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A Kunitz proteinase inhibitor from *Archidendron ellipticum* seeds: Purification, characterization, and kinetic properties

Arindam Bhattacharyya [a,c,*], Suman Mazumdar [c], Sudeshna Mazumdar Leighton [b], Cherukuri R. Babu [a,b]

[a] *Centre for Environmental Management of Degraded Ecosystems, University of Delhi, Delhi 110 007, India*
[b] *Department of Botany, University of Delhi, Delhi 110 007, India*
[c] *Malaria Research Group, International Centre for Genetic Engineering and Biotechnology (ICGEB), Aruna Asaf Ali Marg, New Delhi 110067, India*

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Abstract

Leguminous plants in the tropical rainforests are a rich source of proteinase inhibitors and this work illustrates isolation of a serine proteinase inhibitor from the seeds of *Archidendron ellipticum* (AeTI), inhabiting Great Nicobar Island, India. AeTI was purified to homogeneity by acetone and ammonium sulfate fractionation, and ion exchange, size exclusion and reverse phase chromatography (HPLC). SDS–PAGE of AeTI revealed that it is constituted by two polypeptide chains (α-chain, M_r 15,000 and β-chain, M_r 5000), the molecular weight being ~20 kDa. N-terminal sequence showed high homology with other serine proteinase inhibitors belonging to the Mimosoideae subfamily. Both Native-PAGE as well as isoelectric focussing showed four isoinhibitors (p*I* values of 4.1, 4.55, 5.27 and 5.65). Inhibitory activity of AeTI remained unchanged over a wide range of temperatures (0–60 °C) and pH (1–10). The protein inhibited trypsin in the stoichiometric ratio of 1:1, but lacked similar stoichiometry against chymotrypsin. Also, AeTI-trypsin complex was stable to SDS unlike the SDS unstable AeTI-chymotrypsin complex. AeTI, which possessed inhibition constants (K_i) of 2.46×10^{-10} and 0.5×10^{-10} M against trypsin and chymotrypsin activity, respectively, retained over 70% of inhibitory activity after being stored at −20 °C for more than a year. Initial studies on the insecticidal properties of AeTI indicate it to be a very potent insect antifeedant.

Keywords: Kunitz proteinase inhibitor; Mimosoideae; *Archidendron ellipticum*; Purification; Characterization; Stoichiometry; Isoinhibitors; Electrophoresis

1. Introduction

Plants and insects interact in virtually every conceivable niche, but none is so specialized as that of the tropical rain forests, where both the host and the herbivore strives hard to outdo the defense of the other, to survive. Such an evolutionary arms race at molecular level in the defense mechanisms of plants and the detoxification mechanisms of the insects ensures a parallel co-evolution among them (Gatehouse, 2002; Mello and Silva-Filho, 2002). Among an array of such interactions, insect proteinase – plant proteinase inhibitors (PIs) plays a central role in the diversification of both the insect herbivores as well as the host plants.

PIs are ubiquitous, small regulatory proteins generally present at high concentration (5–15% of total protein), and are especially well spread in the seeds of Fabaceae, Brassicaceae, Poaceae as well as in tubers of Solanaceae (Ryan, 1981; Bolter and Jongsma, 1997; Ascenzi et al., 1999; Oliva et al., 2000). These proteins are fundamental in the control and/or the protection against proteolytic action of the digestive enzymes of seed predators (Batista et al., 1996; Shewry and Lucas, 1997). The inhibitory activity of PIs is largely brought about by intramolecular interactions viz., disulfide bond, hydrogen bond, and hydrophobic interaction, which are involved in stabilization

* Corresponding author. Tel./fax: +91 011 27666237.
E-mail address: ady1111@rediffmail.com (A. Bhattacharyya).

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Nomenclature

AeTI	*Archidendron ellipticum* trypsin inhibitor	DEAE	di-ethyl amino ethyl
TAME	tosyl-arginyl-methyl ester hydrochloride	SKTI	soybean Kunitz trypsin inhibitor
BTEE	*n*-benzoyl tyrosine ethyl ester		

of the primary binding loop (reactive site loop) structure, enabling a stable complex with a cognate protease (Bode and Huber, 1992; Iwanaga et al., 2005). Currently, 10 families of plant PIs are recognized based on the protein primary structure (Laskowski and Kato, 1980; Richardson, 1991; De Leo et al., 2002). The best-known group of enzyme inhibitors from seeds are those which affect the activities of serine proteinases such as trypsin, chymotrypsin and subtilisin (Richardson, 1991). Apart from the serine PIs, inhibitors of the sulphydryl, acidic and metallo-proteinases have also gained prominence for their functional significance.

