which statistical analysis has not been done. CD - critical difference. Twenty larvae were used in each bioassay. The weight is the mean of survived larvae in each of the individual experiments. Number in the parenthesis indicates the number of survived larvae. SE - Standard error mean.

Insects are reported to synthesize new or altered midgut proteases *de novo* as a consequence of adaptation to inhibitors supplemented through their diets and dietary pattern (Bhavani et al. 2007; Jongsma and Bolter 1997; Volpicella et al. 2003). *Helicoverpa* gut protease (HGP) activity was 85.67± 2.87TU in control and 34.67 ± 0.94TU in ChTI supplemented diet respectively. Gut enzymes resolved into three activity bands in controls, among them two were absent in ChTI-treated larval gut samples, suggest that ChTI did not elicit *de novo* synthesis of gut proteinases (Fig. 3B). In a gel assay, it showed complete inhibition of gut enzyme of *S. littoralis* larvae fed with a diet containing ChTI (Fig. 3C). The results put together suggest that *H. armigera* and *S. littoralis* depends more on trypsin-like proteinases for metabolizing the dietary proteins and inhibition of gut proteinases causes drastic effect on normal growth and development of larvae (Bhavani et al. 2007; Jongsma and Bolter

Table 2. Effect of ChTI on growth of 3rd instars *Helicoverpa armigera* larvae.

Age of larvae in Days	Mean of the survived larval weight (mg) Control	Mean of the survived larval weight (mg) ChTI	SE	CD
5	-	-	-	-
7	12.7** (20)	10.73**(20)	0.88	5.73
9	45.22** (20)	38.82**(20)	1.74	9.97
11	257.77**(20)	49.06**(20)	9.62	55.03
13	322.92**(20)	68.51**(20)	11.42	65.36
15	445.53**(20)	75.35**(17)	9.99	58.39
17	401.42**(20)	82.74**(15)	12.06	71.79
19	-	79.88 (10)	-	-
Pupal weightΦ	305.90	No Pupation	-	-
Mortality (%)	0	100	-	-

ChT1: Artificial diet treated with 5000 TIU/ml of ChTI,
** - significance at 1%. Φ - Data for which statistical analysis has not been done. CD-critical difference. The weight is the mean of 20 larvae. The weight is the mean of survived larvae in each of the individual experiments. Number in the parenthesis indicates the number of survived larvae. SE - Standard error mean.

1997; Volpicella et al. 2003; Srinivasan et al. 2005). Artificial diets are very nutritive with vitamins and mineral supplements, and allow faster growth of larvae, hence require a large amount of inhibitors to bring in a significant reduction in larval growth. Whereas the presence or expression of small amounts of inhibitory proteins would be enough to bring control on polyphagous insects like *H. armigera* since the natural hosts will have limited nutrients in additions to the presence of inhibitor. In a separate study, ChTI was tested on growth, development and mortality of the coleopteran larvae, cowpea weevil, and *Callosobruchus maculates*. There was no mortality observed even at 1% (w/w) ChTI concentration (data not shown). This could be because the cowpea weevils use cysteine and acid proteinases to digest their dietary protein in the gut (Murdock et al. 1987; Terra and Ferreira 1994), hence ChTI, a serine protease inhibitor is unlikely to cause any growth retardation. These results indicate that ChTI is a specific inhibitor of serine proteinases and causes significant reduction in growth and development of lepidopteron larvae which predominantly have Trypsin like serine proteinase in their gut.

Bioassay with fungal and yeast strains

Proteinase inhibitors exhibit a wide spectrum of activity including suppression of pathogenic nematodes like *Globodera tabaccum*, *G. pallida*, and *Meloidogyne* incognita (Williamson and Hussey 1996), inhibition of spore germination and mycelium growth (Ng et al. 2003; Wang et al. 2006; Ye and Ng 2002). ChTI in range 5-20µg was used to carry out zone inhibition assay against fungal phytopathogens (Fig.4A). The radii of growth inhibition zones were measured in each case after 48h. *Alternaria alternata*, *Aspergillus flavus*, *Colletotrichum capsici*, *Fusarium oxysporum*, *Fusarium solani* (crossandra isolated), *Rhizoctonia oryzae* and *Sclerotia* spp. showed maximum susceptibility to ChTI. *Fusarium solani* (crossandra isolated) was highly susceptible whereas with same concentration of ChTI, *Fusarium solani* (chick pea isolated) showed resistance to ChTI inhibition (Fig.4B). These results indicate broad-spectrum antifungal activity of ChTI and its strain specificity. Our data also

