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Research Article

CHARACTERIZATION OF MONOHEADED TRYPSIN INHIBITORS FROM THE SEEDS OF *ABELMOSCHUS MOSCHATUS* L.

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ABSTRACT

Objective: The objective of the present study was to characterize the monoheaded trypsin inhibitors, *Abelmoschus moschatus* trypsin inhibitor-I (AMTI-I) and AMTI-II from the seeds of *A. moschatus* with respect to their specificity, mode of action, and active site residues.

Methods: Standard methods were followed in determining inhibitory activities of monoheaded inhibitors. IC_{50} values and inhibitory constants (Ki) of AMTI-I and AMTI-II were determined. Studies on complex formation and chemical modification of inhibitors were performed.

Results: AMTI-I and AMTI-II were found to be serpins, strongly active against trypsin, moderately active against porcine elastase, *Staphylococcus aureus* protease, and *Aspergillus oryzae* protease. AMTI-I and AMTI-II have shown non-competitive type of inhibition toward bovine trypsin with K_i values of inhibitors for trypsin found to be 0.25±0.02 nM and 0.22±0.06 nM, respectively. Complex studies revealed the formation of stable 1:1 complex of trypsin with both AMTI-I and AMTI-II. Chemical modification of the functional groups of the inhibitors by selective reagents indicated that arginine residues are essential for their trypsin inhibitory activities.

Conclusion: Investigations on the specificity of protease inhibitors are important for understanding their physiological role, control mechanisms involved in the regulation of proteolysis in biological systems and mode of action.

Keywords: Trypsin inhibitors, *Abelmoschus moschatus*, Serpins, Non-competitive, Monoheaded inhibitors.

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INTRODUCTION

Protease inhibitors, proteins which reversibly, stoichiometrically, and competitively inhibit the catalytic activity of proteolytic enzymes, are widely distributed in nature. These inhibitors interact with their target proteases and form stable protease-inhibitor complexes that are incapable of enzymatic activity [1]. Apart from regulating physiological processes through controlling protease activities, protease inhibitors are also involved in defense mechanisms in plants [2]. The inhibitors also find application in pharmacological and medical fields as therapeutic agents in the treatment of wide range of disorders associated with enhanced proteolytic activities [3], HIV therapy [4], and cancer [5]. Considering their numerous applications, search for multifunctional protease inhibitors with broad spectrum of biological properties is of great interest from plant sources need to be continued for effectively exploiting them in the above areas. Ever since they are recognized as antinutritional factors, protease inhibitors have been isolated and characterized from a number of plant sources [6-13]. These inhibitors may be constitutive or induced on a pathogen attack [14].

Serine protease inhibitors, particularly trypsin inhibitors, have been isolated from numerous plant species which exhibited resistance to insects, fungi, bacteria, and viruses [5,15,16]. Among them, the Kunitz [17,18] and the Bowman–Birk type's [19,20] inhibitors are extensively studied. Kunitz trypsin inhibitors are usually 18–24 kDa heterogeneous proteins, with one or two polypeptide chains and low cysteine content, usually with four Cys residues arranged into two disulfide bridges with a single trypsin reactive site which is generally an arginine residue located in one of the protein loops. On the other hand, Bowman–Birk inhibitors are smaller in size (8–10 kDa), with seven disulfide linkages, high cysteine content, and two independent reactive sites for trypsin and chymotrypsin binding. Existence of multiple molecular forms of protease inhibitors has been reported in several plant tissues [2,21,22].

Two trypsin inhibitors (*Abelmoschus moschatus* trypsin inhibitor-I [AMTI-I] and AMTI-II) isolated and purified from the seeds of *A. moschatus* have been found to be homogenous by the criteria of native PAGE and gel filtration [23]. Investigations on the specificity of protease inhibitors are important for understanding their physiological role, control mechanisms involved in the regulation of proteolysis in biological systems and mode of action. This paper, therefore, present studies related to the specificity, mode of action, complex studies, and chemical modification of AMTI-I and AMTI-II.

MATERIALS AND METHODS

Purification of trypsin inhibitors

Trypsin inhibitors, AMTI-I and AMTI-II, were isolated and purified following ammonium sulfate fractionation, ion exchange chromatography on DEAE-cellulose, and gel filtration on Sephadex G-100 through the procedure described earlier [23].

