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Physico-chemical and Antifungal Properties of Trypsin Inhibitor from the Seeds of *Mucuna pruriens*

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ABSTRACT

The trypsin inhibitor has been purified to apparent homogeneity from the seeds of *Mucuna pruriens* employing ammonium sulfate fractionation, cation exchange chromatography on CMcellulose and gel-permeation chromatography on Sephadex G-100. The purified *Mucuna pruriens* trypsin inhibitor (MPTI) showed a specific inhibitor activity of 474.66, fold purity of 99.51 and the yield obtained was 22.08%. The homogeneity of the purified preparation was confirmed by PAGE, IEF and SDS–PAGE. The molecular weight determined by gel-permeation chromatography and SDS–PAGE were 12 and 11.6 kDa respectively. The pI of MPTI was found to be 5.6 ± 0.05. MPTI was found to be stable at all temperatures lying between 0 and 90 ºC and pH 2.0 – 10 and hence exhibited high stability. The MPTI was inhibited both trypsin and chymotrypsin showing the double headed nature. Antifungal studies showed inhibitory activity of MPTI against *Aspergillus niger* and *Trichoderma viridae.*

Key words: *Mucuna pruriens*, Trypsin inhibitor, Purification, Characterization, Antifungal properties.

INTRODUCTION

Protease inhibitors (PIs) are proteins or peptides capable of inhibiting the catalytic activity of proteolytic enzymes and are widely distributed in plants, animals and microorganisms. Plants are the most abundant sources of PIs of which most of them studied and characterized were serine protease inhibitors (Rao and Suresh, 2007). These PIs are concentrated in seeds and tubers of plants belonging to Gramineae, Leguminosae and solanaceae families (Connors *et al*., 2002). Among the seed legumes, two major families of PIs, Bowman – Birk inhibitors (BBI) and Kunitz type inhibitors (KTI) have been studied extensively (Lingaraju and Gowda, 2008). A Bowman – Birk inhibitor (BBI) was first isolated from soybeans by Bowman and its biochemical properties were studied by Birk (Birk, 1985). Subsequently, many BBIs have been isolated and characterized from legumes (only Fabaceae family), gramineae and many other plants (Ikenaka and Norioka, 1985).

PIs play essentials roles in biological system including the blood coagulation system, compliment cascade, apoptosis, cell cycle and hormone processing pathways (Lingaraju and Gowda, 2008). They are also involved in the

treatment of human pathologies such as inflammation, hemorrhage (Oliva *et al*., 2000) and cancer (Kennedy, 1998). Plant seeds are rich sources of proteinacious protease inhibitors, which impair the nutritional quality by reducing protein digestibility and absorption (Liener and Kakade, 1980). Plant PIs also play essential role in the regulation of endogenous proteinases and involved in defense mechanisms against insects, fungi and other pathogenic microorganisms (Valueva and Mosolov, 1999; Carlini and Grossi-de-sa, 2002; Kim *et al*., 2005; Breiteneder and Radauer, 2004). Interest in understanding the physiological importance of PIs has increased due to their involvement in regulation of many biological processes that involved preoteolytic enzymes such as intracellular protein breakdown, transcription, cell cycle, cell invasion (Kataoka, Itoh & Koono, 2002) and apoptosis (Thompson & Palmer, 1998; Fumagalli *et al*., 1996; Kato, 1999). More recent studies indicated that PIs have been employed as new drugs in highly active antiretroviral combination therapy (HAART), increasing life expectancy in HIV-positive patients (Asztalos *et al*., 2006; Yeni, 2006; Lopes *et al*., 2009).