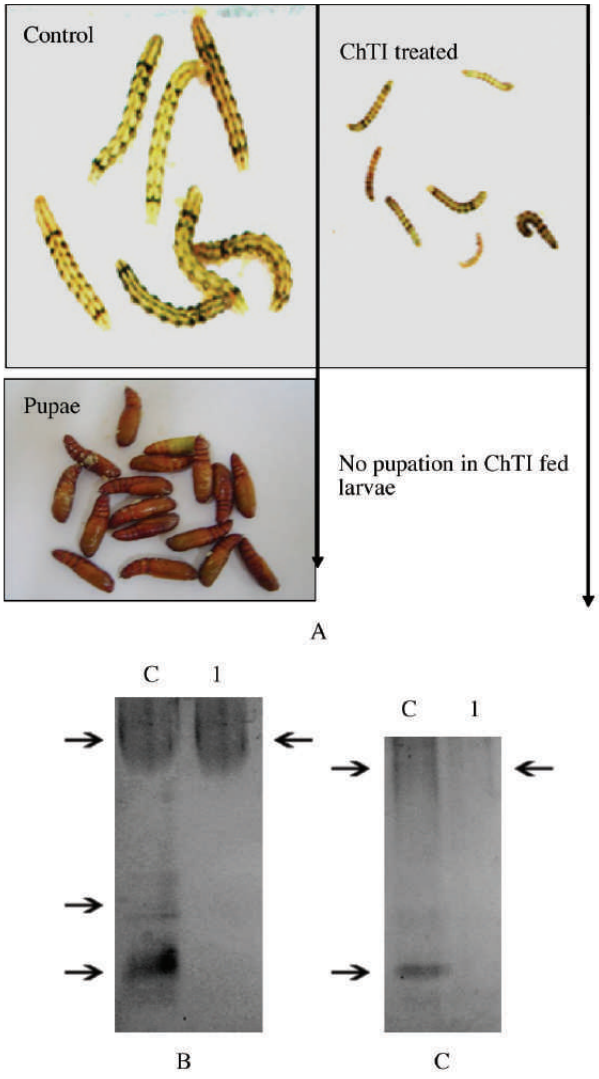

Fig. 3. Effect of ChTI on growth of *Helicoverpa* and *Spodoptera* larvae. The second instar larvae were fed with artificial diet containing ChTI (5000TIU) and control on same diet without ChTI. Fifteen-day old gut extract were used for assay (A) Control and ChTI fed larvae. (B) Activity staining of gut proteolytic enzymes of *Helicoverpa armigera*: lane 1. control and lane 2. Fed with ChTI. (C) Activity staining of gut proteolytic enzymes of *Spodoptera littorals*: lane1.control and lane 2. Fed with ChTI.

