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Isolation and Purification of Trypsin Inhibitors from the Seeds of *Abelmoschus moschatus* L.

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Abstract Four trypsin inhibitors, AMTI-I, AMTI-II, AMTI-III, and AMTI-IV, have been isolated and purified to homogeneity from the seeds of *Abelmoschus moschatus* following ammonium sulphate fractionation, DEAE-cellulose ion exchange chromatography and gel permeation on Sephadex G-100, and their molecular weights were determined to be 22.4, 21.2, 20.8 and 20.2 kDa respectively by SDS-PAGE. While all the four inhibitors were very active against bovine trypsin, two of them (AMTI-III and AMTI-IV) showed moderate activity towards bovine chymotrypsin. AMTI-I and AMTI-II were found to be glycoproteins with neutral sugar content of 2.8 and 4 %, respectively, and all the four inhibitors were devoid of free sulphhydryl groups. The inhibitors were quite stable up to 80 °C for 10 min and were not affected at alkaline as well as acidic conditions tested. Treating them with 8 M urea and 1 % SDS for 24 h at room temperature did not result in any loss of their antitryptic activities. However, they lost considerable antitryptic activity when treated with 6 M guanidine hydrochloride. Activities of the inhibitors were unaffected even after their reduction with DTT suggesting that disulphide bonds are not needed for their inhibitory activities.

Keywords *Abelmoschus moschatus* · Antitryptic · Trypsin inhibitors

Introduction

Protease inhibitors, proteins capable of inhibiting catalytic activities of proteolytic enzymes, are widely distributed among plants, animals and microorganisms [1]. They are known to regulate proteolytic processes and participate in defence mechanisms in plants against attack by a large number of insects, fungi and other pathogenic microbes [2]. Serine protease

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inhibitors (serpins) have been isolated, purified and characterized from various plant sources [3–6], and the presence of their isoinhibitors has also been demonstrated in some plants [7–12].

Protease inhibitors attracted the attention of nutritionists due to their abundance in valuable plant foods and their involvement in nutritive properties. They have been studied as model systems for elucidating proteinase inhibition mechanisms, as well as protein-protein associations [13]. In pharmacological and medical fields, investigations have been made into the potential of these inhibitors as therapeutic agents in the treatment of wide range of disorders associated with enhanced proteolytic activities like pancreatitis, shock, allergy, and inflammation [14]. They also find application in HIV therapy [15] and cancer [16]. Protease inhibitors with associated antimicrobial and lectin activities will find lot of applications in medical and agricultural fronts. Search for such multifunctional protease inhibitors in plant sources needs to be carried out.

Abelmoschus moschatus (L.) Medic family *Malvaceae* is an aromatic and medicinal plant popularly known as Mushkdana/Kasturi bhendi. The unripen pods, leaves and new shoots are eaten as vegetables. The seeds are rich in protease inhibitors, and they are used to check excessive thirst, cure for stomatitis, dyspepsia, urinary discharge, gonorrhoea, leucoderma and itchiness. Not much work has been done on protease inhibitors from these seeds. This paper, therefore, deals with the isolation and purification of trypsin inhibitors from the seeds of *Abelmoschus moschatus*.

Materials and Methods

Source

Abelmoschus moschatus plants bearing pods of uniform size were selected in and around Visakhapatnam District. Pods were collected at the ripening stage, and seeds removed from the pods were used for the isolation and purification of trypsin inhibitors.

Chemicals and Reagents

Bovine pancreatic trypsin (1× crystallized, DCC-treated, type xi), bovine pancreatic α-chymotrypsin (3× crystallized, type ii), bovine serum albumin (BSA), chymotrypsinogen A, ovalbumin, lysozyme, phosphorylase b and soybean trypsin inhibitor (type I-S) were purchased from Sigma Chemical Company, St. Louis, Missouri, USA.

N-Acetyl-L-tyrosine ethyl ester (ATEE), α-*N*-benzoyl-DL-arginine-*p*-nitoanilide-HCl (BAPNA), DEAE-cellulose, *N*,*N*-dimethylsulfoxide, dithiothreitol (DTT), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and sodium dodecyl sulphate (SDS) were also from Sigma Chemical company, St. Louis, Missouri, USA. Sephadex G-100 and Sephadex G-200 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals used were of analytical grade.

Purification of *Abelmoschus moschatus* Trypsin Inhibitors

Twenty-five grams of the seeds were homogenized with 150 ml of 0.1 M sodium phosphate buffer (pH 7.6), and the extract made up to 250 ml with the buffer was centrifuged at 5600 rpm for 15 min at 4 °C. The supernatant treated with four volumes of ice-cold acetone for 1 h was centrifuged at 2500 rpm for 15 min at 4 °C. The precipitate was resuspended in 230-ml buffer, the extract was subjected to heat treatment for 10 min at 70 °C, then quickly cooled in ice, then

centrifuged at 5600 rpm for 15 min at 4 °C, solid ammonium sulphate was added gradually to the supernatant with constant stirring at 4 °C to obtain 60 % saturation, and after overnight standing at 4 °C, the precipitate collected after centrifugation at 3000 rpm for 10 min at 4–6 °C was dissolved in the buffer and dialysed against the same buffer.