The genus Mucuna belongs to the family Fabaceae (Leguminoceae) which contains up to 150 species of annual and perennial legumes of pan tropical distribution. Mucuna is extensively used as cover crop to control insects and weeds in agriculture. Mucuna pods are covered with reddish-orange hairs, which readily dislodge and cause intense skin irritation and itch due to presence of a chemical called Mucunain. Many varieties and accessions of the wild legume, Mucuna are in great demand in food and pharmaceutical industries. The nutritional importance of Mucuna seeds as a rich source of protein supplement in food and feed has been well documented (Siddhuraju, Becker and Makkar, 2000; Siddhuraju and Becker, 2001; Bressani, 2002, Chandrashekharaiah, Ramachandra Swamy and Siddalinga Murthy, 2011). It is used in Ayurvedic medicine. The plant and its extracts have been long used in tribal communities as a toxin antagonist for various snakebites. Research on its effects against *Naja* spp. (cobra) (Tan *et al*., 2009), *Echis* (Saw scaled viper) (Guerranti, 1999), Calloselasma (Malayan Pit viper) and *Bangarus* (Krait) (Meenatchisundaram and Michael, 2010) have shown it has potential use in the prophylactic treatment of snakebites. *M. pruriens* seeds have also been found to have antidepressant properties in cases of depressive neurosis when consumed (Daniel, 2006) and formulations of the seed powder have shown promise in the management and treatment of Parkinson's disease (Katzenschlager *et al*., 2004). In view of the importance of PIs and seeds of *Mucuna* being a potential source of PIs, the present investigation was undertaken to study the biological properties of protease inhibitors form the soaked seeds of *Mucuna pruriens*. In the present study, purification, characterization and properties of trypsin inhibitor isolated from the soaked seeds *Mucuna pruriens* has been described.

MATERIALS AND METHODS

Materials

Seeds of *Mucuna pruriens* were collected from Siddarabetta, Tumkur District and Thorekempohalli, Nelamangala Taluk, Bangalore Rural District, Karnataka, India. Trypsin, Chymotrypsin, N-benzoyl-L-arginine pnitronilide (BAPNA), N-acetyl-DL-phenylalanine ± naphtyl ester (APNE), BSA, ampholites (pH 3 – 10), acrylamide and bis-acrylamide 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 according to the reported method of Shibata *et al*., (1986) using BAPNA as substrate. Trypsin was dissolved in 0.001 N HCl containing 20 mM CaCl2 at a concentration 200 ¼g per ml. The assay mixture containing 100 ¼l of the trypsin solution, 900 ¼l 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 according to the reported method of Shibata *et al*., (1986) using BAPNA as substrate by estimating the remaining hydrolytic activity of trypsin. The assay mixture containing 100 ¼l of trypsin solution, 400 ¼l 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), 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 according to the method of Kakade *et al*. (1969a). 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 according to the method of Kakade *et al*. (1969b). 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 according to the procedure of Reisfield *et al*. (1962). 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 ² 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. SDS–PAGE (10% T, 2.7% C) was performed after denaturing the proteins with SDS and ²-mercaptoethanol. Gel-electrofocussing was performed by the method of Wrigley (1969), in 8% polyacrylamide gels. The electrophoresis was performed at 4 °C for 2 h. After the run, the gels were removed and stained for esterase activity as described earlier. The gels were stained for proteins using 0.02% coomassie brilliant blue G-250 (w/v) in 3.5% (w/v) perchloric acid and destained in distilled water.

Gel localization of Trypsin Inhibitors

Visualization of trypsin and chymotrypsin inhibitor in polyacrylamide gel was performed according to Filho and Moriera (1978). After electrophoresis, native gel was incubated in 0.1 M Sodium Phosphate buffer, pH 8 containing Trypsin (40 ¼g/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

performed at 4 ^{0}C unless otherwise stated. To the crude trypsin inhibitor extract, solid ammonium sulphate was added to 0–80% saturation at 4 ^{0}C. The precipitate obtained was removed by centrifugation at 10,000 rpm for 30 min. The precipitate thus obtained was redissolved in 0.025 M sodium acetate buffer, pH 5.0 and dialyzed against the same buffer. The dialyzed fraction was loaded onto a CM-cellulose column (2.5 x 22 cm) pre-equilibrated in 0.025 M sodium acetate buffer, pH 5.0 at a flow rate of 30 ml/h. 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.