Plant serine PIs are grouped into Kunitz, Bowman Birk, Potato I and II, and Squash families of inhibitors. Seeds of Leguminosae are particularly rich in Kunitz and Bowman Birk families of PIs. Kunitz type inhibitors are proteins of $M_r \sim 20$ kDa that contains four cysteine residues forming two disulfide bridges and possess a single reactive site (an arginine residue) located in one of the protein loops (Richardson, 1991). Bowman Birk type inhibitors on the other hand have $M_r \sim 8$–10 kDa, with high cysteine content and two reactive sites (Richardson, 1991). Previous studies on the distribution pattern of PIs among the seeds of leguminous trees clearly suggested an evolutionary relationship between the PI family and the legume sub-families (Norioka et al., 1988; Macedo et al., 2000). Interestingly, Kunitz type PIs which are usually single polypeptide chains, cleaves at residue 140 or in its vicinity and shows two polypeptide chains in the inhibitors prepared from Mimosoideae sub family, following reduction of disulfide bridges (Oliva et al., 2000).

Although, many transgene systems have been developed for evolving insect resistant transgenic plants (Hilder et al., 1987; Boulter et al., 1989; Koo et al., 1992; Xu et al., 1996; Cipriani et al., 1999; Confalonieri et al., 1998; Lee et al., 1999), the selection pressure exerted by the rigidity of the PIs compels the insect to up-regulate the synthesis of new proteinases that are largely insensitive to these PIs (Broadway, 1995; Jongsma et al., 1995). Consequently, one way to obtain better inhibitors of insect proteinases is, to search among plant species unrelated to host plant for PI families that do not exist in the host plants (Jongsma et al., 1996).

Archidendron ellipticum (Mimosoideae) is an infrequently occurring tree in open areas/treefall gaps, extending from the interior plain forests to the lower hill slope forests of Great Nicobar Island, India. Incidences of herbivore attack on the seeds of *A. ellipticum* were very sporadic. The present paper describes the purification, characterization and partial NH_2-terminal sequence of a strong trypsin inhibitor from *A. ellipticum* (AeTI).

2. Results and discussion

2.1. Purification and characterization of AeTI

AeTI was partially purified by acetone and ammonium sulfate precipitation (0–30%, 30–40%, 40–50% and 50–65% saturation). Subsequently, the 50–65% ammonium sulfate precipitated protein fraction was subjected to anion exchange chromatography on DEAE 52 (Fig. 1a). Three major protein peaks were eluted using a linear NaCl gradient, all possessing some degree of inhibitory activity. The protein eluted with 0.15 M NaCl (fractions 5–12) demonstrating a strong trypsin inhibitory activity (Table 1) was pooled, lyophilized and loaded on a Superdex S-75 column for gel filtration chromatography. AeTI resolved into a minor trailing peak and a major leading peak following gel filtration (Fig. 1b), the latter showing ~98% trypsin inhibition. On submitting the gel filtrated inhibitor fraction to size exclusion HPLC on Bio-select SEC 125-5 column (300×7.8 mm) a retention time of 9.23 min was recorded (data not shown). Also, the dimeric form of AeTI noted during gel filtration chromatography was absent on size exclusion HPLC. Since AeTI was run in a straightforward phosphate buffer during size exclusion HPLC it prevented dissociation of any dimer previously present. Finally, a purity level of ~90% was achieved by subjecting AeTI to reverse phase HPLC on C_{18} Bondapak column (data not shown). Purified AeTI represented ~8% of the total seed proteins of *A. ellipticum*.

The monomeric form of AeTI from gel filtration chromatography possessed an apparent molecular weight of ~20 kDa. Analyses by dodecylsulfate polyacrylamide gel electrophoresis without 2-mercaptoethanol yielded a single band with an apparent $M_r \sim 20$ kDa (Fig. 1b). In the presence of the reducing agent however, AeTI yielded two lower molecular weight bands corresponding to ~15 and 5 kDa (Fig. 1b), suggesting that AeTI is probably composed of two polypeptide chains linked by one or more disulfide bonds. Similar results have been reported previously for the $M_r \sim 20$ kDa Kunitz proteinase inhibitors found in seeds of *Leucaena leucocephala* (Oliva et al., 2000), *Enterolobium contortisiliquum* (Batista et al., 1996), *Dimorphandra mollis* (Macedo et al., 2000), *Albizia julibrissin* (Odani et al., 1979), *Prosopis juliflora* (Negreiros et al.,

