



Characterization and Potent Application of *Pleurotus floridanus* Trypsin Inhibitor (PfTI)

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Abstract – Characterization and *in vitro* inhibition studies of protease inhibitor from the mushroom *Pleurotus floridanus* (PfTI) towards the pest *Papilio demoleus* is studied. The addition of 1 mM Mn²⁺, Na²⁺, Ba²⁺ and Ni²⁺ enhanced the PfTI activity. The ICP-atomic emission spectrum showed the presence of Ca²⁺, Mg²⁺ and Zn²⁺ in the PfTI. Surfactants SDS and CTAB at a concentration of 1% reduced the PfTI activity whereas, the nonionic detergents Triton X and Tween 80 increased the activity. The inhibitory activity gradually decreased with increase in concentration of DMSO and H₂O₂. The activity was increased by dithiothreitol up to a concentration of 80 μM and inactivated at 140 μM. The activity of PMSF modified PfTI was drastically reduced to 0.234 U/mL at 4 mM concentration and similar results were obtained for modification of cysteine by *N*-Ethylmaleimide at slightly higher concentrations. The complex of trypsin and PfTI showed complete loss in fluorescence intensity at 343 nm compared with control. *In vitro* inhibition studies of PfTI with midgut proteases isolated from citrus pest *P. demoleus* with protease activity of 1.236 U was decreased to 0.613 U by 50 μL (0.1 mg/mL) of the inhibitor. Inhibitor was stable up to 0.04 M concentration of HCl.

Keywords – Trypsin inhibitor, *Pleurotus floridanus*, Characterization

Introduction

Protease inhibitors have been gained significant attention in recent years for their role as defense molecules against plant pests and pathogens.¹⁻³ The insect gut contains trypsin and trypsin like proteases which are serine proteases and have also been identified in digestive tract extracts from many families of insects, particularly those of Lepidoptera.⁴ Protease inhibitors reportedly inhibit gut proteases of insects and pests leading to non-availability of amino acids necessary for the growth and development.⁵ The inhibition of this group of enzymes therefore has anti-nutritional effect that could impair the insect development, adult survival and fecundity.^{6,7}

A Kunitz bifunctional inhibitor (ApTI) from Brazilian Carolina seeds (*Adenanthera pavonina* L.) has been characterized which inhibits serine and cysteine proteases and is effective against coleopteran as well as lepidopteran

pests.^{6,8} It has been reported that ApTI incorporated in artificial diet is efficient in controlling of larval development of sugarcane borer and both the short- and long-term *D. saccharalis* sensitivity to ApTI was evaluated.⁹ Biotechnological tools enabled proteinase inhibitors to be engineered into plants to provide resistance to insect pests. A *Beta vulgaris* serine proteinase inhibitor gene (BvSTI) was over expressed in *Nicotiana benthamiana* plants to study its effect on lepidopteran insect pests.¹⁰

Recently we identified an inhibitor from edible mushroom *Pleurotus floridanus* (PfTI) and reported its purification and application against serine protease of microbial origin.¹¹ The present communication discusses the efficacy of PfTI against insect gut proteases and its characterization.

Experimental

General experimental procedures – *N*_α-Benzoyl-DL-arginine *p*-nitroanilide hydrochloride (BAPNA), Sodium dodecyl sulphate (SDS) Cetyltrimethyl ammonium bromide

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(CTAB), Hydrogen peroxide, Dimethyl sulfoxide, β -mercaptoethanol, dithiothreitol (DTT), *N*-Ethylmaleimide, Diethyl pyrocarbonate, *N*-Bromosuccinamide and Phenyl methyl sulfonyl fluoride (PMSF) were purchased from Sigma-Aldrich, India. Trypsin (from Bovine pancreas), Ethylene diamine tetraacetic acid (EDTA) disodium salt dihydrate, Triton X-100, Tween-80 and Tween-20 were purchased from SRL, India.