Enzyme assay methods

Measurement of trypsin and trypsin inhibitory activity

Trypsin activity was assayed by the method of Kakade [24] using BAPNA as the substrate.

For trypsin inhibitory activity, trypsin (30 µg) was preincubated at 37°C for 10 min with aliquots of the inhibitor, and the residual trypsin activity was taken as an index of the inhibitory activity. Suitable controls were included to correct for the presence of endogenous proteinase activity in the extract. One trypsin unit is defined as an increase in 0.01 absorbance unit at 410 nm for trypsin under the assay conditions. One trypsin inhibitory unit is defined as the number of trypsin units inhibited under assay conditions.

Assay of chymotrypsin and chymotrypsin inhibitory activity

Esterolytic activity of chymotrypsin was assayed by the method of Prabhu and Pattabiraman [25] using ATEE as the substrate. For chymotrypsin inhibitory activity, assay mixture containing chymotrypsin (1 µg) was

preincubated at 37°C for 10 min with suitable aliquots of the inhibitor solution, and the residual trypsin activity was taken as an index of the inhibitory activity. One chymotrypsin unit (CU) is arbitrarily defined as the increase in 0.01 absorbance unit at 530 nm under the conditions of assay. One chymotrypsin inhibitory unit is defined as the number of CU inhibited under assay conditions.

Assay of elastase and elastase inhibitory activity

Esterolytic activity of elastase was assayed according to the method of Naughton and Sanger [26] using elastin Congo red as the substrate. For the assay of inhibitory activity, 25 µg of the enzyme was preincubated with 10–50 µg of inhibitors and the residual activity was measured.

Assay of papain and papain inhibitory activity

The proteolytic activity of papain was assayed according to the method of Arnon [27] using casein as the substrate. Inhibitory activity assay was carried out by incubating 20 µg of enzyme with 10–50 of the inhibitors before the addition of substrate.

Assay of pepsin and pepsin inhibitory activity

The activity of pepsin was assayed by the method of Anson [28] using denatured hemoglobin as the substrate. For the inhibitory activity assay, 20 µg of pepsin was incubated with 10–50 µg inhibitors in 1 ml of 1 mM HCl for 10 min before the addition of hemoglobin solution.

Assay of pronase and pronase inhibitory activity (Streptomyces griseus protease)

Amidolytic activity of pronase was determined using BAPNA as the substrate [24] by the same method described for the activity of trypsin. For inhibitory activity, the assay mixture contained varying amounts of the inhibitor (0.5–5.0 µg) and suitable aliquots of the enzyme in 2 ml of water. After preincubation for 10 min at 37°C, the residual enzyme activity was measured.

Assay of esterolytic and esterolytic inhibitory activity of subtilisin

The esterolytic activity of subtilisin was assayed using ATEE as the substrate based on the method suggested by Schwert and Takenaka [29]. The inhibitory activity assay was carried out by incubating 20 µg of enzyme and 5–40 µg of inhibitors in 1 ml of buffer for 5 min. The residual activity was assayed by adding the substrate and recording the change in absorbance at 237 nm.

Assay of thermolysin and thermolysin inhibitory activity

Caseinolytic activity of thermolysin was assayed according to the method of Matsubara [30]. For the assay of thermolysin inhibitory activity, thermolysin solution (4 µg) was incubated with 10–40 µg of inhibitors for 10 min before the addition of substrate and the residual thermolysin activity was assayed.

Assay of α-amylase and α-amylase inhibitory activity

Pancreatic α-amylase was assayed by the method of Saunders and Lang [31]. For the inhibitory activity assay, inhibitors (5–10 µg) in 0.1 ml of water were added to buffer solution containing 5 µg of pancreatic α-amylase**.** The mixture was made up to 2 ml with buffer and incubated at room temperature for 30 min and the residual α-amylase activity was assayed.

Units for pepsin, papain, pronase, subtilisin, thermolysin, and α-amylase

One enzyme unit is defined as the amount of enzyme that catalyzes the conversion of 1 µmole of substrate per min. One inhibitor unit is defined as the quantity of inhibitor that reduces the rate of conversion of substrate by 1 µmol/min under standard conditions**.**

Protein estimation

Protein was estimated by the method of Lowry *et al.* [32] using bovine serum albumin (BSA) as the standard.