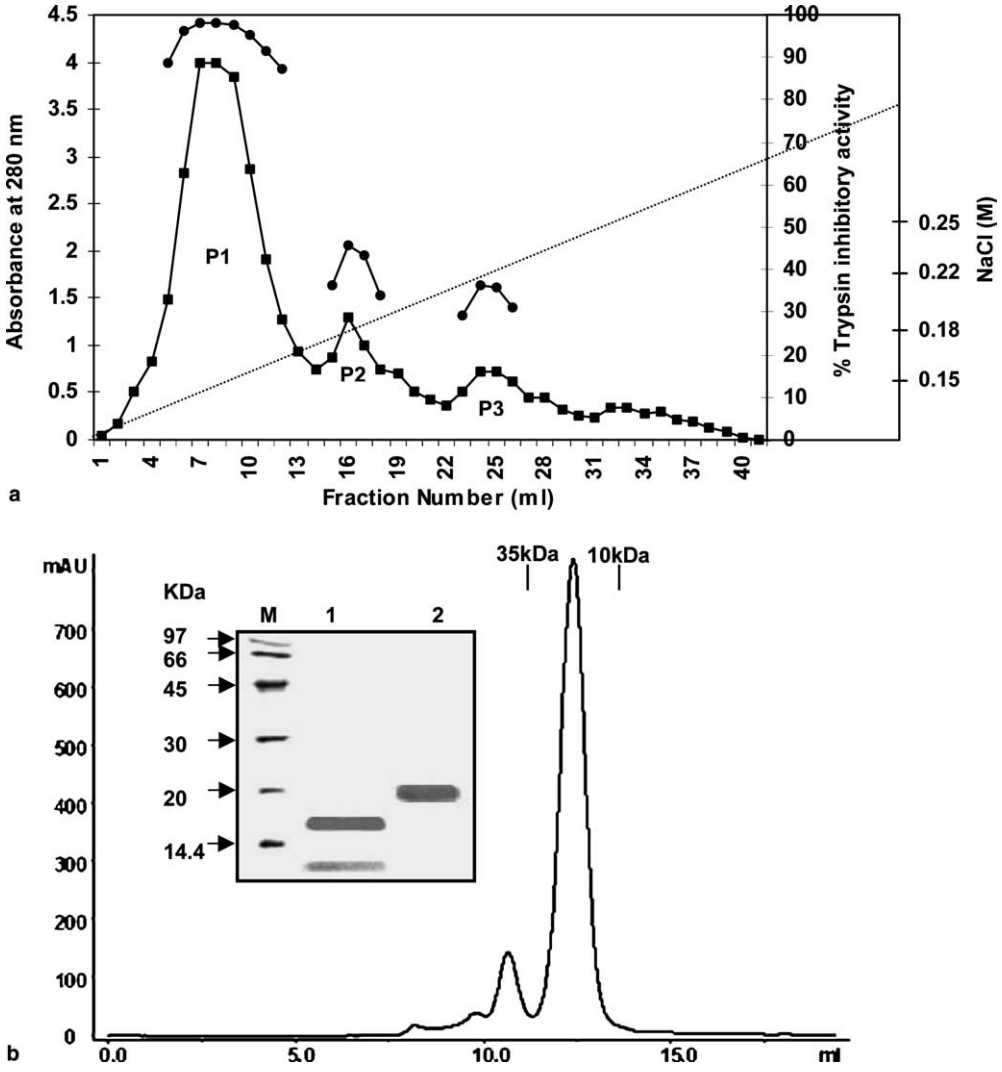

Fig. 1. Purification profile of AeTI by chromatographic procedures. (a) Isolation of AeTI forms by ion exchange chromatography (Abs_{280} [■—■]; inhibition of trypsin [●—●]). Ammonium sulfate fractionated (50–65%) AeTI was loaded onto the anion exchange column packed with DEAE 52 cellulose. Protein elution was performed using a NaCl gradient (0.1–0.25 M). Fractions 5–12 (peak P1) showed the maximum trypsin inhibitory activity. (b) Gel-filtration chromatography and SDS–PAGE analyses of purified AeTI. Anion exchanged fractions (5–12) of AeTI were pooled, lyophilized and loaded on a Superdex S-75 gel-filtration column. AeTI migrates as a monomer with a very small fraction of dimer. (Inset) SDS–PAGE profile of AeTI. Following reduction with DTT and alkylation with iodoacetic acid AeTI shows two bands of ~5 and 15 kDa (lane 2); under non-reducing condition AeTI migrates as a single band of ~20kDa (lane 3) and molecular weight markers (lane 1).

1991), *Adenanthera pavonia* (Richardson et al., 1986) and *Acacia alata* (Kortt and Jermyn, 1981), all species belonging to Mimosoideae.

Native gel electrophoresis (Fig. 2) as well as isoelectric focusing revealed the presence of four isoforms or isoinhibitor forms. The four isoinhibitors had p*I* of 4.1, 4.55, 5.27 and 5.65, respectively. An acidic nature and the presence of isoinhibitors are common characteristics of Kunitz inhibitors (Richardson, 1991). Although the physiological role for the existence of isoinhibitors is not clearly defined, it appears to be a part of strategies adopted by the host plant to ensure its survival.

Over 90% trypsin inhibitory activity was maintained between 25–55 °C following temperature treatment of both 30 min and 1 h duration. Endurance to high temperature is generally associated with high disulfide content. Apart from very alkaline pH (>12) where AeTI lost ~34% of its potency after 2 h and ~65% after 4 h, extremes of pH had negligible effects on AeTI activity. The unusually high thermostability and high pH amplitude of AeTI has also been observed for other serine PIs, such as the chymotrypsin inhibitors isolated from *Enterolobium contortisiliquum* (Batista et al., 1996), *Schizolobium parahyba* (Souza et al., 1995) and *Psophocarpus tetragonolobus* (Gruen et al., 1984). Barring an initial fall of ~20% inhibitory activity during the first three months, AeTI retained over 70% trypsin inhibition upon storage at −20 °C for over a period of one year. Similar results have been obtained by (Belew et al., 1975), for the trypsin and chymotrypsin inhibitors of *Cicer arietinum*.