The dialysed sample (60 ml containing 424.8 mg protein) was loaded on DEAE-cellulose column (2.2×34 cm) previously equilibrated with the buffer, and the unbound proteins were eluted with 350 ml of the equilibration buffer. Fractions of 8 ml were collected at a flow rate of 60 ml/h and were assayed for protein by measuring their absorbance at 280 nm as well as the inhibitory activity against trypsin and chymotrypsin using BAPNA and ATEE as the substrates. Subsequently, the elution of the bound protein was done with a linear gradient of 0.1 to 1.0 M NaCl, and fractions of 8 ml were collected at a flow rate of 60 ml/h. The active fractions were pooled and lyophilized.

The unbound sample and the lyophilized material obtained after elution with 0.1 M NaCl, 0.25 M NaCl and 0.5 M NaCl were separately dissolved in the buffer and then loaded on to Sephadex G-100 column (1.9×63 cm) previously equilibrated with the buffer and eluted with the same buffer. Two-millilitre fractions were collected at a flow rate of 12 ml/h, and the protein was monitored and trypsin and chymotrypsin inhibitory activities of the fractions were assayed.

Protein Estimation

Protein was estimated by the method of Lowry [17] using bovine serum albumin as the standard.

Determination of Molecular Weight

Molecular weights of the inhibitors were determined by SDS-PAGE using the method of Laemmli [18] and also by gel filtration on Sephadex G-200 column.

Measurement of Trypsin and Trypsin Inhibitory Activity

Trypsin activity was assayed by the method of Kakade [19] using BAPNA as the substrate. Trypsin (30 μg) in 2 ml water was incubated with 7 ml of substrate solution at 37 °C for 10 min. The reaction was stopped by adding 1 ml of 30 % (*v*/*v*) acetic acid. The absorbance of the solution was measured at 410 nm against an incubated blank containing 2 ml of water instead of trypsin solution.

For trypsin inhibitory activity, trypsin (30 μg) was pre-incubated 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 enzyme unit is defined as an increase in 0.01 absorbance unit at 410 nm for trypsin under the assay conditions. One enzyme inhibitory unit is defined as the number of enzyme units inhibited under these conditions.

Assay of Chymotrypsin and Chymotrypsin Inhibitory Activity

Esterolytic activity of chymotrypsin was assayed by the method of Prabhu and Pattabiraman [20] using ATEE as the substrate.

Aliquots of chymotrypsin solution containing 0.2–2.0 μg of the enzyme in 1 ml 0.1 M phosphate buffer (pH 7.5) were incubated with 1ml ATEE solution at 37 °C for 10 min. The

reaction was stopped by adding 5 ml of ethyl acetate. A 0.5 ml of aqueous layer was assayed for *N*-acetyl-L-tyrosine by the method of Lowry (30). Optical density measurements were made at 530 nm in photoelectric colorimeter.

For chymotrypsin inhibitory activity, the assay mixture contained in 1 ml, 1 μg of chymotrypsin and suitable aliquots of the inhibitor solution. After incubating at 37 °C for 10 min, the residual chymotrypsin inhibitory activity was measured. 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 (CIU) is defined as the number of CU inhibited under these conditions.

Estimation of Carbohydrate Content

Neutral sugar content of the inhibitors was determined by phenol sulphuric acid method of Dubois [21] using D-mannose (5 mg/100 ml water) as the standard. The hexosamine content of the inhibitors was determined by the modified Morgan-Elson method [22] using *N*-acetyl D-glucosamine (5 mg/100 ml water) as the standard.

Estimation of Thiol Groups

The free thiol groups in the four inhibitors were estimated following the procedures of Ellman [23] and Habeeb [24] using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB).

Determination of Tryptophan

Tryptophan content of the inhibitors was determined according to the spectrophotometric method of Edelhoch [25].

Effect of Protein Denaturing Conditions/Agents

pH

In order to determine the pH stability of each of the inhibitor, 1 mg/ml solution of the inhibitor in an appropriate buffer (10 mM) was kept at 5 °C for 24 h. Aliquots of the inhibitors were diluted with phosphate buffer (pH 7.6) and assayed for trypsin and chymotrypsin inhibitory activities.

Temperature

Three-millilitre samples of 100 μg/ml solution of each of the inhibitor in 0.1 M sodium phosphate buffer (pH 7.6) were separately incubated in a water bath at different temperatures for 10 min. After the heat treatment, the solutions were quickly cooled in ice, and appropriate aliquots were used for the assay of inhibitory activity against trypsin and chymotrypsin.

Denaturants

To determine the stability of inhibitors, 1 mg/ml solution of each of the inhibitor was prepared in 0.1 M sodium phosphate buffer (pH 7.6) containing 8 M urea, 6 M guanidine hydrochloride and 1 % SDS and was incubated at 5 °C for 24 h. The solutions were diluted with the buffer and the appropriate aliquot of this diluted solution was used for the assay of trypsin and

chymotrypsin inhibitory activities. The control assay mixture had the same amount of denaturant as was present in diluted inhibitor solutions.