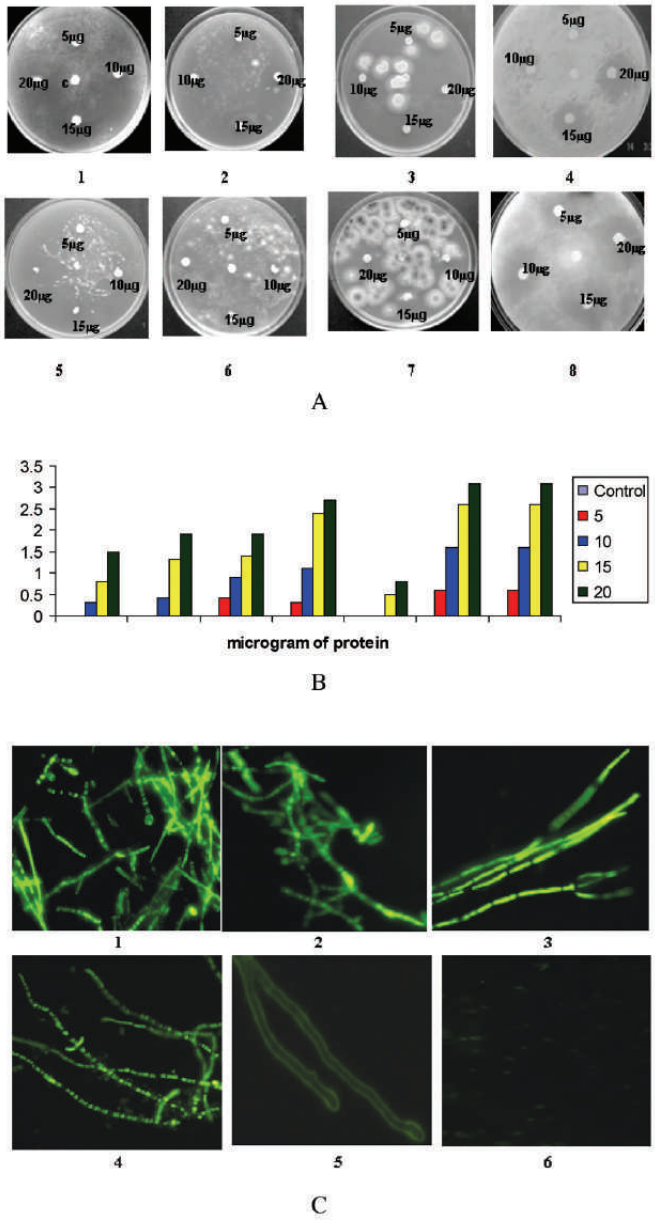

Fig. 4. Antifungal assay using fungal isolates. (A) Zone Inhibition assay of ChTI against Phytopathogens. 1. *Alternaria alternata* 2. *Aspergillus flavus* 3. *Colletotrichum capsici* 4. *Fusarium oxysporum* 5. *Fusarium solani* (crossandra isolated) 6. *Fusarium solani* (chick pea isolated) 7. *Rhizoctonia oryzea* 8. *Sclerotia sp.* (B) Graphical representation of the fungal inhibition zone radii (cm) corresponding to microgram of ChTI protein (C) Fluorescence Micrographs of Fungus cells treated with ChTI.
1- *Colletotrichum capsici* 2- *Aspergillus flavus* 3- *Rhizoctonia oryzae* 4- *Fusarium solani* (crassandra isolated) 5- *Fusarium solani* (chickpea isolated) 6- Control (*Colletotrichum capsici*)

showed that higher inhibitory effect was observed with higher concentrations of ChTI used in zonal inhibition assay. Since ChTI is predominantly a serine proteinase inhibitor, fungal pathogens having more of other classes of proteinase other than serine proteinases, show poor response to ChTI (Fig.4B). Phytopathogens infect their host by secreting hydrolytic enzymes to hydrolyze complex substrates into small organic molecules (Campbell et al. 1999). It is reasonable to suggest that the over-expression of ChTI protein in transgenic plant can inhibit the function of proteinases secreted by phytopathogens through a similar biochemical mechanism.

Although inhibitory effects differ with the type of enzyme, viz., serine *vs*. thiol proteases, the detailed mechanisms under which pathogen become resistant are poorly understood. One of the studies with osmotin on yeast strain offers clues on the possible basis for specificity in defense actions. Osmotin is a plant defense (PR-5) protein. It has a broad spectrum of antifungal activity, but exhibits specificity for certain fungal targets. The structural base for this specificity remains unknown. But sensitivity of *Saccharomyces cerevisiae* cells to osmotin was shown to be dependent on the function of MNN2, MNN4 and MNN6 (Ibeas et al. 2000; Yun et al. 1997), which encode the cell wall mannans. We hypothesized that ChTI may have a similar mechanism of action as that of osmotin on yeast cells. In an attempt to test this hypothesis, yeast strains (W303a, BWG7 and mutants