Determination of IC_{50} and inhibitory constants (Ki) of AMTI-I and AMTI-II

The amidolytic activity of bovine trypsin (30 µg) was determined with various concentrations of BAPNA (0.8–5.0 µmol) in the absence of each of the inhibitor. The assays were then repeated in the presence of 10 µg, 15 µg, and 20 µg of inhibitors in the reaction mixture. Dixon plot analysis was employed to determine the constants of inhibition for bovine pancreatic trypsin with increasing concentrations of AMTI-I and AMTI-II. Kinetic analysis of AMTI-I and AMTI-II was carried out following the standard protocol, and the Ki values of the two inhibitors were calculated from Dixon plots [33].

Studies on complex formation

Isolation of trypsin-inhibitor complex

The trypsin-inhibitor complex was isolated by gel filtration on Sephadex G-200. A mixture of the inhibitor (2 mg) and trypsin (5 mg) in 0.1 M phosphate buffer, pH 7.6, was allowed to stand at room temperature for 15 min and then chromatographed on a Sephadex G-200 column (1.6 cm × 94 cm) at 4–6°C. The absorbance of the fractions at 280 nm was measured and the trypsin inhibitory activity in the fractions was determined.

Molecular weight determination of enzyme-inhibitor complexes

The molecular weights of trypsin-AMTI complexes on Sephadex G-200 column were determined using phosphorylase b (97 kDa), BSA (67 kDa), ovalbumin (45 kDa), and chymotrypsinogen A (25 kDa), soybean trypsin inhibitor (SBTI) (20.1 kDa) lysozyme (14 kDa) as standard proteins for calibration. The elution volume of the standard proteins was determined at 4–6°C. Molecular weight of enzyme-inhibitor complexes was calculated from the calibration curve.

Effect of dithiothreitol (DTT)

To determine the stability of trypsin inhibitors, AMTI-I and AMTI-II were incubated separately with an equal volume of 1 mM and 10 mM DTT in the same buffer for time intervals ranging from 20 to 180 min at 37°C. The reaction was terminated by adding iodoacetamide at twice the amount of each DTT concentration and the mixture was diluted with 20 mM Tris-HCl buffer, pH 8.2, and 20 mM $CaCl_2$, to achieve a final inhibitor concentration of 20 µg/ml. Residual trypsin inhibitory activity was evaluated after incubation at two different DTT concentrations followed by carbamidomethylation.

Chemical modification of inhibitors

Modification of amino groups by acetylation

Acetylation was done according to the method of rice [34] using acetic anhydride. Extent of acetylation was determined using trinitrobenzenesulfonic acid (TNBS) by the method of Haynes *et al*. [35].

Modification of arginyl residues

The arginyl residues in the inhibitor were chemically modified with 1, 2-cyclohexanedione according to the method of Liu *et al*. [36].

RESULTS

Purification of trypsin inhibitors

Two trypsin inhibitors (AMTI-I and AMTI-II) isolated and purified from the seeds of *A. moschatus* have been found to be homogenous by the criteria of native PAGE and gel filtration. The molecular weights of AMTI-I and AMTI-II, as determined by SDS-PAGE, were found to be 22.4 kDa and 21.2 kDa, respectively (Fig. 1). These are close to the corresponding values of 22.8 kDa and 22.4 kDa obtained for the inhibitors by gel filtration on Sephadex G-200. All the inhibitors after treatment with 2-mercaptoethanol showed a single sharp band on gels supporting the monomeric nature of the proteins. Yields of AMTI-I and AMTI-II were 11.21% and 16.81%, respectively. The inhibitors were quite stable up to 80°C for 10 min and were not affected at alkaline as well as acidic conditions tested. AMTI-I and AMTI-II were found to be glycoproteins with 2.8% and 4% carbohydrate content, respectively [23].