Molecular weight determination

The apparent molecular mass of the native enzymes were determined according to the method of Andrews (1970) using Sephadex G-100 (1.13 x 100 cm) pre-equilibrated with 0.025 M sodium phosphate buffer pH 7.0, at a flow rate of 10 ml/h. The column was calibrated using cytochrome-c (12.3 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (BSA) (66 kDa), alcohol dehydrogenase (150 kDa) and b-amylase (200 kDa). Blue Dextran (2000 kDa) was used to determine the void volume (Vo). The molecular weight of the trypsin inhibitor was determined from the plot of log molecular weight versus K_{av}. The molecular weight of the purified trypsin inhibitor was also determined by SDS–PAGE from the plot of log molecular weight against relative mobility.

Effect of pH and temperature

Determination of pH stability

The effect of pH on the activity of the purified *Mucuna pruriens* trypsin inhibitor was studied using the buffers, Glycine – HCl buffer (0.2 M, pH 2), Sodium acetate buffer (0.2 M, pH 4), Sodium citrate buffer (0.2 M, pH 5.5), Sodium phosphate buffer (0.2 M, pH 6.5), Tris - hydrochloride buffer (0.2 M, pH 8.0), Sodium borate buffer (0.2 M, pH 10). The pH stability was determined by pre-incubating the purified *Mucuna pruriens* trypsin inhibitor (MPTI) with above buffers for 30 min. Trypsin inhibitor assay was performed as described earlier.

Determination of temperature stability

The effect of temperature on the activity MPTI was studied at different temperatures ranging between 0 - 90 ºC. The temperature stability of purified MPTI was studied by pre-incubating, the purified MPTI at different temperatures (0 - 90 ºC) for 30 min. The incubated samples were rapidly cooled and assayed at room temperature. Trypsin inhibitor assay was performed as described earlier.

Determination of IC_{50}

The inhibitory activity of purified *Mucuna pruriens* trypsin inhibitor against trypsin was determined using increasing concentrations of MPTI. Trypsin inhibitor assay was performed as described earlier and a graph of percentage residual trypsin activity versus concentration of MPTI (nM) was plotted and the IC50 value was determined.

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 DISCUSSIONS

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 (Kennedy, 1998; Jouanin *et al*. 1998; Schuler *et al*. 1998). In the present study, we described the isolation, purification and characterization of novel trypsin inhibitor from the seeds of *Mucuna pruriens.* MPTI was purified to homogeneity using conventional protein purification methods such as ammonium sulphate fractionation, ion exchange chromatography using CM-cellulose and gel-permeation on Sephadex G-100. The ammonium sulphate fractionation resulted with a specific activity of 8.025 and yield of 77.35 %. The ammonium sulphate fraction was subjected to ion exchange chromatography on CM Cellulose and resulted in the elution of three peaks of inhibitor activities. The elution profile of CM Cellulose chromatography is shown in the Fig.1a. Three peaks of trypsin inhibitor activities eluted were designated as fraction I, fraction II and fraction III. Fraction I was not adsorbed onto the column and eluted with the starting buffer. Fraction II was eluted by 0.1 M NaCl and fraction III by 0.3 M NaCl in starting buffer. The CM Cellulose fraction III containing appreciable level of inhibitory activity was pooled separately and dialyzed against double distilled water and then concentrated using ammonium sulphate. The concentrated CM Cellulose fraction III was subjected to gel filtration on Sephadex G-00 column. The elution profile of Sephadex G-75 chromatography is as shown in the Fig. 1b. The trypsin inhibitor activity was eluted in a single peak. 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% (Table 1).

Criteria of homogeneity

The homogeneity of the purified trypsin inhibitor was established by native PAGE (Fig. 2a), Isoelectric focusing (Fig. 2b) and SDS PAGE (Fig. 2c). Native PAGE of the peak fraction of sephadex G-100 showed single trypsin inhibitor (Fig. 2a (i)) corresponding to a single protein band (Fig.2a (ii)). IEF of MPTI showed a single trypsin inhibitor corresponding to a single protein band. SDS–PAGE in the presence and absence of ²-mercaptoethanol showed single protein bands, suggesting the monomeric nature of the Mucuna pruriens trypsin inhibitor.