Results

Isolation and Purification of Trypsin Inhibitors

Trypsin inhibitors from *Abelmoschus moschatus* seeds were purified following conventional methods of protein purification. When the ammonium sulphate fraction was subjected to DEAE-cellulose column chromatography, trypsin inhibitory activity was found to be associated not only with protein present in the void volume (8–38 fractions) but also with the proteins bound to the matrix. A weakly bound protein eluted by 0.1 M NaCl (58–84 fractions) showed trypsin inhibitory activity, and moderately and firmly bound proteins eluted with 0.25 M NaCl (100–118 fractions) and 0.5 M NaCl (130–152 fractions), respectively, exhibited both trypsin and chymotrypsin inhibitory activities. The elution profile of DEAE-cellulose chromatography is shown in Fig. 1. The inhibitors were designated as *Abelmoschus moschatus* trypsin inhibitors, AMTI-I, AMTI-II, AMTI-III and AMTI-IV, in the order of their elution from DEAE-cellulose column. All the four inhibitors eluted out as a single protein when subjected to gel filtration on Sephadex G-100. The purification of these inhibitors is summarized in Table 1.

The molecular weights of AMTI-I, AMTI-II, AMTI-III and AMTI-IV as determined by SDS-PAGE (Fig. 2) were found to be 22.4, 21.2, 20.8 and 20.2 kDa, respectively. These values were close to those obtained with gel filtration on Sephadex G-200 by plotting Ve/Vo versus log molecular weight—22.8, 22.4, 21.6 and 21.2 kDa. Trypsin inhibitors gave a single sharp band on SDS-PAGE even in the presence of 2-mercaptoethanol supporting the monomeric nature of the protein.

AMTI-I and AMTI-II were found to be glycoproteins with neutral sugar content of 2.8 and 4 %, respectively, and they were devoid of amino sugars (Table 2). On the contrary, AMTI-III and AMTI-IV were found to be free of carbohydrate moieties. In the results of the experiments not presented, it was observed that the trypsin inhibitors were devoid of free sulphhydryl groups possibly due to their involvement in the formation of disulphide bonds. The tryptophan content was found to be low (~two residues) in the four trypsin inhibitors.

Stability of the Purified Inhibitors After Different Treatments

All the four inhibitors were quite stable up to 80 °C for 10 min. However, they lost 25–40 % of their antitryptic activity at 90 °C when incubated for 10–20 min. AMTI-III and AMTI-IV lost 30–50 % of their antichymotryptic activity when treated with the same conditions. Keeping them in a boiling water bath for 10–20 min resulted in further loss of their trypsin inhibitory activity. In contrast, there was a complete loss of chymotrypsin inhibitory activity for both AMTI-III and AMTI-IV when subjected to same treatment. Incubation of inhibitors for 30 min at boiling temperature caused the total loss of their trypsin and chymotrypsin inhibitory activities, and autoclaving at 1.04 kg/cm^2 for 10 min resulted in the total loss of both the activities of the inhibitors (Table 3).

Antitryptic and antichymotryptic activities of the inhibitors were not affected at alkaline as well as acidic conditions tested (Table 4). The inhibitors were found to be quite stable in the presence of chaotropic agent (urea), and contact with this agent for 24 h did not affect both the inhibitory activities (Table 5). Treatment of inhibitors with 1 % SDS did not cause any loss of