Assay of protease inhibitor activity using BAPNA – Protease inhibitor activity of *P. floridanus* crude extract and purified inhibitor preparations were measured using synthetic substrate BAPNA.¹² Three seventy-five microlitre of the protease inhibitor diluted with 0.01 M phosphate buffer (pH 7.5) was incubated with 25 μ L of 0.1 mg/mL trypsin in phosphate buffer pH 7.5 for 10 min at 37 °C. Then 50 μ L, 2 mM freshly prepared BAPNA was added and incubated at 37 °C for 30 min. The reaction was stopped by adding 500 μ L of 30% acetic acid. The absorbance of *p*-nitroaniline released was read at 410 nm (Shimadzu, Japan). The difference in absorbance was calculated by assaying trypsin activity in the absence and presence of inhibitor. One unit of inhibitory activity (U) was defined as the amount of inhibitor needed to inhibit the release of 1 μ mol of *p*-nitroaniline per mL per min at pH 7.5 and at 37 °C.

Effect of various metal ions on protease inhibitor activity – The activity of protease inhibitor in the presence of various metal ions was evaluated by incubating PfTI with 1 mM concentrations of Na⁺, Ca²⁺, Mg²⁺, Fe³⁺, Mn²⁺, Ni²⁺, Hg²⁺, Ba²⁺, Cd²⁺, Mo⁶⁺ and Al³⁺ for 30 min, followed by measuring the protease inhibitory activity.

Metal chelation of protease inhibitor using EDTA – The presence of metal ion concentration and its role on inhibitory properties was determined by metal chelation using 30 mM EDTA (SRL, India).¹³ PfTI (0.1 mg/mL) was dialyzed extensively against 30 mM EDTA over night at 4 °C. Protease inhibitor assay was carried out after dialyzing the samples against deionised water with frequent changes.

Metal ion concentration of protease inhibitor – Metal ion concentrations of PfTI were determined by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES, PerkinElmer, USA). An aliquot of protease inhibitor dialyzed extensively against deionised water and another dialyzed extensively against 30 mM EDTA, overnight at 4 °C.

Effect of various detergents on protease inhibitor activity – Effect of various non-ionic and ionic detergents on protease inhibitor activity was determined by incubating the PfTI with Triton X-100, Tween-80, Tween-20, SDS

and CTAB (1% each w/v) for 30 min, dialyzed against 0.01 M phosphate buffer pH 7.5 and estimated the residual inhibitory activity.

Effect of oxidizing agents on protease inhibitor activity – Effect of oxidizing agents on the activity of PfTI was tested using hydrogen peroxide (1-2%, v/v) and dimethyl sulfoxide (1-5%, v/v) for 30 min and measuring the residual inhibitory activity.

Effect of reducing agents – The effect of reducing agents on the activity of PfTI was studied by incubating the protease inhibitor with β -mercaptoethanol (50, 100, 150, 200, 250, 300, 350 μ M) and dithiothreitol (20, 40, 60, 80, 100, 120 μ M) for 30 min and measuring the residual inhibitory activity.

Chemical modification of amino acids in protease inhibitor – The reactive sites of inhibitor molecule and its role on inhibitory activity were evaluated by chemical modification of selected amino acids. Chemical modifications of amino acids of PfTI were carried out using *N*-Ethylmaleimide¹⁴, Diethyl pyrocarbonate¹⁵, PMSF¹⁶ and *N*-Bromosuccinamide¹⁷ under their respective reaction conditions. Hundred microlitre of PfTI (0.1 mg/mL) was used for this study. The PfTI was dialyzed against phosphate buffer after incubation with different concentrations ranging from 5, 10, 15, 20 and 25 mM of each modifier, and the residual activity was estimated.

Effect of acid treatment on protease inhibitor – Sensitivity of PfTI in an acidic environment was evaluated by incubating it with different concentrations of HCl ranging from 0.02, 0.04, 0.06, 0.08 & 0.1 M for 30 min. The pH was neutralized with 0.1M Tris-HCl buffer (pH 9) and the residual protease inhibitory activity was estimated.

Effect of protease treatment on protease inhibitor – PfTI sensitivity to higher concentration of trypsin was assessed with different concentrations of trypsin (from Bovine pancreas, SRL, India) ranging from 0.02, 0.04, 0.06, 0.08 and 0.1% for 30 min at 37 °C.

Intrinsic fluorescence spectroscopy – Fluorimetry was performed on a Cary Eclipse spectrofluorimeter (Make, country) using a slit width of 5 mm and an excitation wavelength of 278 nm. The emission wavelength was observed from 290 nm with Phosphate buffer, pH 7.5 as control. Protease inhibitor having an A₂₈₀ value, 0.1 was used for the emission spectra. PfTI was complexed with Trypsin in a ratio of 1: 1 concentration. The emission spectra for PfTI and trypsin alone were also carried out.