Table 1
Purification chart of a trypsin inhibitor from *A. ellipticum* (AeTI)

Step/characteristics	Total protein (mg)	% Inhibition	Specific activity TIU/A_{280}	% Recovery	Purification factor
Crude inhibitor	654.3	77.4	0.4	100	1.0
Acetone precipitated	122.0	80.6	0.6	18.6	1.4
Ammonium sulfate precipitation					
0–30%	25.2	84.2	0.8	3.8	1.8
31–40%	15.6	83.8	1.0	2.4	2.3
41–50%	47.7	92.0	4.2	7.3	9.6
51–65%	62.1	96.0	15.7	9.5	35.7
DEAE cellulose chromatography					
0.1 M	1.1	23.2	6.9	0.2	15.6
0.15 M	36.0	97.5	28.9	3.5	65.7
0.18 M	12.7	40.6	12.0	1.9	27.4
0.22 M	5.5	34.5	10.2	0.8	23.2
0.25 M	3.2	28.7	8.5	0.5	19.4
Superdex S 75 gel filtration	5.1	98.0	54.5	0.8	123.9

Note. One trypsin or chymotrypsin unit is defined as 1 μmol of substrate hydrolyzed per minute of reaction. One inhibition unit is defined as unit of enzyme inhibited. Specific activity is defined as inhibition units per absorbance unit, at 280 nm, of the inhibitor.
TIU, trypsin inhibitory units; A_{280}, Absorbance at 280 nm.

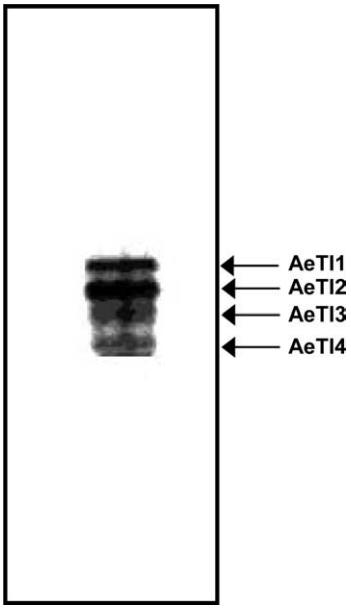

Fig. 2. Native-PAGE profile of AeTI showing four bands representing four isoinhibitors differing minutely in the *e*/*m* ratio.

Fig. 3. Comparative N-terminal sequence alignment of the first 20 amino acids of related Kunitz inhibitors. *A. ellipticum* (AeTI), *Leucaena glauca* (LgTI) [6], *Enterolobium contortisiliquum* (EcTI) [7], *Acacia confusa* (AcTI) [32], *Albizzia julibrissin* (AjTI) [33], *Prosopis juliflora* (PjTI) [34] and *Adenanthera pavonia* (ApTI) [35].

2.2. Sequence alignment and homology analyses

N-terminal sequence analyses of total AeTI for the first 20 amino acids indicated ~85% sequence homology with *Enterolobium contortisiliquum* Trypsin Inhibitor (EcTI) (Batista et al., 1996) and over 70% identity with *Acacia confusa* (Wu and Lin, 1993), *Albizzia julibrissin* (Odani et al., 1979) and *Prosopis juliflora* (Negreiros et al., 1991) inhibitors (Fig. 3). Homology (>50%) was also observed with trypsin inhibitors from other Mimosoid legumes *Adenanthera pavonia* (Richardson et al., 1986) and *Leucaena glauca* (Oliva et al., 2000). Our observation provides support for the evolutionary theory of Norioka et al. (1988), which states that only Kunitz family inhibitors are found in the relatively primitive plants of Mimosoideae and Caesalpinoideae, whereas those of a more advanced family (Papilinoideae) revealed the presence of only Bowman-Birk inhibitors.

2.3. Kinetics of inhibition and inhibition constant

The tryptic activity of bovine trypsin was ~2 times more strongly inhibited by AeTI than the chymotryptic activity of chymotrypsin. Inhibition of trypsin and chymotrypsin at pH 8 by increasing amounts of AeTI is shown in Fig. 4a. The titration curve for trypsin was linear up to ~90% inhibition and fits to a slow-tight binding mechanism with 1:1 stoichiometry (Fig. 4a). Dixon plot analysis revealed the inhibition constants for trypsin and chymotrypsin inhibition, and was 2.4×10^{-10} and 0.5×10^{-10} M, respectively (Fig. 4b and c). Somewhat restrictive, the Dixon plot cannot be used to calculate K_m or V_{max}. Analysis of both the Dixon plots demonstrated that, because the