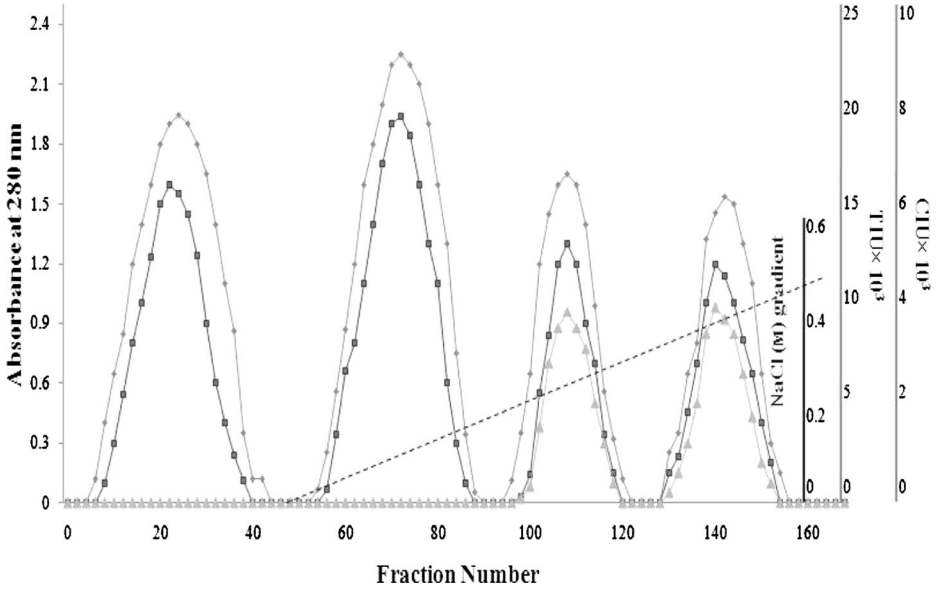

Fig. 1 Ion exchange chromatography of ammonium sulphate fraction on DEAE- cellulose. A 424.8 mg of the ammonium sulphate fraction (0–60 %) was loaded on to the column (2.2×34 cm) in 0.1 M phosphate buffer (pH 7.6) and the adsorbed proteins were eluted with 0.1–1.0 M NaCl in the buffer. Fractions, each 8.0 ml, were collected at a flow rate of 60 ml/h (elutants of 0.6–1.0 M NaCl did not exhibit trypsin and chymotrypsin inhibitory activities, hence not shown in the figure). Protein was monitored by absorbance at 280 nm (♦——————♦). Trypsin inhibitory activity (TIU) (■——————■). Chymotrypsin inhibitory activity (CIU) (▲——————▲)

antitryptic and antichymotryptic activities. The inhibitors have lost their trypsin inhibitory activity in the presence of guanidine hydrochloride. When kept at room temperature with 6 M guanidine hydrochloride for 24 h, AMTI-I, AMTI-II, AMTI-III and AMTI-IV had lost 45, 35, 50 and 55 % of trypsin inhibitory activity, respectively, and the last two inhibitors had lost 50 % of their chymotrypsin inhibitory activity. The inhibitors retained 25 % of their proteinase inhibitory activities even after keeping in boiling water bath for 2 min with 6 M guanidine hydrochloride (Table 5).

A meager decrease of trypsin inhibitory activity for the four inhibitors was observed after 30 min of incubation at 37 °C in the presence of 10 mM DTT, while at 1 mM DTT, all the four inhibitors completely retained their trypsin inhibitory activity. Similar results were obtained with AMTI-III and AMTI-IV for their chymotrypsin inhibitory activity when treated with DTT.

Discussion

Four trypsin inhibitors have been isolated from *Abelmoschus moschatus* seeds in an apparently pure form, while two of them were active only against trypsin and the other two were found to be active against both trypsin and chymotrypsin. The observation that trypsin and chymotrypsin inhibitory activities in the crude extracts of the seeds is stable at 70 °C for 10 min has led to the use of this treatment as the first step in the purification of the inhibitors. About 52 % of proteins present in the crude extract were removed by this step. When the ammonium sulphate

Table 1 Summary of purification of trypsin inhibitors from seeds of *Abelmoschus moschatus* (25 g of seeds)

Preparation	Volume (ml)	Total protein (mg)	Total activity units		Specific activity (units/mg protein)		TIA/CIA	Yield%	Fold purification
			TIU × 10^3	CIU × 10^3	TIA × 10^2	CIA × 10^2			
Crude extract	250	2087.5	788.4	421.18	3.77	2.01	1.90	100	1.00
Acetone treatment	230	1988.2	771.5	414.4	3.89	2.08	1.86	97.86	1.03
Heat treatment	215	1016.4	626.4	328.4	6.16	3.23	1.94	79.45	1.63
Ammonium sulphate (60 %) fractionation	60	424.8	482.8	238.2	11.36	5.61	1.96	61.24	3.01
DEAE-cellulose unbound fraction, PI-I	248	55.2	104.4	–	18.91	–	–	13.24	5.01
Sephadex-G-100, PI-I	46	40.8	88.4	–	21.67	–	–	11.21	5.75
DEAE-cellulose NaCl gradient elution									
PI-II (0.1 M NaCl)	216	58.8	136.8	–	23.26	–	–	17.35	6.17
PI-III (0.25 M NaCl)	152	44.2	64.4	34.8	14.57	7.87	1.87	8.17	3.86
PI-IV (0.5 M NaCl)	184	42.8	68.6	35.4	16.02	8.27	1.95	8.70	4.25
Sephadex G-100									
PI-II	50	52.4	132.6	–	25.30	–	–	16.81	6.71
PI-III	46	31.6	60.2	32.4	19.05	10.25	1.86	7.63	5.05
PI-IV	50	30.5	56.8	30.2	18.62	9.90	1.88	7.20	4.94

Yield and fold purification were calculated on the basis of TIU and TIA respectively

TIU trypsin inhibitory units, *TIA* trypsin inhibitory activity, *CIU* Chymotrypsin inhibitory units, *CIA* Chymotrypsin inhibitory activity