Determination of IC50 values of AMTI-I and AMTI-II against bovine pancreatic trypsin

The inhibition of the amidolytic activity of bovine pancreatic trypsin by the two isoinhibitors was linear up to 80% inhibition (Fig. 2). The IC_{50} (inhibitor concentration in µg required to cause 50% inhibition of enzyme activity) values for both AMTI-I and AMTI-II for trypsin inhibition were found to be 7.5 µg. On extrapolation, it was observed that 15.5 µg of AMTI-I and 15 µg of AMTI-II totally inhibited amidase activity of 30 µg of trypsin.

Specificity of AMTI-I and AMTI-II against proteolytic enzymes

The results of investigations on the inhibition specificities of AMTI-I and AMTI-II have shown that they are strongly active against trypsin, moderately active against porcine elastase, *Staphylococcus aureus* protease, and *Aspergillus oryzae* protease. The IC_{50} values of AMTI-I and AMTI-II for elastase inhibition were found to 20.7 µg and 20.1 µg, respectively. AMTI-I and AMTI-II had a strong effect on the caseinolytic activity of *Staphylococcus aureus* protease. An IC_{50} value of 75 µg was obtained for both AMTI-I and AMTI-II. These inhibitors, however, showed a moderate effect on *A. oryzae* proteinase with IC_{50} values of 100 µg for both AMTI-I and AMTI-II. The inhibitors had no effect on pepsin (carboxyl), papain (thiol), thermolysin (metalloproteinase), subtilisin, and pronase (Table 1).

From the results obtained, it is evident that the isoinhibitors specifically inhibited some serine proteinases of mammalian, bacterial, and fungal origin. IC_{50} values for the inhibitors varied depending on the proteinase inhibited. Thus, the isoinhibitors from the seeds of *A. moschatus* exhibited a narrow specificity being active only against serine proteinases and ineffective against other classes of proteinases.

30 µg of bovine trypsin was incubated with varying amounts of each inhibitor for 10 min at 37°C. The percentage residual enzyme activity was assayed using BAPNA as the substrate. The concentration of the inhibitor required to cause 50% inhibition of the enzyme activity was determined from the graph.

A: Inhibition of trypsin by AMTI-I

B: Inhibition of trypsin by AMTI-II.

*IC_{50} - inhibitor concentration in µg required to cause 50% inhibition of trypsin activity

Mode of inhibition of trypsin by AMTI-I and AMTI-II

Trypsin activity in the presence (10, 15, and 20 µg inhibitor) and absence of AMTI-I and AMTI-II was measured at different substrate (0.8–5 µmol BAPNA) concentrations. The inhibitory constant (*Ki*) value and mode of inhibition of AMTI-I and AMTI-II against bovine pancreatic trypsin were determined from Dixon plot. The results obtained from the kinetic studies showed that AMTI-I and AMTI-II exhibited non-competitive mode of inhibition toward bovine pancreatic trypsin and K_i values calculated from the Dixon plot of the two inhibitors were found to be 0.25±0.02 nM and 0.22 ± 0.06 nM, respectively (Fig.3). The low values of Ki for these inhibitors indicate a high affinity between enzyme and the inhibitors.

Inhibition of amidolytic activity of trypsin by trypsin inhibitors was done by incubating 30 µg of trypsin and BAPNA (0.8–5 µmole) with the reaction system containing 10, 15, and 20 µg of A - AMTI-I and B - AMTI-II. The reciprocals of the velocity were plotted against the inhibitor concentration and Ki value was obtained from the intercepts of two lines at different concentration of substrate.

(- ♦ -) with 10 µg of AMTI-I and AMTI-II, (- ■ -) with 15 µg of AMTI-I and AMTI-II, and (- ▲ -) with 20 µg of AMTI-I and AMTI-II.

Complex studies: Isolation of the trypsin-inhibitor complexes

A mixture of 5 mg of trypsin and 2 mg each of AMTI-I and AMTI- II were separately incubated at room temperature for 15 min. In this instance, excess trypsin was used to make sure that all the inhibitors are complexed and then a mixture was applied on to a column of Sephadex G-200 at 5°C, previously calibrated with the inhibitor, AMTI-II and trypsin run separately, give rise to 2 distinct A_{280} absorbing peaks