Inhibition of midgut proteases – The lime swallowtail *P. demoleus* (Lepidoptera: Papilionidae) larvae were collected from the citrus leaves from Kakkandu, Ernakulam,

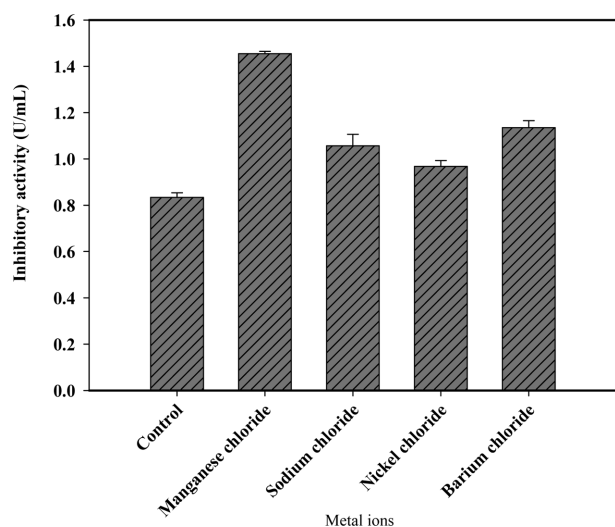


Fig. 1. Effect of metal ions on PfTI. Activity was measured after incubating with 1 mM of metal ion final concentration for 30 min.

India. The midguts of the larvae were transferred into a microfuge tube kept on ice at 4 °C containing 0.01 M phosphate buffer, pH 7.5. The midguts were homogenized at 4 °C and then centrifuged at 12000 g for 10 min at 4°C. The supernatants were used as the crude enzyme (trypsin) extract. Protease inhibitory activity, protein content¹⁸ and specific activity were determined.

Result and Discussion

Protease inhibitors have emerged as a substitute approach for plant protection owing to the adverse effect of chemical pesticide. Analysis of digestive proteases of Lepidopteron insects has revealed the presence of proteases, principally trypsin and chymotrypsin-like enzymes.¹⁹ We have reported previously that PfTI is a serine protease inhibitor with high specificity towards trypsin.¹¹

Data obtained for the studies on the effect of metal ions in the activity of protease inhibitor presented in the Fig. 1. The addition of divalent ions such as Mn^{2+} , Na^{2+} , Ba^{2+} and Ni^{2+} at a concentration of 1 mM enhanced the protease inhibitory activity. Furthermore, it was observed that 1 mM concentration of Mn^{2+} enhanced the inhibitory activity to 1.45 U compared to that of control (0.833 U) and 1 mM concentration of Na^{2+} , Ba^{2+} and Ni^{2+} also led to an increase in protease inhibitory activity reasonably. It was reported that 1 mM concentration of Co^{2+} , Ni^{2+} and Cu^{2+} affect the activity of protease inhibitors.²⁰ None of the other divalent ions had effect on PfTI activity. Involvement of heavy metal ions Cd^{2+} , Zn^{2+} , Fe^{2+} , Cu^{2+} , Hg^{2+} and Pb^{2+} in the inhibitory activity of cysteine

Table 1. Metal ion concentration of PfTI using ICP-AES

Sample	Concentration in ppm		
	Ca	Mg	Zn
Inhibitor dialyzed against deionised water	20.17	30.47	17.78
Demetallised inhibitor	4.66	3.08	4.17

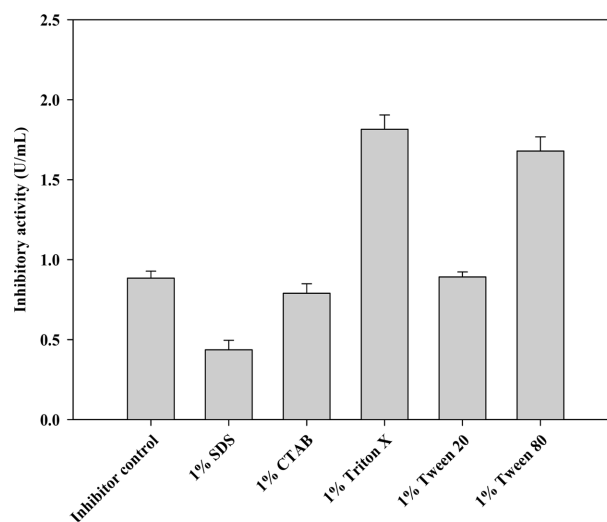


Fig. 2. Effect of detergents on PfTI determined by incubating the PfTI in each detergent for 30 min and estimated the residual inhibitory activity.