Molecular weight

The molecular weight of MPTI as determined by SDS–PAGE was found to be 11.6 kDa both in the presence and absence of ²-mercaptoethanol. This clearly indicates the monomeric nature of MPTI. The monomeric nature of the purified *Mucuna pruriens* trypsin inhibitor (MPTI) was further confirmed by gel-filtration on Sephadex G-100 which indicated molecular weight of 12 kDa. A Bowman-Birk proteinase inhibitor from

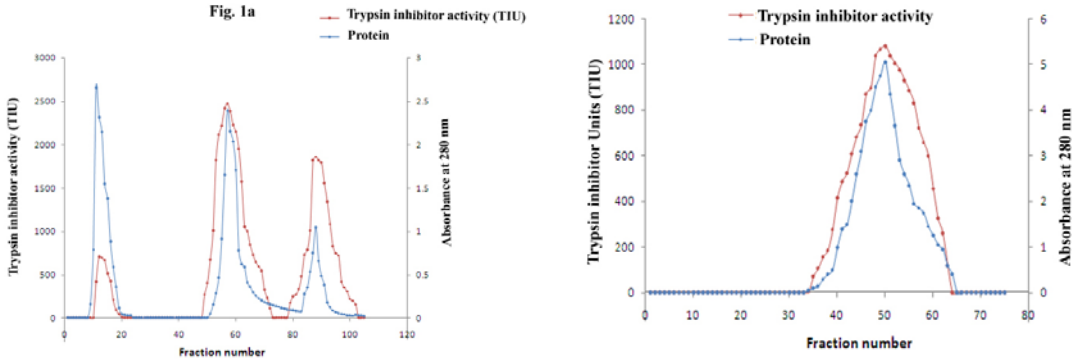

FIG. 1 (a) Elution profile of Trypsin inhibitor from the soaked seeds of *Mucuna pruriens* on CM-Cellulose using 0.025 M sodium acetate buffer pH 5.0 (b) Elution profile of CM-cellulose fraction-II from the soaked seeds of *Mucuna pruriens* on Sephadex G-100 (1.13 x 100 cm)

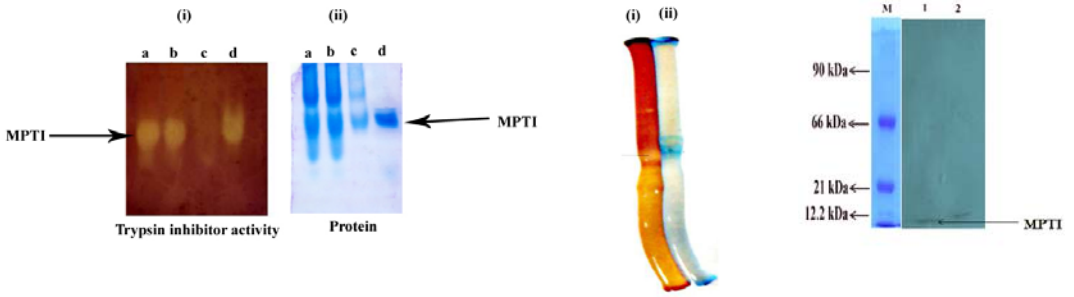

FIG. 2: (a) Native PAGE pattern of Mucuna pruriens trypsin inhibitor (i) and protein (ii) a – Crude, b- ammonium sulphate fractuion, c- ion exchange fraction, d – Gel filtration fraction (b) IEF of purified Mucuna pruriens trypsin inhibitor (i) Trypsin inhibitory activity (ii) Protein (c) SDS – PAGE pattern of (M) standard proteins (1) purified Mucuna pruriens trypsin inhibitor in the presence and (2) absence of ²-mercaptoethanol