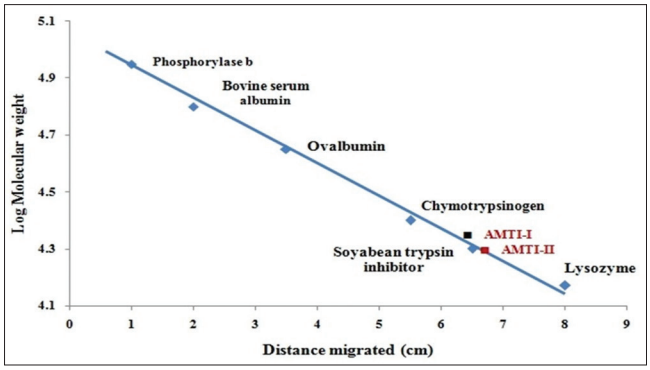

Fig. 1: Molecular weight determination of AMTI-I and AMTI-II by SDS-PAGE on 5–20% gradient slab gel. Plot of distance migrated against log molecular weight of standard proteins (♦) and AMTI - (■) and AMTI-II (■). Standard proteins: (i) Phosphorylase b, 97 kDa. (ii) BSA, 67 kDa. (iii) Ovalbumin, 45 kDa. (iv) Chymotrypsinogen A, 25 kDa. (v) SBTI, 20.1 kDa. (v) Lysozyme, 14 kDa

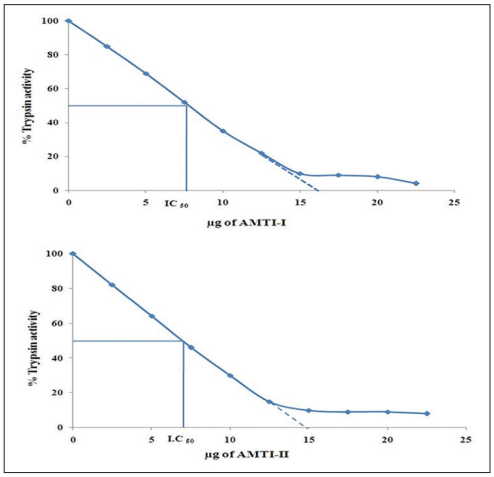

Fig. 2: Inhibitory activity of AMTI (IC_{50})

(Fig. 4). The peak I had an elution volume (41 ml) which is lower than that of free AMTI-II (67 ml). This fraction did not show any trypsin activity by itself nor was there any trypsin inhibitory activity associated with it. AMTI-I also formed complexes with trypsin in a manner analogous to that of AMTI-II (Results not shown).

Molecular weight determination of enzyme-inhibitor complexes

The molecular weight calculated for the trypsin-AMTI-II complex in peak I, based on the calibration curve for standard proteins (Fig.5) gave a value of 46 kDa. Molecular weight of 46 kDa was also obtained for trypsin-AMTI-I complex. This would mean a mole/mole interaction of AMTI-I and AMTI-II with bovine pancreatic trypsin.

Effect of DTT on AMTI-I and AMTI-II

Stability of trypsin inhibitors, AMTI-I and AMTI-II, was checked in the presence of DTT (1–10 mM). At 10 mM DTT, a meager decrease of trypsin inhibitory activity for AMTI-II was observed after 30 min of incubation at 37°C while at 1 mM DTT, the inhibitor completely retained its trypsin inhibitory activity (Fig. 6). Incubation of inhibitor for 180 min in the presence of 10 mM DTT resulted in the loss of 10–20% of its inhibitory activity. AMTI-II did not lose its trypsin inhibitory activity even after reduction with DTT suggesting that disulfide bonds are not needed for its inhibitory activity. Similar results were obtained with AMTI-I when treated with DTT.

2 ml fractions were collected at a flow rate of 10 ml/h. Protein was monitored by measuring the absorbance at 280 nm (-------). Trypsin activity (■------■).