proteases, papain and clostripain have been reported earlier.²¹

The ICP-AES analysis presented in Table 1 confirmed the presence of divalent cations Ca^{2+} , Mg^{2+} and Zn^{2+} in PfTI. It was observed that the demetallized PfTI retained protease inhibitory activity indicating no role for the metal ions Zn^{2+} , Ca^{2+} and Mg^{2+} in inhibitory activity; instead they may be involved in maintaining structural integrity of the inhibitor. The result is in contrary to the previous reports on the removal of a Zn^{2+} atom from cysteine protease inhibitor of pearl millet (CPI) lead in the loss of anti-fungal and protease inhibitor activity.²² Similarly, supplementation of Zn^{2+} improved the activity of protease inhibitor isolated from *Moringa oleifera*.²³

The results obtained for the stability of inhibitor in the presence of detergents is depicted in Fig. 2. The data shows that the anionic detergent SDS and cationic detergent CTAB at a concentration of 1% reduced the inhibitory activity significantly. Whereas, the nonionic detergents Triton X and Tween 80, normally considered as mild detergents increased the inhibitory activity more than two times. Protease inhibitors and detergents are used together in cell lysis buffers to inhibit proteolysis in protein purification procedures. Nonionic detergents

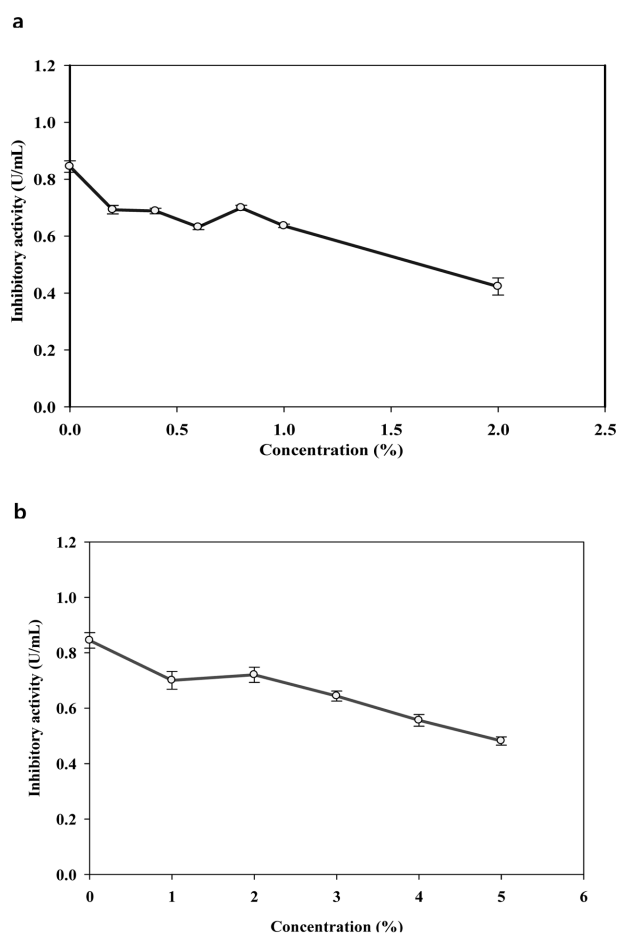


Fig. 3. Effect of oxidizing agents on PfTI. **a**) In the presence of H_2O_2 . **b**) In the presence of DMSO. PfTI incubated with hydrogen peroxide (1-2%, v/v) and dimethyl sulfoxide (1-5%, v/v) for 30 min and measured the residual inhibitory activity.