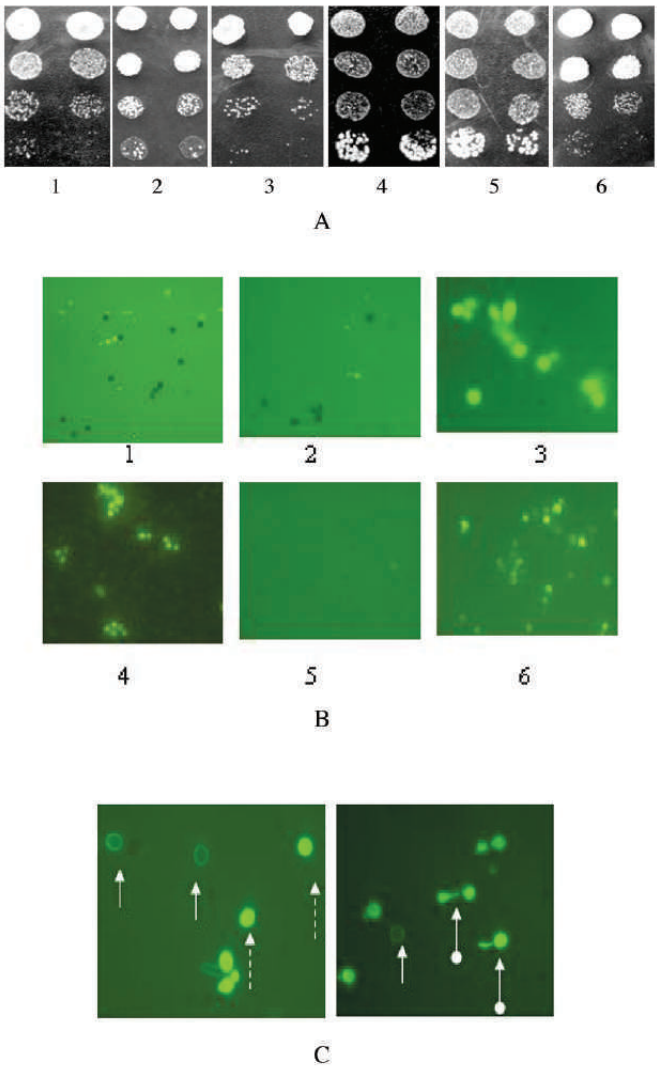

Fig. 5. Antifungal assay using yeast as a model system. (A) Serial dilution assay for Inhibition activity of ChTI against Yeast strains. 1. ΔYNK, 2. W303a, 3.MNN1,4. MNN4, 5. MNN6, 6. BWG7. (B) Fluorescence Microphotographs of yeast mutants incubated with FITC- ChTI.1. MNN6, 2. MNN4, 3. MNN1, 4. ΔYNK, 5. W303, 6. BWG7. (C) Comparison of budding, young and mature MNN1 cells by FITC-ChTI-binding studies. Filled arrows illustrate matured cells, broken arrows illustrate young cells and ball with filled arrows illustrates budding cells.

ΔYNK, MNN1, MNN4 and MNN6) that differ in sensitivity to osmotin were screened for their sensitivity to ChTI. ChTI was most effective against ΔYNK and MNN1 followed by BWG7. MNN6 showed moderate inhibition while the wild strains W303a and MNN4 did not respond (Fig.5A). Significant levels of binding to FITC-ChTI with ΔYNK, MNN1 and MNN6 was observed (Fig.5B). ΔYNK is mutant of W303a, and lacks the gene that codes for nucleotide diphosphokinase. This suggests that the nucleotide diphosphokinase, a member of MAPK signaling cascade (Moon et al. 2003), may have a role in mitigating the antifungal activity of ChTI. Yun et al. (1997) demonstrated that osmotin subverts target cell signal transduction as part of its mechanism of action to weaken the cell wall barriers and increase its cytotoxic efficacy. Actively growing cells will have a more porous cell wall than the non-growing/mature cells (De Nobel and Barnet 1991). FITC-ChTI showed extensive binding to the germinating tips of the mycelia as well as the conidia of phytopathogens studied (Fig. 4C). As spores progress in maturity, they begin with only a mannan layer, then have both mannan and glucan, followed by mannan, glucan, and chitosan (Huang et al.2005). Young and budding cells, therefore show more fluorescence binding with ChTI compared to the mature cells (Fig.5C). MNN1 which lacks the expression of gene encoding for 1, 3 mannosyl transferase that adds mannosyl residues to the outer cell wall, has exposed mannosyl phosphodiester linkages, which seem to be potential target sites for ChTI binding. MNN6 responded moderately while MNN4 showed resistance to ChTI. In both of these mutants the outer mannan layer is intact masking the mannosyl phosphodiester linkages. Null MNN4 or MNN6 mutants lacking phosphomannans were found to be defective in binding osmotin to the fungal cell wall (Ibeas et al. 2000). Their results demonstrated the causal relationship between cell surface phosphomannan and susceptibility of yeast mutants to osmotin. Further, cell surface polysaccharides of invading pathogens were shown to have the determinants for interaction with the antifungal proteins especially the ones similar to PR-5 family of proteins (Ibeas et al. 2000; Yun et al 1997). Strain-specific recognition by ChTI is consistent with the observations made with osmotin like antifungal proteins.