Table 1: Specificity of AMTI-I and AMTI-II on various classes of proteinases

Enzyme	AMTI-I		AMTI-II	
	Inhibitory effect	IC_{50}* value	Inhibitory effect	IC_{50}* value
Trypsin	Positive	7.5	Positive	7.5
Chymotrypsin	Negative	-	Negative	-
Elastase	Positive	20.7	Positive	20.1
Pronase	Negative	-	Negative	-
Staphylococcus aureus protease	Positive	75	Positive	75
Subtilisin BPN	Negative	-	Negative	-
Subtilisin Carlsberg	Negative	-	Negative	-
Proteinase K	Negative	-	Negative	-
Aspergillus oryzae protease	Positive	100	Positive	100
Thermolysin	Negative	-	Negative	-
Papain	Negative	-	Negative	-
Pepsin	Negative	-	Negative	-

Negative: Inhibition not observed even at a concentration of 500 µg of the inhibitor. *IC_{50} value represents µg of inhibitor required to cause 50% inhibition of the enzyme activity

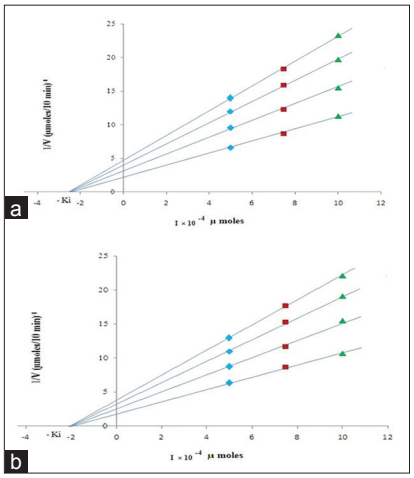

Fig. 3: (a and b) Dixon plot for the determination of inhibition constants (Ki) of AMTI-I and AMTI-II

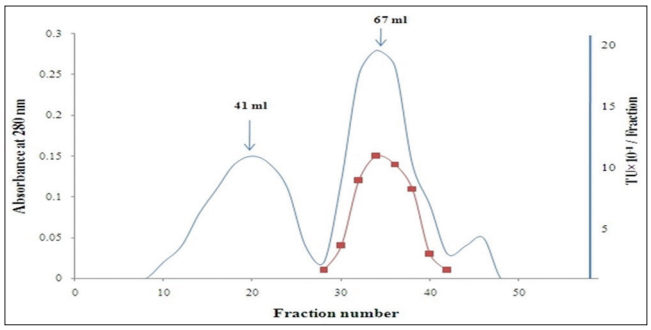

Fig. 4: Elution pattern of trypsin-AMTI-II complex on Sephadex G-200 column

Plot of Ve/Vo against log molecular weight of standard proteins (■), AMTI-II (■), Trypsin - AMTI-II complex (▲). Standard proteins: (i) Phosphorylase 97 kDa. (ii) BSA, 67 kDa. (c) Ovalbumin 45kDa. (d) Chymotrypsinogen A 25 kDa. (e) Lysozyme, 14 kDa

Chemical modification of AMTI-I and AMTI-II

Modification of amino groups by acetylation

Acetylation of AMTI-I and AMTI-II with acetic anhydride and TNBS did not result in the loss of trypsin inhibitory activity, thus confirming that the amino groups of inhibitors are not needed for their trypsin inhibitory effect.

Modification of arginyl residues

Modification of arginyl residues of the inhibitors was done using 1, 2-cyclohexanedione. It can be seen from Table 2 that both AMTI-I and AMTI-II had lost 46 and 56% of their antitryptic activities when treated with 1 mM CHD. Raising the concentration of CHD to 5 mM resulted in a loss of about 80% trypsin inhibitory activity of both the inhibitors. The results obtained suggest the involvement of arginine in the reactive site of AMTI-I and AMTI-II. The chemical modification studies of the inhibitors confirmed that both the inhibitors are of arginine type but not lysine type.

Arginyl residues of AMTI-I and AMTI-II were chemically modified with CHD. Trypsin inhibitory activities of the modified inhibitors were assayed using BAPNA as the substrate.

DISCUSSION

The results of investigation of the inhibitory specificity of AMTI-I and AMTI-II have shown them to be serpins and strongly active against trypsin, moderately active against porcine elastase, *Staphylococcus aureus* protease, and *A. oryzae* protease. Both the inhibitors were ineffective against pepsin (carboxyl), papain (thiol), thermolysin (metalloproteinase), subtilisin, and pronase. Majority of plant proteinase inhibitors isolated so far have been found to be specific for serine proteases, but in certain cases, they have been found to inhibit a range of other serine proteinases such as elastase [37,38], thrombin [39,40], plasmin and kallikrein [41-43], and α-amylases [44,45].