normally do not interact extensively with the protein surface, whereas ionic detergents, in particular SDS, generally bind non-specifically to the protein surface, which usually lead to protein denaturation.²⁴ The effect of ionic detergents on PfTI activity shall be due to the unfavorable electrostatic interactions. The interaction of mild detergents with hydrophobic amino acids of the inhibitor probably changed its conformation in such a way to facilitate strong binding with the trypsin. *Cajanus cajan* inhibitor to detergents showed any major conformational change in the presence of either sodium dodecyl sulfate (SDS), sodium deoxycholate (DOC). Whereas, the inhibitory activity of the PI was reduced in the presence of DOC.²⁵

The results depicted in the Fig. 3 testify that the PfTI activity decreased along with increase in concentrations of H_2O_2 and DMSO. It was detected that the protease inhibitory activity gradually decreased along with increase

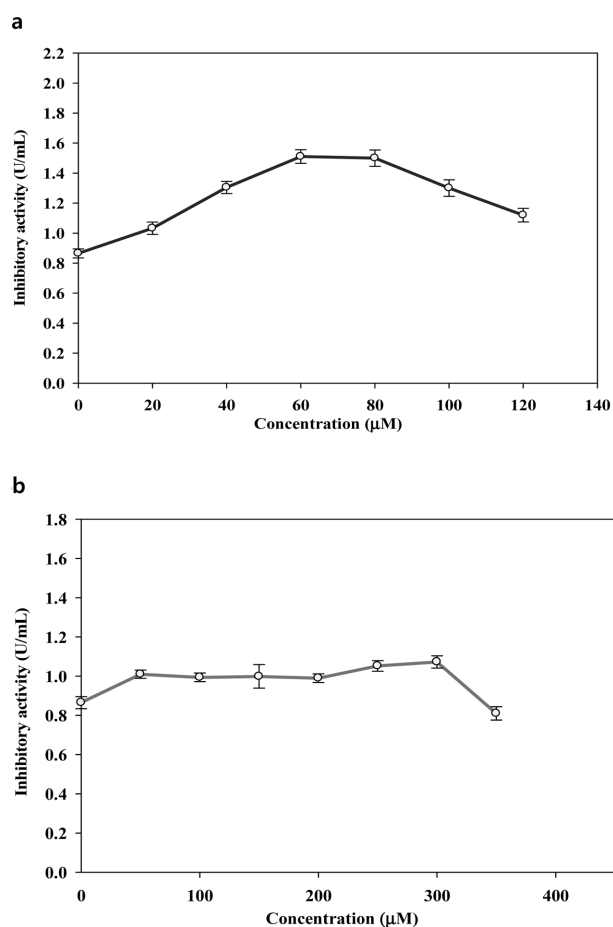


Fig. 4. Effect of reducing agents on PfTI **a**) Effect of dithiothreitol on protease inhibitor. **b**) Effect of β -mercaptoethanol on inhibitor. PfTI incubated with dithiothreitol (20-120 μ M) and β -mercaptoethanol (50-350 μ M) for 30 min and measured the residual inhibitory activity.

in concentration from 1% to 5% of DMSO and complete loss of activity at 6% of DMSO. Whereas, oxidation of PfTI by H_2O_2 was stronger than that of DMSO since complete loss of activity was shown at a concentration above 2% of H_2O_2 . The results indicated the involvement of methionine residue at the reactive site of PfTI isolated from *P. floridanus*. Oxidation of one of the eight methionine residues of α 1-protease inhibitor (α 1-PI) (Met358) resulted a near complete loss of α 1-PI activity toward its primary biological target, elastase.²⁶ Similarly, the activity of protease inhibitor isolated from *M. oleifera* was declined in response to an increase in the concentration of oxidizing agents DMSO and H_2O_2 .²³

The effect of reducing agents on PfTI activity was studied using dithiothreitol and β -mercaptoethanol is shown in Fig. 4. It was inferred that the activity was increased by dithiothreitol up to a concentration of 80 μ M and a concentration of above 140 μ M dithiothreitol caused

complete inactivation of the inhibitor. Whereas in the case of β -mercaptoethanol inactivation occurred at a concentration of 400 μ M compared to dithiothreitol. Intra disulfide bonds are vital for the proper folding and stability of many proteins. Leech carboxypeptidase inhibitor (LCI) is a 67-residue composed of a compact domain with a five-stranded β -sheet and a short α -helix that are strongly stabilized by four disulfide bonds. The designed multiple mutants of LCI were displaying a highly unstructured conformation and a very low inhibitory capability indicated the importance of disulfide bonds in LCI for the correct folding and functional structure.²⁷ In contrary, lower concentration of dithiothreitol (DTT) had no effect on protease inhibitors isolated from *Peltophorum dubium* and *Erythrina caffra*, a Kunitz type trypsin inhibitor.^{28,29}