The results of our study provide interesting clues on a new inhibitor that might be an interesting target for the development of genetically modified crop plants resistant to insect-pests and foliar diseases. Further studies are required to further evaluate its therapeutic application for treating infectious diseases.

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References

Badole S, Patel N, Bodhankar S, Jain B, Bhardwaj S. 2006. Antihyperglycemic activity of aquous extract of leaves of *C. hirsutus* (L). Diels in Alloxan-induced diabetic mice. Indian J. Pharmacol. 38: 49-53

Bhattacharyya A, Mazumdar S, Leighton SM, Babu CR. 2006. A Kunitz proteinase inhibitor from *Archidendron ellipticum* seeds: purification, characterization, and kinetic properties. Phytochem. 67: 232-241

Bhavani P, Bhattacharjee C, Prasad DT. 2007. Bioevaluation of partially purified subabul proteinase inhibitors on *Helicoverpa armigera*. Arthropod Plant Interac. 1: 255-261

Bishop D, Pearce G, Bryant JE, Ryan CA. 1984. Isolation and

characterization of the proteinase inhibitor inducing factor from tomato leaves: Identity and activity of poly and oli gogalacturonide fragments. J. Biol. Chem. 259: 13172-13177

Brzin J, Kidric M. 1995. Proteinases and their inhibitors in plants: role in normal growth and in response to various stress conditions. Biotechnol. Genet. Eng. Rev. 13: 420- 467

Campbell NA, Reece JB, Mitchell LG. 1999. Fungi: Mycology. In Biology, Ed5 Benjamin/ Cummings, Essex.

Chrispeels MJ, Raikhel NV. 1991. Lectins, lectin genes and their role in plant defense. Plant Cell 3:1-9

De Leo F, Volpicella MW, Licciulli F, Liuni S, Gallerani R,Ceci LR. 2002. PLANT-PIs: a database for plant protease inhibitors and their genes. Nucl. Acids Res. 30:347-348

De Nobel JG, Barnett JA.1991. Passage of molecules through yeast cell walls: A brief essay. Yeast 7: 313-323

Deshimaru M, Watanabe A, Suematsu K, Hatano M, Terada S. 2003. Purification, amino acid sequence, and cDNA cloning of trypsin inhibitors from onion (*Allium cepa* L.) bulbs. Biosci. Biotech. Biochem. 67:653-659

Ferry N, Jouannin L, Ceci LR, Muligan EA, Emami K, Gatehouse JA, Gatehouse AM. 2005. Impact of oilseed rape expressing the insecticidal serine proteinase inhibitor, mus tard trypsin inhibitor-2 on the beneficial predator *Pterostichus madidus*. Mol. Ecol. 14: 337-349

Filho JX, Moreira RDA. 1978. Visualization of proteinase inhibitors in SDS-polyacylamide gels. Anal. Biochem. 84: 296-299

Franco OL, dos Santos RC, Batista JA, Mendes AC, de Araujo MA, Monnerat RG, Grossi-de-Sa MF, De Freitas SM. 2003. Effects of black-eyed pea trypsin/chymotrypsin inhibitor on proteolytic activity and on development of *Anthonomus gran dis*. Phytochem. 63: 343-349

Giri AP, Harsulkar AM, Deshpande VV, Sainani MN, Gupta VS, Ranjekar PK. 1998. Chickpea defensive proteinase inhibitors can be inactivated by pod-borer gut proteinase. Plant Physiol. 116: 393-401