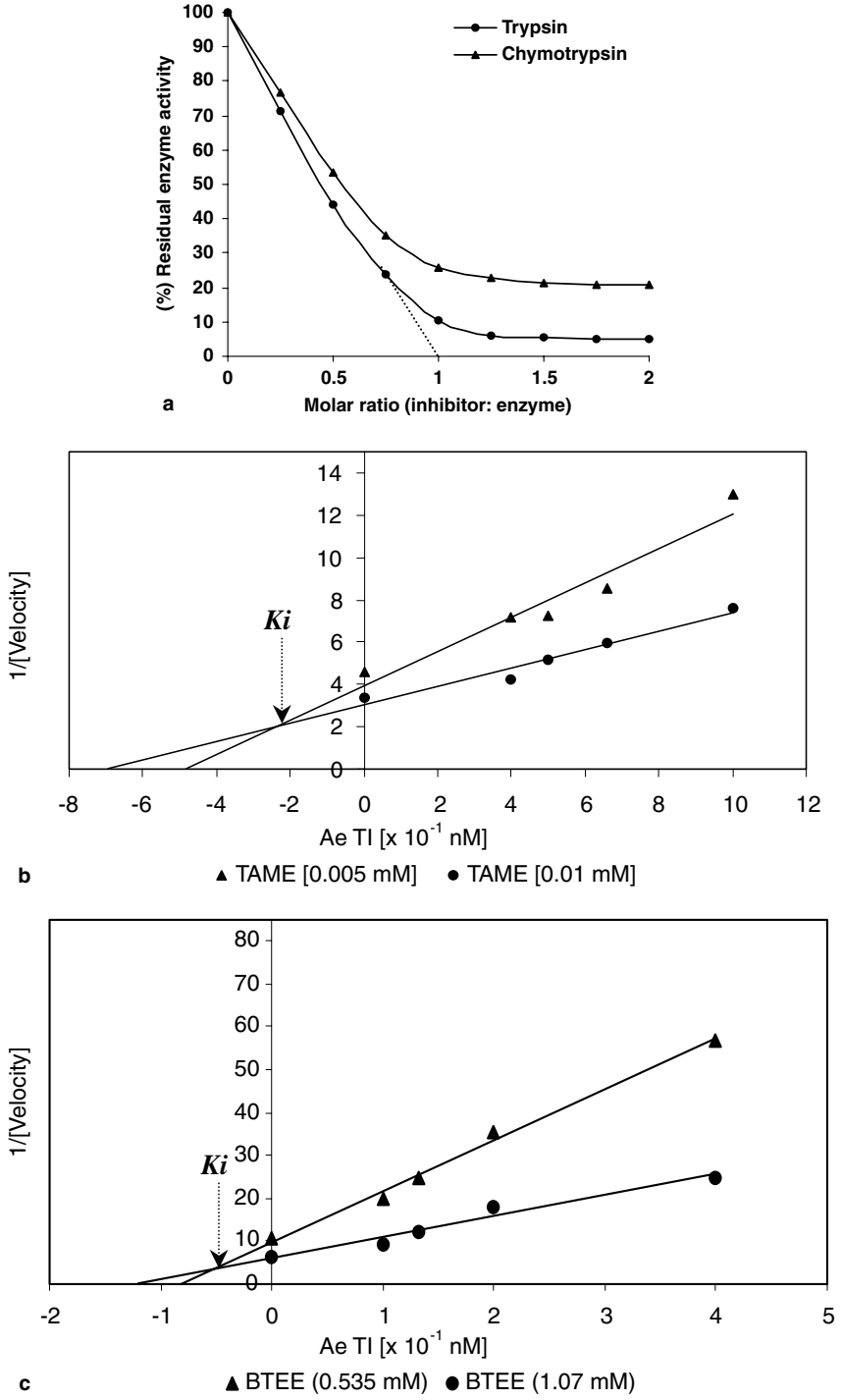

Fig. 4. Kinetic analysis of trypsin and chymotrypsin inhibition by AeTI activity. Inhibition of bovine trypsin (●—●) and α-chymotrypsin (▲—▲) at pH 8.0 by increasing amounts of purified AeTI. (a) Dixon plot for the determination of the inhibition constant (K_i) of AeTI against either (b) trypsin or (c) chymotrypsin. Enzyme assays were carried out with trypsin and chymotrypsin in the presence of increasing concentrations of AeTI at two different concentrations of TAME and BTEE, respectively. The reciprocals of velocity were plotted against the AeTI concentration, and the K_i value was obtained from the intercepts of two lines at two concentrations of substrate.

lines do not intersect at the *x*-axis, inhibition was competitive unlike the non-competitive nature of other similar Kunitz inhibitors (Macedo et al., 2004; Araújo et al., 2005). However, true nature of inhibition by AeTI can only be established after the determination of K_m and V_{max} values. Affinity of AeTI for inhibition of chymotryptic activity was slightly weaker than that of trypsin, the titration curve for enzyme inhibition being up to ~70% linear (Fig. 4c). In fact, AeTI inhibits α-chymotrypsin with a stoichiometry of inhibition of >1. More or less similar values were recorded

for PIs isolated from *Enterolobium contortisiliquum* (Batista et al., 1996), *Schizolobium parahyba* (Souza et al., 1995) and *Glycine max* (Freed and Ryan, 1980). The titration curve obtained with bovine trypsin suggests that 100% inhibition has not taken place. Similar non-stoichiometric plots were obtained for a chymotrypsin inhibitor from *Psophocarpus tetragonolobus* (Kortt, 1980). Notably, most of the other Mimosoid trypsin inhibitors inhibit bovine trypsin in a 1:1 molar ratio viz., *Enterolobium contortisiliquum* (EcTI) (Batista et al., 1996), *Leucaena leucocephala* (LlTI) (Oliva et al., 2000), *Dimorphandra mollis* (DmTI) (Macedo et al., 2000), *Albizia julibrissin* (AjTI) (Odani et al., 1979), *Adenanthera pavonia* (ApTI) (Richardson et al., 1986) and *Acacia alata* (AaTI) (Kortt and Jermyn, 1981).