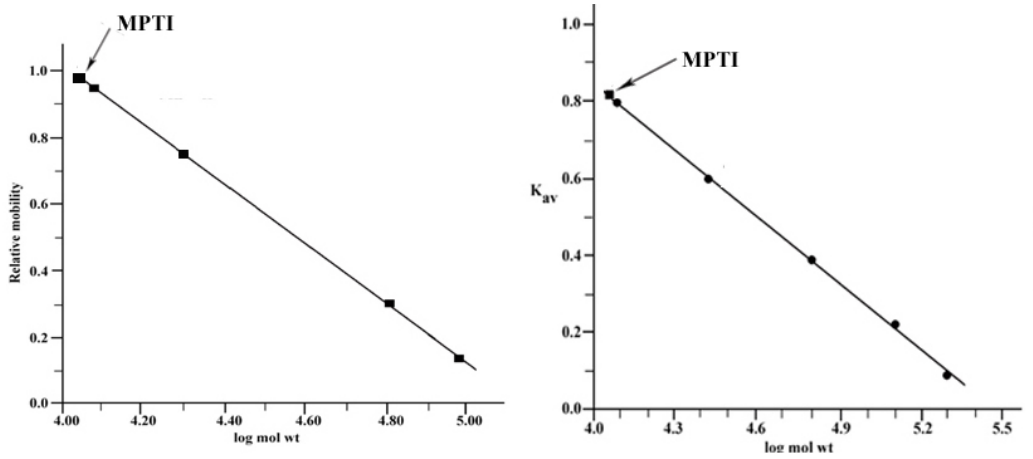

FIG. 3. (a) Determination of molecular weight of purified trypsin inhibitor by gel filtration chromatography (b) Determination of molecular weight of purified trypsin inhibitor by SDS – PAGE

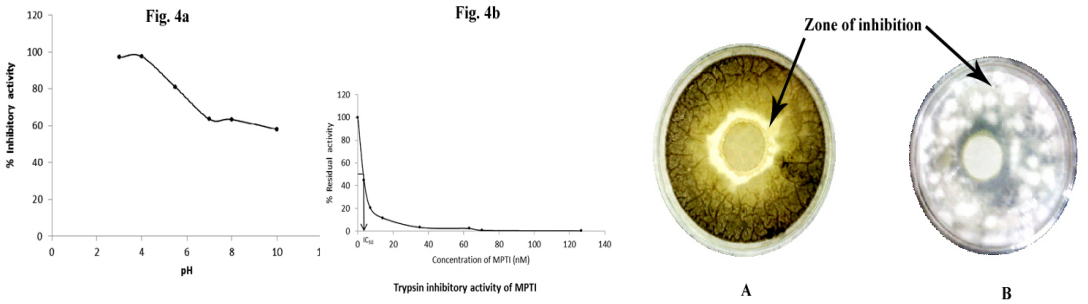

FIG. 4. (a) Effect of pH on the stability of purified trypsin inhibitor (b) Determination of IC_{50} (c) Antifungal fungal activity of purified trypsin inhibitor
A - Aspergillus niger B - Fusarium moniliforme hyphae

the seeds of *Vigna mungo* purified and characterized by Prasad *et al*. (2010) had a molecular weight of 8 kDa on an 18 % gel under non-reducing conditions. Scarafoni *et al*. (2008) purified a Bowman-Birk trypsin inhibitor from *Lupinus albus* seeds which consisted of a single polypeptide chain having a molecular weight of 6 kDa. Torres Castillo *et al*. (2009) purified and characterized a highly stable trypsin like proteinase inhibitor from the seeds of *Opuntia sterptacantha* which showed a low molecular mass of 6.2 kDa. The results for MPTI is also comparable with protease inhibitor from *Lupinus bogotensis* seeds purified and characterized by Diana Molina *et al*. (2010) which was a single polypeptide chain having a molecular weight of 12 kDa. Generally, the Bowman – Birk type inhibitor has a lower molecular weight compared with Kunitz type. The Kunitz type inhibitors have a molecular weight of >20 kDa with low cysteine content and a single reactive site, whereas the Bowman – Birk type inhibitors have a molecular weight of 8 – 10 kDa , as well as a high cysteine content and two reactive sites (Richardson, 1977). Two protease inhibitors of Bowman – Birk type with molecular weights of 15,000 and 10,500 kDa were found in pigeon pea (Godbole *et al*., 1994). From the result, Mucuna pruriens trypsin inhibitor belongs to Bowman – Birk type. However, the amino acid sequence is needed to verify the classification of this inhibitor.

