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Antimicrobial Activity of Trypsin-Chymotrypsin Inhibitor from the Seeds of *Mucuna pruriens*

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Protease inhibitors are proteins or peptides capable of inhibiting the catalytic activity of proteolytic enzymes and plants are the most abundant sources of which most of them studied and characterized were serine protease inhibitors. Protease inhibitors have been studied with a focus on their potential for biotechnology based pest control for agriculture. The trypsin –chymotrypsin inhibitor has been purified from the seeds of *Mucuna pruriens* employing ammonium sulfate fractionation, CM-cellulose chromatography and Sephadex G-100 chromatography. The purified *Mucuna pruriens* trypsin-chymotrypsin inhibitor showed a specific inhibitor activity of 474.66, fold purity of 99.51 and the yield obtained was 22.08%. The *Mucuna pruriens* trypsin-chymotrypsin inhibitor was inhibited both trypsin and chymotrypsin showing the double headed nature. Antifungal studies showed inhibitory activity against *Aspergillus niger* and *Trichoderma viridae*.

Key words: Mucuna pruriens, seeds, Trypsin inhibitor, antifungal properties.

Plants lack an immune system and phytopathogen attack represents a major problem for agricultural crops. Fungal pathogens are responsible for significant crop losses worldwide, resulting from the infection of growing plants and the destruction of harvested crops[1, 2]. Plants for their protection produce number of compounds that act as natural defenses against pests and pathogens. These include inhibitory compounds such as phenols, melanins, tannins or phytoalexins as well as accumulation of proteins that can directly inhibit microbial growth[3]. Anti-microbial peptides or proteins provide the first line of defense against pathogens in both plants animals and these have been isolated from various organisms including animals, bacteria, insects and plants. Recently, several antimicrobial plant proteins and peptides that inhibit the growth of ergonomically important pathogens have been isolated from plant sources which include chitinases, β-1, 3-glucanases, thaumatin-like (TL) proteins, proteinase inhibitors, endoproteinases, peroxidases, ribonuclease-like proteins and plant defensins. Plants have evolved multiple and complex defense mechanism to combat against various fungi with different infection strategies.

Protease inhibitors are ubiquitous in nature and widely distributed in all living organisms. Proteinase inhibitors are one of the most important groups of defense proteins identified from broad range of plants and largely described in leguminous plants. These protease inhibitors are generally believed to act as storage and defense proteins in biological systems and involved in many biological functions including regulation of proteolytic processes and participate in defense mechanisms against attack by insects,

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fungi and other pathogenic microorganisms[4, 5]. Proteinase inhibitors are generally present in high amounts in storage tissues, but can also be induced in response to attacks by insects and pathogenic microorganisms. The mechanism of defense involves the inhibition of proteases of microorganisms, causing the reduction in the availability of the amino acids necessary for their growth and development[2].

The genus *Mucuna* belongs to family fabaceae (leguminoceae) which contains up to 150 species of annual and perennial legumes of pantropical distribution. *Mucuna* is growing extensively as cover crop to control the weeds and pests or insects in agriculture. *Mucuna* are in great demand in food and pharmaceutical industries. Nutritional importance of *Mucuna* seeds as a rich source of protein supplement in food and feed has been well documented[6, 7, 8]. Eight South Indian accessions of *Mucuna* consist of high amount of crude protein (16.2 – 29%) [9]. All parts of Mucuna plant are known to possess high medicinal value and seeds constitute excellent raw material for indigenous ayurvedic drugs and medicines[10, 11]. More than 200 medicinal have been identified and the notable drug is L-DOPA, which serves as a potential drug as anti-parkinson's disease[12, 13, 14].

In the present study, antifungal activity of trypsin inhibitor isolated from the seeds of *Mucuna pruriens* against *Trichoderma viridae* and *Asprgilus niger* has been described.

MATERIALS AND METHODS

Mucuna seeds

Seeds of *Mucuna pruriens* were collected from Siddarabetta, Tumkur District and Thorekempohalli, Nelamangala Taluk, Bangalore Rural District, Karnataka, India. The seeds were soaked and dehulled. The dehulled seeds were homogenized with chilled acetone (10%) in a warring blender for 5 minutes and the homogenate was filtered using suction pump. The cake obtained was dried at 37°C, powdered and stored at 4°C until further use.