SDS–PAGE analysis was also carried out to study the stability and dissociation patterns of the strong proteinase-PI complex. A series of mixtures of AeTI (5 μg) with increasing amounts of α-trypsin and α-chymotrypsin, respectively, were incubated at 37 °C for 30 min. and subsequently run on a SDS–PAGE. Three major bands, one at $M_r \sim 45$ kDa and the other two at $M_r \sim 20$ kDa were observed in case of trypsin. The high molecular weight band represented the inhibitor-enzyme complex whereas the other two bands corresponded to free AeTI and free trypsin (Fig. 5a). With increasing amounts of trypsin, there was a substantial increase in the amount of free trypsin and a simultaneous decrease of free AeTI as seen in the SDS–PAGE profiles (Fig. 5a). Also, high levels of trypsin led to partial digestion of AeTI as well as AeTI-trypsin complex as evident from several smaller sized proteins (cleavage products of AeTI). A characteristic feature of the proteinase-PI complex is that it is stable to both heat and SDS treatment, implying covalent bond formation between enzyme and PI (Stavridi et al., 1996; Plotnick et al., 1996). However, unlike the usually stable trypsin-AeTI complex, AeTI-chymotrypsin complex was unstable towards SDS denaturation, as shown by SDS–PAGE analysis (Fig. 5b). This implies that may be either the distortion of the enzyme active site within the complex was not appropriate or the covalent proteinase-PI linkage was somehow exposed to an attacking nucleophile or water, or both.

2.4. Enzymatic assays of AeTI against digestive proteinases from S. litura larvae

Inhibitory assays were performed on the trypsin like proteases from the midguts of fifth instar larvae of *Spodoptera litura* using the substrate TAME. Remarkably, AeTI showed an increase of ~25% in the midgut trypsin inhibitory activity as compared to that of the well-known soybean Kunitz trypsin inhibitor (SKTI) (Fig. 6). Similarly, AeTI also showed ~1.5-fold increase in the suppression of bovine trypsin as compared to SKTI (Fig. 6). However, the levels of inhibitory activity varied markedly for bovine trypsin and midgut trypsin of fifth instar larvae of *S. litura*. For example, while AeTI showed a fall of ~20% activity

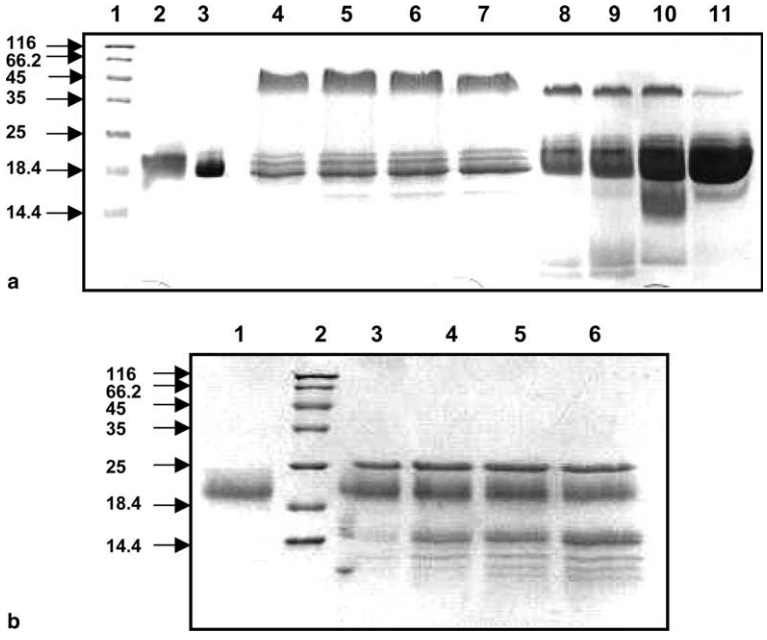

Fig. 5. SDS–PAGE demonstrating the effect of treating purified AeTI with various concentrations of serine proteases in a buffer (0.05 M Tris–HCl; 0.001 M $CaCl_2$, pH 8.0), incubated at 37 °C for 30 min. (a) Typically, increasing amounts of trypsin (T) (molar ratios 1:1–1:20) were incubated with AeTI (5 μg) (A) and subsequently run on a SDS–PAGE. An AeTI-trypsin complex was observed. Lane 1: molecular weight standards; lane 2: AeTI; lane 3: bovine trypsin; lane 4: A + T (1:1); lane 5: A + T (1:1.5); lane 6: A + T (1:3); lane 7: A + T (1:5); lane 8: A + T (1:7.5); lane 9: A + T (1:10); lane 10: A + T (1:15); lane 11: A + T (1:20). (b) Different concentrations of α-chymotrypsin (C) (molar ratios 1:1–1:5) were also incubated with AeTI (A). An AeTI-chymotrypsin complex was not observed. Lane 1: AeTI; lane 2: molecular weight standards; lane 3: A + C (1:1); lane 4: A + C (1:2); lane 5: A + C (1:3) and lane 6: A + C (1:5).