Modification of amino acids at the reactive site by specific chemicals proved to be a valuable tool in protein-protein interactions. Among the four chemical modifiers used, PMSF inactivated the PfTI even at lesser concentration of the modifier. Inhibitory activity of the PfTI was drastically reduced to 0.234 U/mL at 4 mM concentration PMSF compared to the control activity 0.892 U/mL. Similar effects were shown for *N*-Ethylmaleimide at slightly higher concentrations. An abrupt reduction in the PfTI activity from 0.892 U/mL to 0.345 was observed at 10 mM concentration of *N*-Ethylmaleimide. Modification of histidine residue with DEPC inactivated PfTI at concentrations of above 25 mM. The PfTI activity declined initially to 0.68 U/mL at 5 mM concentration of the modifier and was found stable up to 20 mM concentration of DEPC. But the PfTI activity was reduced to 0.4768 U/mL at 25 mM concentration of DEPC followed by complete inactivation at 30 mM concentration. *N*-Bromosuccinamide, tryptophan modifier had no effect on PfTI activity. The results showed further the presence of serine, cysteine and histidine residue in the reactive site of the PfTI. In contrast, the modification of tryptophan with *N*-Bromosuccinamide had no effect on the activity of the inhibitor.

In Kunitz-domains, primary sites interacting with the target proteases (and determining their protease-specificity) are found as a short segment containing conserved cysteine, and also includes residues adjacent to the fourth conserved cysteine contacting the target proteases.³⁰ The modification of cysteine, arginine or aspartic/glutamic acid residues resulted the loss of anti-fungal activity of pearl millet cysteine protease inhibitor (CPI), while CPI activity was selectively enhanced by modification of arginine or histidine residues.³¹ Involvement of an arginine

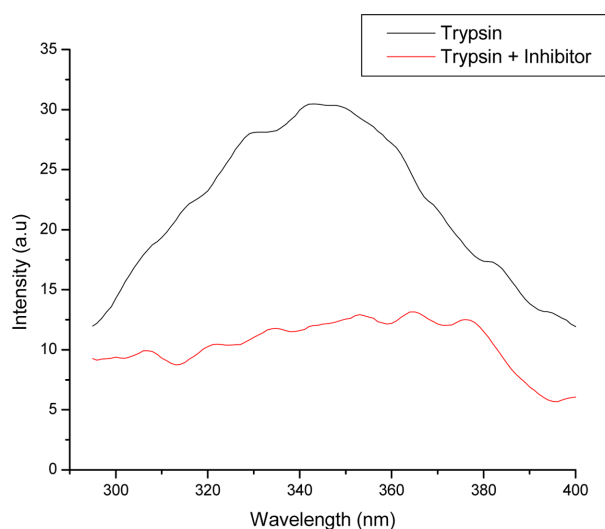


Fig. 5. Fluorescence analysis of trypsin and trypsin-inhibitor complex. Excited at 278 nm and emission was observed from 290 nm. Phosphate buffer, pH 7.5, served as control. Trypsin was complexed with PfTI in a ratio of 1:1 concentration.

residue in amylase inhibitor activity isolated from barley has been reported.³² Lentinus proteinase inhibitor, purified from the fruiting bodies of the edible mushroom, *Lentinus edodes*, suggested the presence of one or more arginine residues in the inhibition of trypsin.³³

The pretreatment of inhibitor with increasing concentration of HCl and trypsin exhibited a decrease in activity shows that PfTI is sensitive towards higher concentration of HCl and trypsin. The PfTI was stable up to 0.04 M concentration of HCl (0.75 U/mL). Pretreatment of PfTI with increasing concentration of trypsin reduced the protease inhibitory activity. It was observed that the PfTI activity was started to decline for the concentrations above 0.06% trypsin.