Chemicals

Trypsin, Chymotrypsin, N-benzoyl-L-arginine pnitronilide (BAPNA), N-acetyl-DL-phenylalanine ± naphtyl ester (APNE), BSA, ampholites (pH 3 – 10), acrylamide and bis-acrylamide, CM-cellulose, Sephadex G -100, ammonium per sulphate were obtained from Sigma-Aldrich chemical company, St. Louis, USA and all other chemicals used were of analytical grade.

Preparation of crude trypsin inhibitor extract

Mucuna pruriens seeds were soaked overnight in distilled water and crushed with a mortar and pestle using 100 ml (10% w/v) of chilled 0.05 M Glycine - HCl buffer, pH 3. The homogenates were filtered using muslin cloth and the filtrates were centrifuged at 10,000 rpm for 30 min at 4 °C. The supernatants were collected.

Trypsin Activity

Trypsin assay was performed using BAPNA as substrate[15]. Trypsin was dissolved in 0.001 N HCl containing 20 mM $CaCl_2$ at a concentration 200 µg per ml. The assay mixture containing 0.1 mL of the trypsin solution, 0.9 mL of 0.1 M Tris Hydrochloride buffer, pH 8 and 1mL of 5 mM BAPNA (BAPNA was dissolved in 2.5% DMSO and volume was made up to appropriate using 0.05 M Tris Hydrochloride buffer, pH 8). The reaction was terminated after 10 min using 30% acetic acid. The color developed was read at 410 nm against reagent blank. Trypsin units: 1 Trypsin unit (TU) is defined as increase in the OD of 0.01 at 410 nm.

Typsin Inhibitor Activity

Trypsin inhibitor assay was performed using BAPNA as substrate by estimating the remaining hydrolytic activity of trypsin[15]. The assay mixture containing 0.1 mL of trypsin solution, 0.4 mL of 0.1 M Tris Hydrochloride buffer, pH 8 and 0.5 mL of appropriately diluted inhibitor extract was incubated for 10 min at room temperature. 1mL of 5 mM BAPNA solution was added. The reaction was terminated after 10 min using 30% acetic acid. The color developed was read at 410 nm against reagent blank. Trypsin inhibitor units: One Trypsin inhibitor unit (TIU) is defined as decrease in the OD by 0.01 at 410 nm. Protein concentration was determined according to the method of Lowry et al (1951)[16], using bovine serum albumin (BSA) as standard. The protein content in the eluents obtained from chromatographic columns was routinely monitored by measuring absorbance at 280 nm.

Chymotrypsin assay

The chymotrypsin activity was determined using casein as the substrate[17]. Twenty

four µg of chymotrypsin was taken in 2.0 ml of sodium phosphate buffer, pH 7.6 containing 0.15 M NaCl. The reaction was initiated by the addition of 2.0 ml of 2% casein at 37°C. The reaction was stopped after 20 minutes by the addition of 6% trichloroacetic acid (6.0 ml) and after standing for 1 hr, the suspension was filtered through whatman no. 1 filter paper. Absorbance of the filtrate was measured at 275 nm using spectrophotometer. One (chymotrypsin unit is arbitrarily defined as an increase in absorbance by 0.01 275 nm under conditions of assay.

Chymotrypsin inhibitor assay

The chymotrypsin inhibitor activity was determined using casein as the substrate[18]. Enzyme solution 24 µg of chymotrypsin was preincubated with known aliquots of the inhibitor extract at 37°C for 10 min in 0.01 M sodium phosphate buffer, pH 7.6, containing 0.15 M NaCl. The residual enzyme activity was determined as described above.

Polyacrylamide gel electrophoresis (PAGE)

Non-denaturing PAGE (7.5% T, 2.7% C) was performed at pH 4.3[19]. The slab gel (7.5% separating gel and 4% spacer gel) was prepared and gel was placed into the electrophoretic chamber. The electrode chambers were filled with electrode buffer of pH 4.5 (3.12 g β alanine + 0.8 ml glacial acetic acid diluted to 600 ml with distilled water). The samples suitably diluted with 20 % sucrose containing methyl green were loaded onto each sample well and subjected to electrophoresis in cold (4 ºC) applying a current of 20-25 mA for 3 hr.