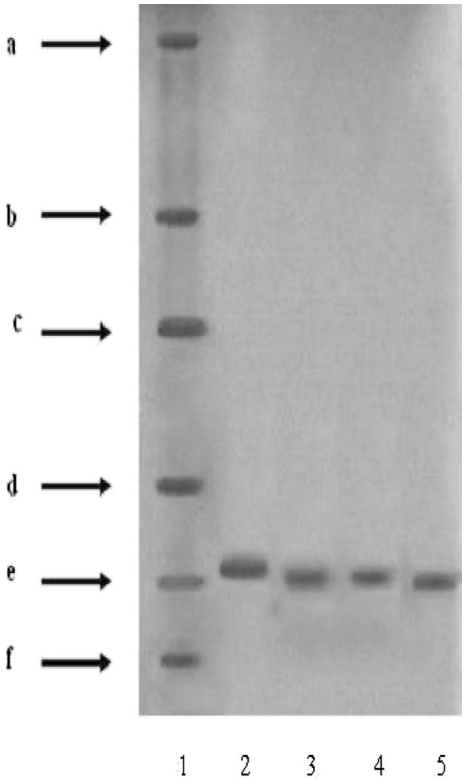

Fig. 2 Molecular weight determination of trypsin inhibitors by SDS-PAGE on 5–20 % gradient slab gel. Standard proteins (*1*). Phosphorylase b (97 kDa) (*a*). Bovine serum albumin (67 kDa) (*b*). Ovalbumin (45 kDa) (*c*). Chymotrypsinogen A (25 kDa) (*d*). Soybean trypsin inhibitor (20.1 kDa) (*e*). Lysozyme (14 kDa) (*f*). Purified AMTI-I (*2*). AMTI-II (3). AMTI-III (*4*). AMTI-IV (*5*)

fraction was subjected to DEAE-cellulose column chromatography, trypsin inhibitory activity was found to be associated with protein present in the void volume and also with the proteins bound to the matrix. A weakly bound protein eluted by 0.1 M NaCl showed trypsin inhibitory activity, and moderately and firmly bound proteins eluted with 0.25 M NaCl and 0.5 M NaCl exhibited both trypsin and chymotrypsin inhibitory activities. All the four inhibitors eluted out as a single protein when subjected to gel filtration on Sephadex G-100.

During the purification, the ratio of trypsin inhibitory activity (TIA) to chymotrypsin inhibitory activity (CIA) in the latter two inhibitors remained nearly constant suggesting that the two inhibitory activities might reside in the same protein molecule.

Table 2 Estimation of carbohydrate content of AMTI-I and AMTI-II

Type of sugar	AMTI-I (mg sugar/g protein)	AMTI-II (mg sugar/g protein)
Neutral sugar	2.8±0.06	4.0±0.14
Amino sugars	0	0

Neutral sugar content of the inhibitor was estimated by the method of Dubois et al. (1956) using D-mannose as the standard. Amino sugars of the inhibitor was determined by the modified method of Morgan and Elson (Reissig et al. 1955) using *N*-acetyl D-glucosamine as the standard

Table 3 Effect of heat treatment on *Abelmoschus moschatus* trypsin inhibitors

Temperature (°C)	Time (min)	AMTI-I	AMTI-II	AMTI-III		AMTI-IV	
		TIU/mg of AMTI-I×10^2	TIU/mg of AMTI-II×10^2	TIU/mg of AMTI-III×10^2	CIU/mg of AMTI-III×10^2	TIU/mg of AMTI-IV×10^2	CIU/mg of AMTI-IV×10^2
25	10	21.56	25.28	19.02	10.24	18.44	9.82
37	10	21.64	25.34	19.24	10.34	18.62	9.94
50	10	21.34	25.22	19.18	10.36	18.34	9.78
60	10	21.48	25.16	19.22	10.16	18.12	9.84
70	10	21.38	25.18	19.32	10.22	18.46	9.92
80	10	21.44	25.36	19.16	10.28	18.38	9.76
90	10	16.24	22.56	14.34	7.46	13.56	7.08
90	20	12.98	19.84	11.52	5.12	11.42	4.96
100	10	6.62	7.68	6.22	0	5.54	0
100	20	3.28	3.86	2.94	0	2.72	0
100	30	0	0	0	0	0	0
121[a]	10	0	0	0	0	0	0

Inhibitory activity at 25 °C was taken as 100 %

TIU trypsin inhibitory units, *CIU* chymotrypsin inhibitory units

[a] Autoclaving at 1.04 kg/cm^2 pressure

The trypsin inhibitors isolated from the seeds of *Abelmoschus moschatus* have been found to be homogenous by the criteria of native PAGE and gel filtration. Each one of the four inhibitors showed a single band on gelatin PAGE corresponding to silver/Coomassie brilliant blue stainable protein band.

The molecular weights of AMTI-I, AMTI-II, AMTI-III and AMTI-IV, as determined by SDS-PAGE, were found to be 22.4, 21.2, 20.4 and 20.8 kDa, respectively. These are close to

Table 4 Effect of pH on *Abelmoschus moschatus* trypsin inhibitors

pH	Name of the buffer	AMTI-I	AMTI-II	AMTI-III		AMTI-IV	
		TIU/mg of AMTI-I×10^2	TIU/mg of AMTI-II×10^2	TIU/mg of AMTI-III×10^2	CIU/mg of AMTI-III×10^2	TIU/mg of AMTI-IV×10^2	CIU/mg of AMTI-IV×10^2
3	Glycine-HCl	21.42	25.20	19.12	10.28	18.42	9.68
5	Sodium citrate	21.68	25.38	19.28	10.30	18.68	9.90
7	Sodium Phosphate	21.26	25.44	19.32	10.32	18.22	9.82
9	Tris-HCl	21.34	25.24	19.40	10.12	18.18	9.80
12	Glycine-NaOH	21.32	25.18	19.22	10.18	18.28	9.84