Results depicted in the Fig. 5 suggest that the binding of PfTI with trypsin was strong. The PfTI and trypsin were mixed in 1:1 ratio and excited at 278 nm. The emission spectrum of trypsin at an excitation wavelength of 278 nm revealed a major peak at 343 nm indicating the intrinsic fluorescence of tryptophan amino acid residue. The emission spectrum of the PfTI trypsin complex showed complete loss in fluorescence intensity at 343 nm compared with PfTI alone, suggesting that binding of inhibitor results in the quenching of tryptophan fluorescence. A decrease in fluorescence intensity of pearl millet cysteine protease inhibitor (CPI) was associated with the quenching of tyrosine fluorescence.³¹

The proteases isolated from midgut of citrus pest, the lime swallowtail *P. demoleus* (Lepidoptera: Papilionidae) was inhibited by the PfTI *in vitro*. Results obtained for the

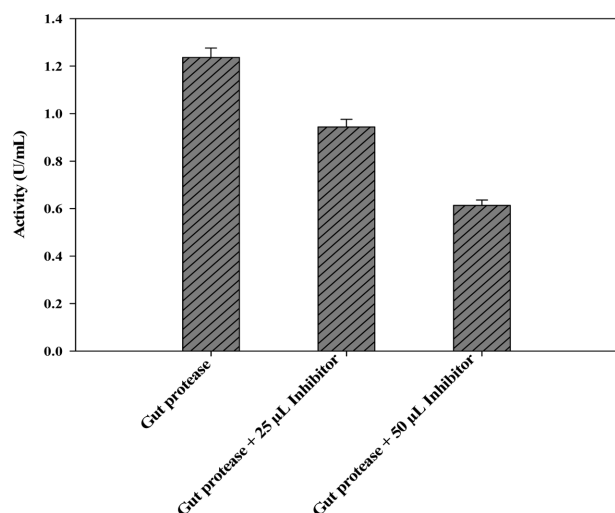


Fig. 6. Inhibition of midgut protease of *P. demoleus*. Twenty five microlitre (0.1 mg/mL) of the midgut proteases isolated from citrus pest was pre incubated with 25 µL and 50 µL of the inhibitor (0.1 mg/mL) and activity was measured.

in vitro inhibition studies of PfTI with midgut proteases isolated from citrus pest, the lime swallowtail *P. demoleus* (Lepidoptera: Papilionidae) is depicted in the Fig. 6. Twenty five microlitre (0.1 mg/mL) of the midgut proteases isolated from citrus pest was pre incubated with 25 µL and 50 µL of PfTI (0.1 mg/mL). Considerable inhibition of the midgut protease was detected with 50 µL of PfTI. Midgut protease of the *P. demoleus* showed a protease activity of 1.236 U which was reduced to 0.613 U by 50 µL of PfTI. The inhibitory activity of the PfTI was stable in the pH range of 4-10 and the remarkable specificity against trypsin signified its possible use as biopesticide.¹¹ The alkaline gut of lepidopteran and dipteran larvae primarily relies on serine proteases like trypsin and chymotrypsin for the digestion of plant material.³ Serine proteases have been isolated in extracts from the digestive tracts of insects from many families, particularly those of lepidoptera and many of these enzymes are inhibited by protease inhibitors.³⁴ The caterpillars of *P. demoleus* can completely defoliate young citrus trees and it was observed that the mean leaf area consumed by all instars of a single larva was 247.6 cm²; and 83.6% of this area was consumed in the fifth instar, 12.7% in the fourth, 2.3% in the third and less than 2% in the first two instars.³⁵

Protease inhibitors function as plant defense molecules and there are reports on biochemical tests and inhibitor incorporated feeding assays demonstrated that these inhibitors impaired insect digestion, and could be good candidate proteins to be used in transgenic plants resistant

to insect pests.³⁶⁻³⁸ Proteinaceous protease inhibitor of fungal origin as a valuable antinutritional agent is limited to the cysteine protease inhibitors from the edible parasol mushroom macrocypins (family I85) and clitocypin of *Clitocybe nebularis*.³⁹ Hence, the *in vitro* inhibition study of the serine protease inhibitor, PfTI against *P. demoleus* suggested the potential of *P. floridanus* inhibitor for use as a biocontrol agent against lepidopteran insect pests.

Conflict of interest

The authors declare that they have no conflict of interest.

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