Gel localization of Trypsin Inhibitors

Visualization of trypsin and chymotrypsin inhibitor in polyacrylamide gel was performed according to Filho and Moriera (1978)[20]. After electrophoresis, native gel was incubated in 0.1 M Sodium Phosphate buffer, pH 8 containing Trypsin (40 µg/ml) for 1 hr and visualized with APNE (1 mg/ml) in DMSO and Fast blue B salt (7 mg/10 ml). The appearance of clear transparent bands against pink background indicates the presence of Trypsin inhibitor. Proteins were stained on polyacrylamide gels using 0.5 % solution of coomassie brilliant blue R-250 in 25 % methanol and 7.5 % acetic acid in water for 1 hr. The gel then was destained in 25 % methanol and 7.5 % acetic acid in water overnight. The gels were stored in 7.5 % acetic acid.

Purification

All the purification procedures were carried out at 4°C unless otherwise stated.

Crude trypsin inhibitor extract: was prepared as described previously and it was subjected to 0 – 40% and 40 – 80% ammonium sulphate precipitation. The pellet obtained after 40 – 80% saturation was dialyzed against 0.025 M sodium acetate buffer, pH 5.5 and subjected to CM-cellulose chromatography on CM-cellulose column (2.5 x 22 cm) equilibrated previously with 0.025 M sodium acetate buffer, pH 5.5. The column was washed with the starting buffer and the bound proteins were eluted by stepwise increase in ionic strength using start buffer containing 0.1 and 0.3 M NaCl with a fraction volume of 10 ml. The CM-cellulose fraction III containing trypsin inhibitor activity were pooled, concentrated and applied to a Sephadex G-100 column (1.0 x 140 cm) pre-equilibrated with 0.025 M sodium phosphate buffer pH 7.0. The proteins were eluted with the same buffer and fractions of 2.0 ml were collected at a flow rate of 12 ml/h. The trypsin inhibitor activity was eluted in a single peak.

Determination of anti-fungal activity

Potato Dextrose Agar (infusion from 200 g of potatoes + 20 g dextrose and 15 g agar in 1000 ml of distilled water) was prepared. The solution was heated to boiling to dissolve the medium completely. The media and glass wares were sterilized by autoclaving at 121 ºC at 15 psi for 15 min. The molten media was poured into two sterilized petriplates. The inoculums of *Trichoderma viridae* and *Aspergillus niger* were prepared by serial dilution. 0.1 ml of the 10-4 dilution sample was spread-plated over the solidified agar under aseptic conditions. The purified *Mucuna pruriens* trypsin inhibitor discs were prepared by adding 0.1 ml of the enzyme drop-wise onto a 2 cm Whatman filter paper disc and allowing to air dry. The discs were placed on the centre of the inoculated petriplate and incubated at room temperature for 3days. The diameters of the inhibition zones were measured.

RESULTS AND DISCUSSION

Protease inhibitors were purified and characterized from different sources including plants, animals, and microorganisms. There is a

growing interest in the identification of novel protease inhibitors because of their involvement in many biological processes including their potent activity in preventing carcinogenesis both in vivo and in vitro systems and their use in developing pest resistance in otherwise susceptible plants[21, 22, 23]. In the present study, we described the purification and antifungal activity of novel trypsin inhibitor from the seeds of *Mucuna pruriens*. *Mucuna pruriens* trypsin inhibitor was purified employing conventional protein purification methods such as ammonium sulphate fractionation, CM-cellulose chromatography and Sephadex G-100 chromatography. The trypsin inhibitor activity was eluted from the gel-permeation chromatography in a single peak (Fig. 1). The specific activity of the crude trypsin inhibitor extract towards bovine trypsin was 4.77 and 474.66 was obtained for purified trypsin inhibitor. Similarly, the fold purification of purified trypsin inhibitor after gel-filtration chromatography was 99.51 and yield obtained was 22.08%.