Kunitz family inhibitors have major inhibiting activity against serine proteinases, mainly trypsin and chymotrypsin. However, some inhibitors are also able to inhibit other proteinases such as aspartic and cysteine proteinases [46,47]. A 9 kDa proteinase inhibitor isolated from the seeds of ginkgo (*Ginkgo biloba*) inhibited not only pepsin (acidic proteinase) but also papain, a cysteine proteinase [48].

Serpins inhibit proteinases by competitive or non-competitive mechanisms. As regard to mechanism of action, the inhibitors have shown non-competitive type of inhibition toward bovine pancreatic trypsin. Although a few such as Kunitz SBTI, inhibitors from *Entada scandens, Archidendron ellipticum*, *Putranjiva roxburghii, Pithecellobium dumosum,* and *Piptadenia moniliformis* have shown competitive type of inhibition [9,49-53], majority of the inhibitors including those from *Inga laurina, Vicia faba*, *Adenanthera pavonina,* and *Achyranthes aspera* followed non-competitive inhibition kinetics [8,54-56].

K_i values of AMTI-I and AMTI-II for trypsin, calculated from the Dixon plot, were found to be 0.25±0.02 nM and 0.22±0.06 nM, respectively. The low values of Ki for these inhibitors indicate a high affinity

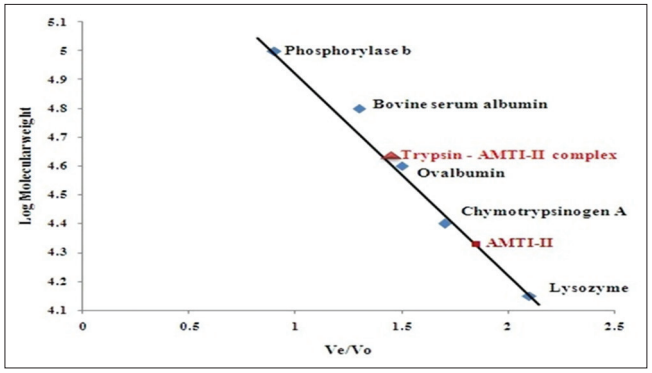

Fig. 5: Molecular weight determination of trypsin-AMTI-II complex by gel filtration on Sephadex G-200

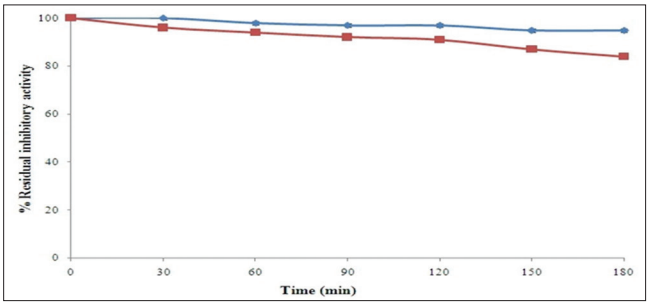

Fig. 6: Effect of DTT on trypsin inhibitory activity of AMTI-II. (♦-------♦) 1 mM DTT, (■---------■) 10 mM DT

Table 2: Effect of modification of arginine on antitryptic activities of AMTI-I and AMTI-II

CHD	AMTI-I AMTI-II TIU/mg×10^2 TIU/mg×10^2	
Control	21.60	25.30
1 mM	11.71	11.38
5 mM	4.32	5.66

CHD: 1,2-cyclohexanedione, TIU: Trypsin inhibitory units

between enzyme and the inhibitors and are a regular hallmark of legume Kunitz trypsin inhibitors from *Leucaena leucocephala, A. ellipticum*, *Adenanthera pavonina*, *Enterolobium contortisiliquum*, and *Dimorphandra mollis* [18,49,57-59].

The formation of stable trypsin-inhibitor complexes has been demonstrated by Sephadex gel filtration studies and an estimate of their approximate size of the complex is made. The results obtained suggest that both the isoinhibitors are capable of binding to trypsin in a 1:1 molar ratio. Results of kinetic experiments indicate that one mole of AMTI-I/AMTI-II binds to two moles of trypsin in which case a molecular weight of 70 kDa should have been obtained for trypsin-inhibitor complex. However, the isolated trypsin-inhibitor complex on gel filtration gave a molecular weight of 46 kDa suggesting that trypsin is complexed with one mole of the inhibitor. It is possible that extra trypsin molecule might have dissociated from the complex during its passage through the column or could be due to the non-specific inactivation of the enzymes induced by the inhibitor during the assay conditions.