Giri AP, Harsulkar AM, Ku MS, Gupta VS, Deshpande VV, Ranjekar PK, Franceschi VR. 2003. Identification of potent inhibitors of *Helicoverpa armigera* gut proteinases from winged bean seeds. Phytochem. 63: 523-532

Habu Y, Fukushima H, Sakata Y, Abe H, Funada R. 1996. A gene encoding a major Kunitz proteinase inhibitor of storage organs of winged bean is also expressed in the phloem of stems. Plant Mol. Biol. 32: 1209-121

Huang LS, Hugh K, Doherty HK, Herskowitz I. 2005. The Smk1p MAP kinase negatively regulates Gsc2p, a 1,3-_-glu can synthase, during spore wall morphogenesis in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA. 102: 12431-12436

Ibeas JI, Lee H, Damsz B, Prasad DT, Pardo JM, Hasegawa PM, Bressan RA, Narasimhan ML. 2000. Fungal cell wall phos phomannans facilitate the toxic activity of a plant PR-5 pro tein. Plant J. 23: 375-383.

Johnson ED, Mille EA, Anderson MA. 2006. Dual location of a famiy of proteinase inhibitors within the stigmas of *Nicotiana alata*. Planta. 225:1265-1276.

Jongsma MA, Bolter C. 1997.The adaptation of insects to plant protease inhibitors. J. Insect Physiol. 4l: 885-895

Kakade ML, Simons N, Liener IE. 1969. An evaluation of natur al and synthetic substrates for the antitryptic activity of soy bean samples. Cereal Chem. 46: 518-522

Kim J, Park H, Jung D, Kim S. 2003. Protein immobilization on plasma polymerized ethylenediamine coated glass slides. Anal. Biochem. 313: 41-45

Laemmli UK. 1970.Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680 -685

Leung D, Abbenante G, Fairlie DP. 2000. Protease inhibitors: current status and future prospects. J. Med. Chem. 43:305-341

Lin YH, Li HT, Huang YC, Hsieh YC, Guan HH, Liu MY, Chang T, Wang AH, Chen CJ. 2006. Purification, crystalliza tion and preliminary X-ray crystallographic analysis of rice Bowman-Birk inhibitor from *Oryza sativa*. Acta Crystallograph Sect F Struct. Biol. Cryst. Commun. 62: 522-524

Monteiro S, Barakat M, Piçarra-Pereira MA, Teixeira AR, Ferreira RB. 2003. Osmotin and thaumatin from grape: A putative general defense mechanism against pathogenic fungi Phytopathol. 93:1505-1512

Moon H, Lee B, Choi G, Shin D, Prasad DT, Lee O, Kwak SS, Kim D, Bahk J, Hong JC, Lee SY, Cho MJ, Lim CO, Yun DJ. 2003. Nucleotide diphosphate kinsae2 interacts with two oxidative stress activated mitogen activated protein kinases to regulate cellular redox state and enhances multiple tolerance in transgenic plants. Proc. Natl. Acad. Sci. USA. 100:358-363

Moura DS, Ryan CA. 2001. Wound-inducible proteinase inhibitors in pepper. Differential regulation upon wounding, systemin, and methyl jasmonate. Plant Physiol. 126:289-298.

Murdock LL, Brookhart G, Dunn PE, Foard DE, Kelley S, Kitch L, Shade RE, Shukle RH, Wolfson JL. 1987. Cysteine diges tive proteinases in *Coleoptera*. Comp. Biochem. Physiol. 87: 783-787

Nandeesha P, Prasad DT. 2001. Characterization of serine pro teinase inhibitor from subabul (*Leucaena leucocephala* L.) seeds. J. Plant Biochem. Biotech. 10: 75-78

Nandi AK, Basu D, Das S, Sen SK. 1999. High level expression of soybean trypsin inhibitor gene in transgenic tobacco plants failed to confer resistance against damage caused by *Helicovarpa armigera*. J. Biosci. 24: 445-452

Nayak SK Singhai AK. 1993. Antiinflammatory and analgesic activity of roots of *C. hirsutus*. Indian J. Nat Prod. 9:12-14

Ng TB, Lam SK, Fong WP. 2003. A homodimeric sporamin-type trypsin inhibitor with antiproliferative, HIV reverse tran scriptase-inhibitory and antifungal activities from wampee (*Clausena lansium*) seeds. Biol. Chem. 384: 289-293

Norton G. 1991. Proteinase Inhibitors In JPF D'Mello, CM Duffus, JH Duffus eds, Toxic Substances in Crop Plants The Royal Society of Chem. pp 68:106.