Anti-fungal activity of MPTI was tested on two fungi: *Aspergillus niger* and *Fusarium moniliforme* hyphae, using the disc method as described earlier. An inhibition zone was observed

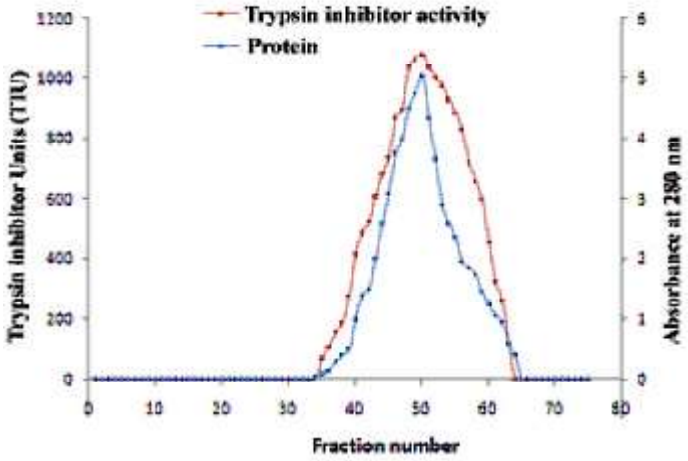
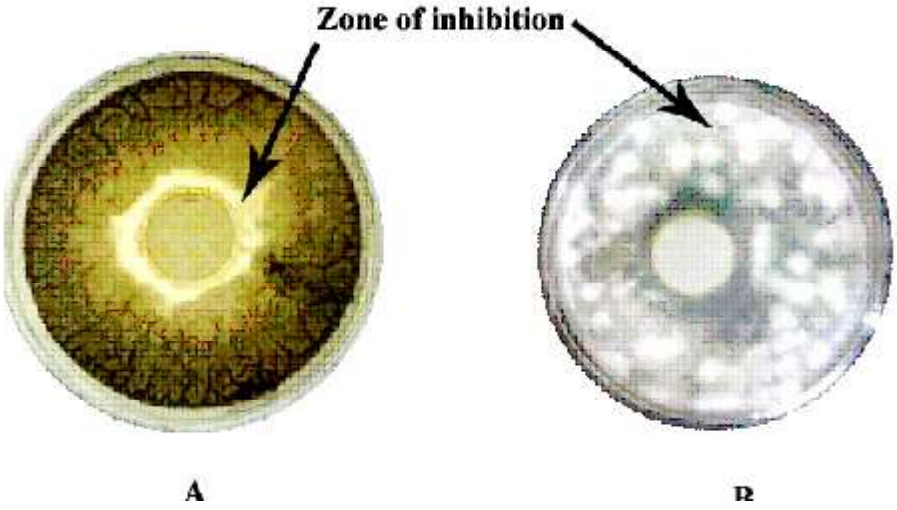


Fig. 1. Elution profile of CM-cellulose fraction from the soaked seeds of *Mucuna pruriens* on Sephadex G-100 (1.13 x 100 cm)