AMTI-I, AMTI-II, AMTI-III and AMTI-IV were separately incubated for 24 h at 4 °C in the respective buffers and assayed for Trypsin and chymotrypsin inhibitory activities. BAPNA was used as the substrate for trypsin and ATEE for chymotrypsin

TIU trypsin inhibitory units, *CIU* chymotrypsin inhibitory units

Table 5 Effect of 8 M urea, 1 % SDS and 6 M guanidine hydrochloride on *Abelmoschus moschatus* trypsin inhibitors

Name of the buffer	AMTI-I	AMTI-II	AMTI-III		AMTI-IV	
	TIU/mg of AMTI-I×10^2	TIU/mg of AMTI-II×10^2	TIU/mg of AMTI-III×10^2	CIU/mg of AMTI-III×10^2	TIU/mg of AMTI-IV×10^2	CIU/mg of AMTI-IV×10^2
Control	21.60	25.30	19.0	10.20	18.60	9.90
8 M urea	20.44	24.62	18.54	9.44	18.02	9.10
1 % SDS	20.26	24.48	18.36	9.34	17.68	9.04
6 M guanidine hydrochloride	11.94	16.24	9.40	5.12	8.68	4.90
6 M guanidine hydrochloride (at 100 °C for 2 min)	5.38	6.48	5.28	2.28	4.68	2.14

AMTI-I, AMTI-II, AMTI-III and AMTI-IV were separately incubated for 24 h at room temperature (29 °C) in 8 M urea, 1 % SDS and 6 M guanidine hydrochloride and assayed for TIA and CIA. BAPNA was used as the substrate for trypsin and ATEE for chymotrypsin

TIU trypsin inhibitory units, *CIU* chymotrypsin inhibitory units

the corresponding values 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. Seeds of *Crotalaria paulina*, *Dimorphandra mollis*, *Murraya koenigii* and *Achyranthes aspera* are reported to contain monomeric trypsin inhibitors with similar molecular weights [26–29]. AMTI-I and AMTI-II were found to be glycoproteins with 2.8 and 4 % carbohydrate content, respectively. Majority of the proteinase inhibitors isolated from plants were non-glycoproteins, but those from *Abelmoschus esculentus*, *Artocarpus integrifolia*, *Peltophorum dubium* and *Carica papaya* were reported to be glycosylated inhibitors [30–33].

All the four inhibitors were devoid of free thiol groups probably due to their involvement in the formation of intra-chain disulphide bridges. The tryptophan content of the inhibitors was found to be low in agreement with the amino acid content in a number of Kunitz type of inhibitors from *Acacia confusa* [34] and *Delonix regia* [35]. Some protease inhibitors such as those from faba bean [36], ridge gourd [37], cow pea [9] and prickly chaff flower [29] are devoid of tryptophan residues.

The unusual stability of proteinase inhibitors in general is their most remarkable property. The inhibitors showed similarities in their stability with protease inhibitors from a number of plant sources. The presence of crude fibre, phytate and tannins might provide some protection to the inhibitors against heat treatment [38]. Trypsin inhibitors purified from several plant sources were found to be stable over a wide pH range (4–10) [39]. The differences in the stability of the trypsin inhibitors are probably due to differences in the nature of the proteins—conformation and bonding involved [40]. The disulphide bridges also contribute to the stability of trypsin inhibitors. However, the low cysteine content in these inhibitors negates the possibility of the stability of the inhibitors rendered due to extensive intra-peptide cross linking.

Most of the Kunitz inhibitors have a direct relationship between reduction of their disulphide bonds and loss of their biological activity. The four inhibitors, on the contrary, did not lose their proteinase inhibitory activities even after reduction with DTT suggesting that disulphide bonds are not needed for their inhibitory activities. This uncommon behaviour has been reported for a few inhibitors from *Entada scandens* [41] and *Piptadenia moniliformis* [42].

Because of their small molecular masses, acidic nature (low pI value) and existence in multiple forms, the inhibitors from *Abelmoschus moschatus* may be categorized as Kunitz inhibitors.

Conclusions

Four trypsin inhibitors, AMTI-I, AMTI-II, AMTI-III and AMTI-IV, have been isolated and purified from the seeds of *Abelmoschus moschatus*, and their molecular weights were estimated to be 22.4, 21.2, 20.8 and 20.2 kDa, respectively by SDS-PAGE. All the inhibitors inhibited bovine trypsin strongly, and two (AMTI-III and AMTI-IV) of them moderately inhibited bovine chymotrypsin. The purified inhibitors were quite stable up to 80 °C for 10 min, and their activities were not affected at alkaline as well as acidic conditions tested. Treatment with 8 M urea and 1 % SDS for 24 h at room temperature did not cause any loss of their antitryptic and antichymotryptic activities. However, the inhibitors have lost their trypsin inhibitory activity in the presence of 6 M guanidine hydrochloride. The four inhibitors did not lose their proteinase inhibitory activities even after reduction with DTT suggesting that disulphide bonds are not needed for their inhibitory activities.