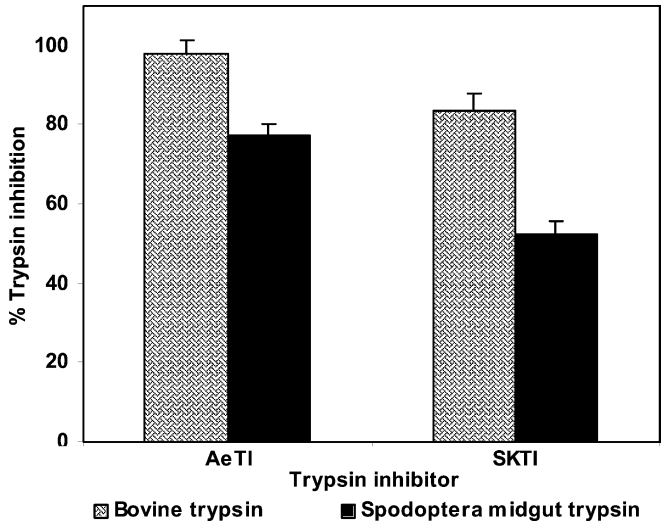

Fig. 6. Effect of AeTI on the bovine and midgut trypsin activity of *Spodoptera litura*. SKTI was used as a reference standard. Five replicates were maintained for each measurement.

against larval trypsin as compared to that against bovine trypsin, SKTI showed almost 30% decrease for the same (Fig. 6). Our observations imply that AeTI is a more potent inhibitor of insect midgut proteases as compared to SKTI. Inhibition caused by AeTI to midgut extracts of *S. litura* was similar to results observed with proteinase inhibitors from other leguminous plants (Broadway and Duffey, 1986; Hilder et al., 1987; Franco et al., 2003; Franco et al., 2004; De Gomes et al., 2005). Detailed work on the effect of AeTI at different dose regimens on the growth and development of different crop pests need to be carried out to establish its full potentiality.

3. Experimental

3.1. Purification procedure of AeTI

Forty gram of air-dried *A. ellipticum* seeds were taken as the starting material. The seeds were soaked in 0.15 N NaCl for 16 h. The swollen cotyledons were then homogenized with 300–350 ml of saline Tris buffer (20 mM Tris, pH 8.0; 0.15 M NaCl) containing 1 mM sodium metabisulfite at 4 °C. After centrifugation at 12,000*g* for 20 min at 4 °C the supernatant (crude extract) was precipitated either with acetone or ammonium sulfate. Cold acetone (80% [v/v]) was added to the crude extracts at 4 °C and allowed to stand for 15 min. Following centrifugation at 12,000*g* for 10 min at 4 °C, the pellet was washed, lyophilized and proteinase inhibitory activity was assessed. For ammonium sulfate precipitation different percentages of $(NH_4)_2SO_4$ fractionation (0–30%, 30–40%, 40–50% and 50–65%) were used. Precipitates from each of the fractions were centrifuged at 12,000*g* for 10 min at 4 °C. These fractions were then dialyzed against deionzed water, lyophilized and submitted to proteinase inhibitory assays. The 51–65% $(NH_4)_2SO_4$ precipitated fraction was applied to an anion exchanger column DEAE 52, pre-equilibrated with 20 mM Tris buffer, pH 8.0. Protein fractions (5 ml) were eluted using a linear gradient (0.15–0.25 M) of NaCl, at a flow rate of 0.5 ml/min and absorbance was recorded at 280 nm. Fractions eluted with 0.15 M NaCl was submitted to gel filtration on Superdex S-75 (pre-equilibrated with 0.1 M Tris buffer (pH 8.0) and 0.1 M NaCl). AeTI active fractions (0.5 ml; flow rate of 20 ml/h) was applied on a silica-based size exclusion (SE) HPLC chromatography column Bio-select SEC 125-5 (300 × 7.8 mm) pre-equilibrated in standard Phosphate-buffered saline (PBS, pH7.4). The column fraction with AeTI activity was finally loaded on a reverse phase Bondapak C_{18} column. The gradient used for elution (0.7 ml fractions; flow rate of 1.0 ml/min for 40 min) was developed using Buffer A (0.05% trifluoroacetic acid in water) and Buffer B (0.05% trifluoroacetic acid in 90% acetonitrile, 10% water).

3.2. Trypsin and chymotrypsin inhibitory assay

Trypsin and chymotrypsin inhibitory assays were carried out by estimating the remaining esterolytic activity of trypsin and chymotrypsin towards the substrate tosyl-arginyl-methyl ester hydrochloride (TAME) and *n*-benzoyl tyrosine ethyl ester (BTEE), respectively, following (Birk, 1976). Both trypsin and chymotrypsin were pre-incubated with AeTI for 5 min at 25 °C, in 1 ml Tris buffer (46 mM) containing 11.5 mM $CaCl_2$, pH 8.1, prior to the reaction. One trypsin or chymotrypsin unit is defined as 1 μmol of substrate hydrolyzed per minute of reaction. One inhibition unit is defined as unit of enzyme inhibited. Specific activity is defined as inhibition units per absorbance unit, at 280 nm, of the inhibitor.