A - *Aspergillus niger* B - *Fusarium moniliforme* hyphae

Fig. 2. Antifungal fungal activity of purified trypsin inhibitor

around both discs, each containing 25 MPTI units. The normal growth and development was suppressed by *Mucuna pruriens* trypsin-chymotrypsin inhibitor (Fig. 2). Antifungal activity of protease inhibitors isolated from *Acacia plumose* was reported by Lopes et al., (2009)[4] and they observed antifungal activity of ApTI against *Aspergillus niger* and *Fusarium moniliforme* hyphae. A 18 kDa leguminous trypsin-chymotrypsin inhibitor Limenin with antifungal activity fron *Phaseolus limensis was* isolated and purified employing a combination of extraction, ammonium sulfate precipitation, ion exchange chromatography on SP-Toyopearl and high performance liquid chromatography (HPLC) on Mono S. The isoelectric point of the purified trypsin inhibitor was 7.6. It exhibited antifungal activity towards *Botrytis cinerea, Alternaria alternata(Fr.) Keissl, and Pythium aphanidermatum*[24]. A Bowman - Birk type trypsin inhibitor from wheat kernel and its antifungal activity was studied. It showed strong antifungal activity against a number of pathogenic fungi and in vito studies proved that wheat kernel trypsin inhibitor (WTI) inhibited spore germination and hyphal growth of pathogens [25]. Many research groups have validated the role of plant trypsin inhibitors (TIs) as a means of plant defense against fungal infection. A trypsin inhibitor from maize was isolated and its antifungal activity against *Aspergillus flavus* was studied. *Aspergillus flavus* is a severe agricultural problem in the southern United States and aflatoxins produced by *A. flavus* are carcinogenic to humans and animals upon ingestion. Antifungal activity of trypsin isolated from maize proved that maize trypsin inhibitor is potential source of resistance to aflatoxin accumulation in maize[26]. Trypsin inhibitor from *Nicotiana tabacum* was purified and tested for antifungal activity. The results revealed the antifungal activity against *Rhizoctonia solani, Rh. Nigricans and P.parasitica var. nicotianae*. Many phytopathogenic fungi are known to produce extracellular proteinases which play an important role in the pathogenicity, virulence and development of deseases. Plants synthesize inhibitory proteins in response to proteinases secreted by phytopathogens and prevent the infection by suppressing the activity of proteinases[2]. Basesd on our results, we propose that when *Mucuna pruriens* is challenged with phytopathogens, *Mucuna* trypsin-chymotrypsin inhibitor inhibits the extracellular protein secreted by phytopathogens, leading to the inhibiton of hyphal growth of phytopathogens.

CONCLUSION

The purification trypsin inhibitor from seeds of *Mucuna pruriens* has been successfully carried out using ammonium sulphate fractionation, ion exchange chromatography and gel-filtration chromatography. Its inhibition towards both bovine trypsin and chymotrypsin suggested that it is BBI type protease inhibitor. Its biological properties such as antifungal activity suggested that it is a potent agent to control unwanted proteolyic processes and as a biopesticide or insecticide in transgenic commercial and food plants

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REFERENCES

1. Whitby, S.N. the potential use of plant pathogens against crops. *Microbes infect*, 2001; **3**: 73 – 80.
2. Hao Huang, Sheng-Dong Qi, Fang Qi, Chang-Ai Wu, Guo-Dong Yang and Cheng-Chao Zheng. NtKTI1, a Kunitz trypsin inhibitor with antifungal activity rom *Nicotiana tabacum*, plays an important role in tobacco's defense response. *The FEBS journal*, 2010; **277**: 4076 – 4086.
3. Chen, Z.Y., Brown, R.L., Lax, A.R., Cleveland,T.E., Russin, J.S. Inhibition of plant pathogenic fungi by a corn trypsin inhibitor over-expressed in Escherchia coli. *Appl.Environ.Microbiol*., 1999; **65**:1320 – 1324.
4. Lopes, J. L. S., Valadares, N. F., Moraes, D. I., Rosa, J. C., Araujo, H. S. S., & Beltramini, L. M. Physico-chemical and antifungal properties of protease inhibitors from Acacia plumose.

Phytochemistry, 2009; **70**: 871-879.