Isoelectric Point

The isolelectric pH of purified MPTI was 5.6±0.05 and it showed binding affinity to a cation exchanger at pH 5.0. Maria Ligia R Macedo *et al*. (2000) purified and characterized a trypsin inhibitor form *Dimorphandra mollis* seeds. The pI values the three isoforms were found to be 5.6, 5.8 and 5.9, comparable to that of MPTI. A highly stable trypsin-like proteinase inhibitor purified and characterized by Torres Castillo *et al*. (2009) from the seeds of *Opuntia sterptacantha,* possessed acidic pI value of 4.5. Bowman-Birk proteinase inhibitors with pI values of 4.3, 4.4, 5.0, 5.3 and 6.0 were purified and characterized by Prasad *et al*. (2010) from the seeds of *Vigna mungo.*

Effect of pH and temperature

The activity of MPTI was found to be stable in the pH range 2 - 10. The inhibitory activity of MPTI was found to be almost same in temperatures ranging from 0-90 ºC. The inhibitory activity was found to be 76.77 % at 0 ºC and 73.42 % at 90 ºC. Scarafoni *et al*. (2008) purified a Bowman-Birk trypsin inhibitor from *Lupinus albus* seeds which showed no significant change in the inhibitory activity after subjecting to various pH and temperature conditions. Trypsin inhibitor from Thai mung bean was also stable between pH 2 - 10. The thermal stability of MPTI is comparable to that of LaBBI. . Torres Castillo *et al*. (2009) purified and characterized a highly stable trypsin-like proteinase inhibitor from the seeds of *Opuntia sterptacantha* which retained inhibitory activity at a temperature of 120 ºC and pH ranging from 3-7. Thermal stability of MPTI was comparable with trypsin inhibitor isolated from Thai mung bean which was stable between 0 – 100 ^{0}C (Sappasith Klomklao *et al*., 2011).

Half-Maximal Inhibitor Concentration

A graph of percentage residual trypsin activity versus concentration of MPTI (nM) was plotted and the IC50 value was found to be 3.6 ± 0.1 nM. A Kunitz trypsin inhibitor from *Entada scandens* seeds purified by Lingaraju *et al*. (2008) was obseverd to have an IC50 value of 23.4 ± 0.001 nM which is higher when compared to that of MPTI.

Anti-Fungal Activity

Anti-fungal activity of MPTI was tested on two fungi: *Aspergillus niger* and *Trichoderma viridae*, using the disc method as described earlier. An inhibition zone was observed around both discs, each containing 25 MPTI units. The normal growth and development was suppressed by MPTI. Antifungal activity of protease inhibitors isolated from *Acacia plumose* was reported by Lopes *et al*., (2009) and they observed antifungal activity of ApTI against *Aspergillus niger* and *Fusarium moniliforme* hyphae.

CONCLUSION

The identification, purification and characterization of a low molecular weight trypsin inhibitor (MPTI) from seeds of *Mucuna pruriens* has been successfully carried out using ammonium sulphate fractionation, ion exchange chromatography and gel-filtration chromatography. This inhibitor is an acidic, 11 – 12 kDa protein. Its

low molecular mass is similar to other trypsin inhibitors previously characterized from plant sources. This inhibitor showed high resistance to heat denaturation and extremes of pH. Hence it is thermo and pH stable inhibitor. Its inhibition towards both bovine trypsin and chymotrypsin and low molecular weight 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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