Most of the Kunitz inhibitors have a direct relationship between reduction of disulfide bonds and loss of biological activity. AMTI-I and AMTI-II, on the contrary, did not lose their proteinase inhibitory activities even after reduction with DTT suggesting that disulfide bonds are not needed for their inhibitory activities. This uncommon behavior has been reported for a few inhibitors from *Erythrina caffra* [60], *P. roxburghii* [50], *E. scandens* [9], *Entada acacia folia* [22], and *P. moniliformis* [53]. For a similar finding with trypsin inhibitor from *E. caffra* seeds, Onesti *et al*. [61] concluded through crystal structure studies that the disulfide bridges were far away from the reactive site, and hydrogen bonds play a key role in maintaining the three-dimensional structure of the loop involved in proteinase binding.

Chemical modification of the functional groups of the inhibitors by CHD indicated that arginine residues in AMTI-I and AMTI-II are essential for the inhibitory activity against trypsin. Thus, AMTI-I and AMTI-II may be classified as arginine-type inhibitors. Protease inhibitors from the seeds of *Glycine max* [62], *Bauhinia variegata* var. *Candida* [63], and *L. leucocephala* [18] have arginine in the active site. All the four isoinhibitors from *Abelmoschus esculentus* also had arginine residue at the active site [21]. Trypsin inhibitor from the seeds of *I. laurina* has lysine in the active site [8]. Serpins without arginine and lysine in the active center are also reported from the seeds of *Delonix regia* and *Swartzia pickellii* [42,64].

There were hardly any differences between AMTI-I and AMTI-II with respect to their molecular weights, inhibitory constants, specificity, and chemical modification studies. Due to their small molecular masses, acidic nature (low pI value) and existence in multiple forms the inhibitors from *A. moschatus* may be categorized as Kunitz inhibitors. The presence of multiple forms of Kunitz inhibitors has been reported in *A. esculentus* [21], *A. pavonina* [57], *Derris trifoliata* [51], and *Entada acacia folia* [22]. A large number of proteinase inhibitors are products of multigene families [65] and the existence of multiple forms has been attributed to expression by distinct genes and/or post-translational proteolytic cleavage of few amino acids from N- or C-terminal end of the inhibitors.

Plants are a rich source a wide array of bioactive molecules, most of which probably evolved as a chemical defense against predation or infection. The increasing use of plant-derived metabolites in the food, cosmetic, and pharmaceutical industries suggests that to find active compounds, a systematic study of plants and their metabolites is very important [66]. Hence, plant-derived secondary metabolites like enzyme inhibitors may play a key role in the treatment of diseases. Dipeptidyl peptidase-4 inhibitors, a novel class of antidiabetic drugs, have been found to show beneficial role in reducing the cardiovascular disease risk in type 2 diabetes mellitus [67]. With potent trypsin inhibitory activity, AMTI-I and AMTI-II can find application in the medical front as therapeutic agents for infections caused by specific bacterial and fungal strains and also as agents of anticancer proteins.

CONCLUSION

The results of investigations on the inhibition specificities of AMTI-I and AMTI-II have shown that they are strongly active against trypsin, moderately active against porcine elastase, *Staphylococcus aureus* protease, and *A. oryzae* protease. AMTI-I and AMTI-II have shown non-competitive type of inhibition toward bovine pancreatic trypsin. Both the inhibitors are monoheaded with a site for trypsin. Chemical modification of the functional groups of the inhibitors by selective reagents (CHD) indicates that AMTI-I and AMTI-II can be classified as arginine-type inhibitors.

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AUTHORS' CONTRIBUTION

Muni Kumar Dokka performed collection of sample, isolation, and purification of trypsin inhibitors, characterization, interpreted data, wrote the manuscript, and acted as first author. Prof. D.Siva Prasad supervised the development of work and helped in the evaluation of the

manuscript. Coauthor, Prof. K.P.J. Hemalatha, helped in interpretation of data and documentation.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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