Odani S, Ikenaka T. 1973. Studies on Soybean Trypsin Inhibitors. J. Biochem. 74: 697-715

Pompermayer P, Lopes AR, Terra WR, Parra JRP, Falco MC, Silva MC.2001. Effect of soybean proteinase inhibitor on development, survival, and reproductive potential of the sugar-cane borer, *Diatraea saccharalis*. Entomol. Exp. Appl. 99:79-85

Raja NRL, Sundaranathavalli S, Ananthi JJ, Nirmaladevi, Kumarappan CT, Kumaraguruparan P, Yaseen K. 2008. Antihyperglycaemic activity of aqueous extract of *Vinca rosea* linn in alloxan induced diabetic rats. Pharmacologyonline. 3: 354-362

Rayas-Duarte P, Bergeron D, Nielson S. 1992. Screening of heat stable trypsin inhibitors in dry beans and their partial purifi cation from great northern beans (*Phaseolus vulgaris*) using anhydrotrypsin- sepharose affinity chromatography. J. Agri. Food Chem. 40:32-43

Rodrigues ML, Machado-Freire MG, Cabrini EC, Toyama MH, Novello JC, Marangoni S. 2003. A trypsin inhibitor from *Peltophorum dubium* seeds active against pest proteases and its effect on the survival of *Anagasta kuehniella* (Lepidoptera: Pyralidae). Biochimica Biophysica Acta. 1621: 170-182

Ryan CA. 1990. Protease inhibitors in plants: genes for improv ing defenses against insects and pathogens. Ann. Rev. Phytopath. 28: 425-449

Ryan CA, Bishop P, Pearce G, Darvill AG, McNeil M, Albersheim P. 1981. Plant Physiol. 68: 616-618

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular Cloning: A Laboratory Manual, Ed 2, Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Sangameswaran B, Jayakar B. 2007. Anti-diabetic and sper matogenic activity of *Cocculus hirsutus* (L) Diels .African J. Biotechnol. 6:1212-1216

Song SC, Yu JH, Hester PY, Kim KI. 1985. Antibodies to the alpha subunit of insulin receptor from eggs of immunized hens. J. Immunol. 135: 3354-3359

Srinivasan A, Giri AP, Harsulkar AM, Gatehouse JA, Gupta VS. 2005. A kunitz trypsin inhibitor from chickpea that exerts antimetabolic effect on pod borer (*Helicoverpa armigera*) lar vae. Plant Mol. Biol. 57: 359-374

Terra WR, Ferreira C. 1994. Insect digestive enzymes proper ties, compartmentalization and function. Comp. Biochem. Physiol. Biochem. Mol. Biol.109: 1-62

Volpicella M, Ceci RL, Cordewener J, America T, Gallerani R, Bode W, Jongsma MA, Beekwilder J. 2003. Properties of purified gut trypsin from *Helicoverpa zea*, adapted to pro teinase inhibitors. Eur. J. Biochem. 270: 10-19

Wang HX, Ng TB. 2006. Concurrent isolation of a Kunitz-type trypsin inhibitor with antifungal activity and a novel lectin from *Pseudostellaria heterophylla* roots. Biochem Biophys. Res. Comm.342:349-353

Williamson VM, Hussey RS.1996. Nematode pathogenesis and resistance in plants. Plant Cell 8: 1735-1745

Ye XY, Ng TB. 2002. A new peptidic protease inhibitor from *Vicia faba* seeds exhibits antifungal, HIV-1 reverse transcrip tase inhibiting and mitogenic activities. J. Peptide Sci. 8: 656-662

Yun DJ, Zhao Y, Pardo JM, Narasimhan ML, Damsz B, Lee H, Abad LR, D'Urzo MP, Bressan RA. 1997. Stress proteins on the yeast cell surface determine resistance to osmotin, a plant antifungal protein. Proc. Natl. Acad. Sci. USA. 94:7082-7087