5. Jin-Young Kim, Seong-Cheol Park, Indeok Hwang, Hyeonsook Cheong, Jae-Woon Nah, Kyung-Soo Hahm, Yoonkyung Park. Protease inhibitors from plants with antimicrobial activity. *International journal of molecular sciences*, 2009; **10**: 2860 – 2872.
6. Siddhuraju, P., Becker, K., Makkar, H. P. S. Studies on the nutritional composition and antinutritional factors of three different germplasm seed materials of an underutilized tropical legume. *Mucuna pruriens* var. utilis. *Journal of Agricultural Food Chemistry*, 2000; **48**: 6048-6060.
7. Siddhuraju, P., Becker, K. Preliminary evaluation of Mucuna seed meal (*Mucuna pruriens* var. utilis) in common carp (*Cyprinus carpio* L.): An assessment by growth performance and feed utilization. *Aquaculture*, 2001a; **196**: 105-123.
8. Bressani, R. Factors influencing nutritive value in food grain legumes: *Mucuna* compared to other grain legumes. In: Food and Feed from *Mucuna*: Current Uses and the Way Forward (Editors, Flores B M, Eilittä M, Myhrman R, Carew L B and Carsky R J), Workshop, CIDICCO, CIEPCA and World Hunger Research Center, Tegucigalpa, Honduras, 2002; 164 -188.
9. Chandrashekharaiah, K.S., Ramachandra Swamy, N.,. Siddalinga Murthy, K.R. Carboxylesterases from the seeds of an underutilized legume, Mucunapruriens; isolation, purification and characterization. *Phytochemistry*, 2011; **72**: 2267 – 2274.
10. Caius, J.F. The Medicinal and Poisonous Legumes of India. Scientific Publishers, Jodhpur, India, 1989; 70-71.
11. Warrier, P. K., Nambiar, V. P. K. and Ramankutty, C. Indian Medicinal Plants, A Compendium of 500 Species. 1996; 4, Orient Longman Limited, Madras, India.
12. Shaw, B. P. and Bera, C. H. A preliminary clinical study to cultivate the effect of vogorex-SF in sexual disability patients. *Indian Journal of Internal Medicine*, 1993; **3**: 165 - 169.
13. Prakash, D. and Tewari, S. K. Variation on L-DOPA content in *Mucuna* species. *Journal of Medicinal and Aromatic Plant Science*, 1999; 21: 343 - 346.
14. Farooqi, A. A., Khan, M. M. and Asundhara, M. Production Technology of Medicinal and Aromatic Crops. 1999; Natural Remedies Private Limited, Bangalore, India.
15. Shibata, H., Hara, S., Ikenaka, T., Abe, J. Purification and characterization of protease inhibitors from winged bean (*Psophocarpus tetragonolobus* (L.) DC.) seeds. *Journal of Biochemistry*, 1986; **99**: 1147 – 1155.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. Protein measurement with the folin-phenol reagent. *J. Biol. Chem*, 1951; **193**(1): 265-275.
17. Kakade, M. L., Simons, N. R. & Liener, I. E. The evaluation of natural vs. synthetic substrates for measuring the antitryptic activity of soybean samples. *Cereal Chem*., 1969a; **46**: 518-526.
18. Kakade, M. L., Simons, N.R. & Liener, I. E. The molecular weight of Bowman and Birk soybean protease inhibitor. *Biochim. Biophys. Acta*, 1969b; **200**: 168-169.
19. Reisfield, R. A., Lewis,V. J., Williams, D. E. Disc electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature*, 1962; **195**: 281-283.
20. Filho, J.X., Moreira, R.A. Visualization of proteinase inhibitors in SDS-Polyacrylamide gels. *Anal. Biochem,* 1978; **84**: 296-303.
21. Kennedy, R. C. The Bowman-Birk inhibitor from soybeans as an anticarcinogenic agent. *Am. J. Clin. Nutr*, 1998; **68**: 1406 -1412.
22. Jouanin, L., Bonade´-Bottino, M., Girard, C., Morrot, G., Giband, M. Transgenic plants for insect resistance. *Plant Sci*, 1998; **131**: 1–11.
23. Schuler, H., T., Poppy, M. G., Denholm. L. Insect-resistant transgenic plants. *Trends Biotechnol,* 1998; **16**: 168–174.
24. Wang Shaoyun, Rao Pingfan. A leguminous trypsin-chymotrypsin inhibitor Limenin with antifungal activity fron Phaseolus limensis. *European Food Research and Technology*, 2010; **231**(2): 331-338.
25. Chilosi, G., Caruso, C., Caporale, C., Leonardi, L., Bertini, L., Buzi, A., Nobile, M., Magro, P., Buonocore, V. Antifungal Activity of a Bowman–Birk type Trypsin Inhibitor from Wheat Kernel. *Journal of Phytopathology,* 2000; **148**(7 8):4 77 – 481.
26. Baker, R. L., Brown, R. L., Z-Y Chen, Cleveland, T. E., Fakhoury, A. M. A maize trypsin inhibitor (ZmTIp) with limited activity against Aspergillus flavus. *Journal of food protection*, 2009; **72**(1): 185-188.