3.3. Estimation of proteins

Protein contents were estimated by Coomassie blue staining (dye-binding method) (Bradford, 1976) and from the absorbance at 280 nm. BSA (1 mg/ml) was used as the standard protein.

3.4. Reduction and S-alkylation

AeTI (5 mg) was reduced with dithiothreitol (0.1 M) and *S*-carboxymethylated with iodoacetic acid according to the procedure of Crestfield et al. (1963). Following desalting, AeTI was redissolved in 0.05 M Tris buffer pH 8.0, and separated on Superdex S75 equilibrated with the same buffer.

3.5. SDS–PAGE and Native-PAGE profiles

HPLC purified AeTI was subjected to SDS–PAGE as well as Native-PAGE using 5–15% and 6% polyacrylamide slab gel system, respectively (Laemmli, 1970). For both

SDS–PAGE and Native-PAGE the protein samples were pretreated with Laemmli's buffer but for the latter Laemmli's buffer was excluded of SDS and β-mercaptoethanol. The gels were stained by Coomassie brilliant blue R250.

3.6. N-terminal sequencing

The first 20 amino acids from the N-terminal end were sequenced by Edman degradation method on an Applied Biosystem Procise Sequencer. Percentage sequence identity with trypsin inhibitors from other members of Mimosoideae sub-family was determined using BCM search launcher for multiple sequence alignment (Worley et al., 1998) and BOXSHADE 3.21 was used for shading of multiple alignment files.

3.7. Chemical properties of AeTI

Kinetic analyses over a range of AeTI concentrations were used to more accurately determine the inhibition constant (K_i) value using a Dixon plot (Segel, 1975). Enzyme inhibition was characterized at two different substrate concentrations ($[S_1]$ and $[S_2]$). TAME (0.005 and 0.01 mM) and BTEE (0.53 and 1.07 mM) were used for trypsin and chymotrypsin activity, respectively. Samples were prepared to achieve inhibitor concentrations (nM) of: 0.4, 0.5, 0.66 and 1. The initial slope v was determined for each inhibitor concentration. The reciprocal velocity ($1/v$) versus [PI], for each substrate concentration, $[S_1]$ and $[S_2]$, was plotted (Dixon plots). A single regression line for each [S] was obtained, and the K_i was calculated from the intersection of the two lines.

Additionally, in order to study the effects of electrophoretic conditions on the proteinase-PI complex, SDS–PAGE was employed. Increasing amounts of trypsin (up to 20-fold) as well as chymotrypsin (up to 5-fold) were incubated with AeTI in a buffer (0.05 M Tris–HCl; 0.001 M $CaCl_2$, pH 8.0) at 37 °C for 30 min and then electrophoresed on SDS–PAGE.

3.8. Biological properties of AeTI

The temperature optima of purified AeTI was determined by the trypsin inhibitory assays, under varying temperatures (5–95 °C, with an increment of 10 °C) in 0.05 M Tris–HCl buffer, pH 8.0. Two incubation periods of 30 and 60 min duration were used for each temperature.

To assess the pH optima of AeTI, trypsin inhibitory assays were carried out under varying pH (1–12). Purified AeTI was mixed with buffers of pH 1, 3, 5, 6, 9 and 12, respectively in the ratio of 1:1 (v/v), and incubated for 2 and 4 h. The buffers used were 0.02 M each of KCl–HCl (pH 0.5–1.5), glycine-HCl (pH 2.0–3.5), sodium-phosphate (pH 6–7.5), Tris–HCl (pH 8.0–10.0) and glycine–NaOH (pH 10.5–13.0). Three replicates were maintained for each temperature and pH condition.

Purified AeTI was stored for over 1 year at −20 °C. Monthly assessment of the trypsin inhibitory activity was carried out to evaluate its potency.

3.9. Isolation of midgut proteases

Spodoptera litura were collected from the Division of Entomology, Indian Agricultural Research Institute (IARI) and reared on fresh leaves of *Ricinus communis* (L.). Midguts from the fifth instar larvae were extruded out and each gut was transferred into an eppendorf tube kept on ice at 4 °C containing 40 μl of 0.001 M HCl. 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 and were stored at −20 °C until further use. Total protein content was measured following Bradford (1976).

3.10. Proteinase inhibitor assays

The crude enzyme extract was diluted in the ratio of 1:12.5 using 46 mM Tris buffer containing 11.5 mM $CaCl_2$ (pH 8.1). The trypsin activity was assayed using TAME as described earlier. The inhibitory potential of AeTI and SKTI (as a reference standard for Kunitz inhibitors) were monitored, after mixing variable concentrations of the crude enzyme extract with each of the two inhibitors, respectively. As a control only the crude gut extract (without any inhibitor) was used. Bovine trypsin was used as a reference proteinase. Five replicates for all the tests were maintained.

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