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References

1. Choi, J. H., Park, P. J., & Kim, S. W. (2002). Purification and characterization of a trypsin inhibitor from the egg of skipjack tuna *Katsuwonus pelamis. Fisheries Science, 68*, 1367–1373.
2. Lopes, J. L. S., Valadares, N. F., Moraes, D. I., Rosa, J. C., Araújo, H. S. S., & Beltramin, L. M. (2009). Physico-chemical and antifungal properties of protease inhibitors from *Acacia plumos*a. *Phytochemistry, 70*(7), 871–879.
3. Laskowski, M., Jr., & Kato, I. (1980). Protein inhibitors of proteinases. *Annual Review of Biochemistry, 49*, 593–626.
4. Richardson, M. (1991). "Seed storage proteins: the enzyme inhibitors,"Methods in Plant Biochemistry. In J. L. Rogers (Ed.), *Amino Acids, Proteins and Nucleic Acids, 5* (pp. 259–305). New York: Academic Press.
5. Annapurna, S. S., & Siva Prasad, D. (1991). Purification of trypsin/chymotrypsin inhibitor from Jack fruit seeds. *Journal of the Science of Food and Agriculture, 54*, 399–411.
6. Fook, J. M. S. L. L., Macedo, L. L. P., Moura, G. E. D. D., Teixeira, F. M., Oliveira, A. S., Queiroz, A. F. S., & Sales, M. P. (2005). A serine proteinase inhibitor isolated from *Tamarindus indica* seeds and its effects on the release of human neutrophil elastase. *Life Sciences, 76*(25), 2881–2891.
7. Tsybina, T. A., Dunaevsky, Y. E., Musolyamov Kh, A., Egorov, T. A., & Belozersky, M. A. (2001). Cationic Inhibitors of Serine Proteinases from Buckwheat Seeds. *Biochemistry (Moscow), 66*(9), 941–947.
8. Wong, R. C., Fong, W. P., & Ng, T. B. (2004). Multiple trypsin inhibitors from*Momordica cochinchinensis* seed, the Chinese drug mubiezhi. *Peptides, 25*, 163–169.
9. Sammour, R. H. (2006). Isolation and characterization of four isoinhibitors from cowpea (*Vigna unguiculata* (L.) Walp.) seeds. *Turkish Journal of Biology, 30*, 207–215.
10. Ee, K. Y., Zhao, J., Rehman, A., & Agboola, S. (2008). Characterization of trypsin and chymotrypsin inhibitors in Australian wattle seed (*Acacia victoriae* Bentham). *Food Chemistry, 107*, 337–343.
11. Sun, J., Hexiang, W. & Tzi Bun, Ng. (2010). Trypsin isoinhibitors with antiproliferative activity toward leukemia cells from *Phaseolus vulgaris* cv "White Cloud Bean". *J Biomed Biotechnol*, 1-8.
12. de Caio Fernando Ramalho, O., Ilka Maria, V., Ricardo, A., das Gracas Machado Freire, M., Paulo Aparecido, B., Marangoni, S., & Maria Lígia Rodrigues, M. (2012). Purification and biochemical properties of a Kunitz-type trypsin inhibitor from *Entada acaciifolia* (Benth.) seeds. *Process Biochemistry, 47*, 929–935.
13. Oliva, M. L. V., & Sampaio, M. U. (2009). Action of plant proteinase inhibitors on enzymes of physiopathological importance. *Annals of the Brazilian Academy of Sciences, 81*(3), 615–621.

14. Richardson, M. (1977). The proteinase inhibitors of plants and microorganisms. *Phytochemistry, 16*, 159–169.
15. Martin, J., Redshaw, S., & Thomas, G. (1995). Inhibitors of HIV proteinase. *Progress in Medicinal Chemistry, 32*, 239–287.
16. Jin-Young, K., Park, S. -C., Hwang, I., Cheong, H., Nah, J.-W., Hahm, K.-S., & Park, Y. (2009). Protease inhibitors from plants with antimicrobial activity. *International Journal of Molecular Sciences, 10*(6), 2860–2872.
17. References and further reading may be available for this article. To view references and further reading you must this Lowry, O.H., Rosebrough, N.J., Farr, A.L. &Randall, R.J. (1951). Protein measurement with Folin phenol reagent. *J.Biol.Chem, 193,* 265-275
18. Laemmli, U. K. (1970). Cleavage of structure proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680–685.
19. Kakade, M. L., Simons, N. R., & Liener, I. E. (1969). An evolution of natural vs synthetic substrates for measuring the antitryptic activity of soybean substrates. *Cereal Chemistry, 1969*(46), 518–526.
20. Prabhu, K. S., & Pattabiraman, T. N. (1977). A colorimetric method for the estimation of the esterolytic activity of chymotrypsin. *Indian Journal of Biochemistry and Biophysics, 14*, 96–98.
21. Dubois, M., Gillis, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry, 28*(3), 350–356.
22. Reissig, J. L., Strominger, J. L., & Leloir, L. F. (1955). A modified colorimetric method for the estimation of *N*-acetylamino sugars. *Journal of Biological Chemistry, 217*, 959–966.
23. Ellman, G. L. (1959). Tissue sulfhydryl groups. *Archives of Biochemistry and Biophysics, 82*, 70–77.
24. Habeeb, A. F. S. A. (1972). Reaction of sulpfhydryl groups with Ellmans reagent. *Methods in Enzymology, 25*, 457–464.
25. Edelhoch, H. (1967). Spectroscopic determination of tryptophan and tyrosine in protein. *Biochemistry, 6*, 1948–1954.
26. Luiza, P. A., Di Ciero, L., Novello, J.´e. C., Oliveira, B., Weder, J. U. K. P., & Marangoni, S. (1999). Isolation and characterization of a new trypsin inhibitor from *Crotalaria paulina* seeds. *IUBMB Life, 48*(5), 519–523.
27. Macedo Maria LoAgia, R., Daniela Gaspar, G., de Matos, Machado, O. L. T., Marangoni, S. A., & Novello, J. A. C. (2000). Trypsin inhibitor from *Dimorphandra mollis* seeds: purification and properties. *Phytochemistry, 54*, 553–558.
28. Tejas, K. S., & Sushma, S. G. (2012). Studies on a trypsin inhibitor from the seeds of *Murraya koenigii. International Journal of Pharma and Bio Sciences, 3*(2), 794–809.
29. Geetha, & Siva Prasad, D. (2013). *International Journal of Advancements in Research & Technology, 2*(8), 215–222.
30. Ogata, F., Imamura, H., Hirayama, K., & Makisumi, S. (1986). Purification and characterization of four trypsin inhibitors from seeds of okra, *Abelmoschus esculentus* L. *Agricultural and Biological Chemistry, 50*(9), 2325–2333.
31. Shankunthala, G.V. (1996). Ph.D Thesis, Andhra University, Visakhapatnam, India.
32. Troncoso, M. F., Cerdá Zolezzi, P., Hellman, U., & Wolfenstein-Todel, C. (2003). A novel trypsin inhibitor from *Peltophorum dubium* seeds, with lectin-like properties, triggers rat lymphoma cell apoptosis. *Archives of Biochemistry and Biophysics, 411*(1), 93–104.
33. Azarkan, M., Dibiani, R., Goormaghtigh, E., Raussens, V., & Baeyens-Volant, D. (2006). Thepapaya Kunitz-type trypsin inhibitor is a highly stable beta-sheet glycoprotein. *Biochimica et Biophysica Acta, 1764*(6), 1063–1072.
34. Lin, J. Y., Chu, S. C., Wu, H. C., & Hsieh, Y. S. (1991). Trypsin inhibitor from the seeds of *Acacia confusa. Journal of Biochemistry, 110*(6), 879–883.
35. Silvana, P. C., Oliva, M. L. V., Sampaio, C. A. M., Di Ciero, L., Novello, J. C., & Marangoni, S. (2001). Primary sequence determination of a Kunitz inhibitor isolated from *Delonix regia* seeds. *Phytochemistry, 57*, 625–631.
36. Asao, T., & Imai, F. (1991). Purification and characterization of BowmanBirk type proteinase inhibitor from faba bean (*Vicia faba* L.). *Agric. Biol.Chem, 55*, 707–713.
37. Haldar, U., Saha, S., Beavis, R., & Sinha, N. K. (1996). Trypsin inhibitors from ridged gourd (*Luffa acutangula* Linn.) seeds: purification, properties, and amino acid sequences. *Journal of Protein Chemistry, 15*(2), 177–184.
38. Benjakul, S., Visessanguan, W., & Thummaratwasik, P. (2000). Isolation and characterization of trypsin inhibitors from some Thai legume seeds. *Journal of Food Biochemistry, 24*, 107–127.
39. Klamklao, S., Benzakul, S., Kishimura, H., Osaka, K., & Tanaka, M. (2010). A heat-stable trypsin inhibitor in adzuki bean (*Vigna angularis*): effect of extraction media, purification and biochemical characteristics. *International Journal of Food Science & Technology, 45*(1), 163–169.
40. Cheftel, J. C., Cuq, J. L. & Lorient, D. (1985). Amino acids, peptides and proteins. *In. Food Chemistry*, 245-369. New York: Marcel Dekker.

41. Lingaraju, M. H., & Lalitha, R. G. (2008). A Kunitz trypsin inhibitor of *Entada scandens* seeds: another member with single disulfide bridge. *Biochimica et Biophysica Acta, (BBA) - Proteins and Proteomics, 1784*(5), 850–855.
42. Cruz Ana, C. B., Massena, F. S., Miglioloa, L., Macedo, L. L. P., Monteiro, N. K. V., Oliveira, A. S., Macedo, F. P., Uchoa, A. F., Maria, F., de Sá, G., Vasconcelos, I. M., Murad, A. M., Francoe, O. L., & Santos, E. A. (2013). Bioinsecticidal activity of a novel Kunitz trypsin inhibitor from Catanduva (*Piptadenia moniliformis*) seeds. *Plant Physiology and Biochemistry, 70*, 61–68.