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RESEARCH ARTICLE

HEMAGGLUTINATING ACTIVITY OF TRYPSIN INHIBITORS FROM THE SEEDS OF *ABELMOSCHUS MOSCHATUS* L.

Muni Kumar Dokka, Geetha Konala and Siva Prasad Davuluri*

Department of Biochemistry, Andhra University, Visakhapatnam – 530 003, Andhra Pradesh, India.

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Corresponding Author

Siva Prasad Davuluri

Abstract

A highly stable and potent trypsin inhibitor (AMTI-I) was purified to homogeneity from the seeds of *Abelmoschus moschatus*, family Malvaceae, following conventional methods of protein purification. AMTI-I exerted strong inhibition towards bovine pancreatic trypsin and it showed moderate inhibition towards elastase. SDS-PAGE analysis, under reducing conditions, indicated the protein consisting of a single polypeptide chain with a apparent molecular mass of 22.4 kDa. The inhibitors, AMTI-I and AMTI-II also isolated and purified from the same source, are unique in that they exhibited hemagglutinating activity towards different erythrocytes. This activity was found to be inhibited by D-galactose only. The lectin activity was stable over a broad pH range (3.0-12.0) and temperatures up to 60^{o}C for 15 min and was also resistant to denaturants. Oxidation of AMTI-I and AMTI-II by sodium metaperiodate or treatment with PNGase F affected their lectin activities. Since trypsin inhibitors and lectins have been shown to provide protection in plants against invading insect pests and pathogens, AMTI-I and AMTI-II possessing both antitryptic and lectin activities may be explored in the agricultural front for developing transgenics after carrying out extensive in vitro studies.

INTRODUCTION

Plants have developed several defense mechanisms that offer protection from predators or from infection by pathogens. One of these defenses involves the production of several proteins such as enzyme inhibitors, lectins, chitinases, ureases and ribosome - inactivating proteins (Carlini and Grossi de Sa, 2002; Lajolo and Genovese, 2002; Murdock and Shade, 2002) and allelochemicals comprising tannins, alkaloids, phytates, antibiotics, cyanogenic glycosides etc. These defensive agents, in addition to proteinase inhibitors, may either act alone or in concert to contribute to overall resistance of plants against invading pests and pathogens. Proteinase inhibitors occur as constitutive part of plant tissues and often are inducible. Protease inhibitors have been the subject of extensive research not only for their potential use as bioinsecticides (Haq et al, 2004; Abdeen et al., 2005) but also for their antimicrobial activities (Park et al., 2005).

Lectins have been shown to exert deleterious effects on a range of important insect pests. Lectins bind to specific receptor sites on the peritrophic membrane of cells lining the gut of insects resulting in disruption of epithelial cells in turn causing impairment in the absorption of nutrients across the intestinal walls (Liener, 1986).

Currently, the two major groups of plant derived genes related to proteinase inhibitors and lectins are used to confer insect resistance on crops. Yao et al. (2003) for the first time reported that transgenic tobacco expressing Pinellia ternata agglutinin (pta) gene induced enhanced level of resistance to Myzus persicae. Some plant lectins such as GNA (Galantus nivalis agglutinin), WGA (Triticum eestivum agglutinin) and Con A (Canavalia ensiformis agglutinin) have been successfully expressed in crops to confer résistance against insect pests (Powell et al., 1995; Down et al., 1996; Bandyopadhyay et al., 2001). The insecticidal activity of protease inhibitors and α-amylase

inhibitors were significantly increased when incorporated with lectin (Murdock and Shade, 2002; Amirhusin et al., 2004; Abdeen et al., 2005).

It is well known that plant seeds are rich in proteinase inhibitors and lectins. Protease inhibitors exhibiting lectin activity have also been reported from the seeds of northern beans, jack fruit, cow pea, taub and soybean (Sathe and Salunkhe, 1981; Shakuntala, 1996; Maia, et al., 2000; Troncoso et al., 2003, 2007).

Abelmoschus moschatus (L.) Medic, family Malvaceae, is an aromatic and medicinal plant popularly known as Mushkdana / Kasturi bhendi. The seeds are rich in protease inhibitors and they are used to check excessive thirst, cure for stomatitis, dyspepsia, urinary discharge, gonorrhea, leucoderma and itchiness. Considering some protease inhibitors possessing intrinsic lectin activity, it is thought worthwhile to investigate whether trypsin inhibitors from *Abelmoschus moschatus* could exhibit hemagglutinating activity or not. Experiments, therefore, have been carried out to demonstrate that trypsin inhibitors from the seeds of *Abelmoschus moschatus* exhibit lectin activity. This paper deals with the isolation, purification of AMTI-I from the seeds of *Abelmoschus moschatus* and to examine hemagglutinating activity of AMTI-I and AMTI-II against different erythrocytes.

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 inhibitor.

Blood was collected from the rabbits and rats maintained in the animal house. Sheep blood was obtained from the slaughter house. Human blood belonging to different groups was collected from healthy volunteers.

Purification of *Abelmoschus moschatus* Trypsin Inhibitor (AMTI-I)

A procedure has been established for the purification of proteinase inhibitor from the seeds of *Abelmoschus moschatus*. 25 g of the seeds were homogenized with 150 ml of 0.1 M sodium phosphate buffer, pH 7.6 and then made up to 250 ml with the same buffer. The extract was then centrifuged at 5,600 rpm for 15 min at 4°C. The supernatant (250 ml) was dialyzed against the buffer for 24h in the cold and rapidly heated to 70°C and maintained at this temperature for 10 min. The extract was quickly cooled in ice and then centrifuged at 5,600 rpm for 15 min at 4°C. To the supernatant, solid ammonium sulfate was added to 60% saturation with constant stirring at 4°C. The mixture was kept overnight at 4°C. The precipitate was collected by centrifugation at 3,000 rpm for 10 min at 4-6°C, dissolved in 0.1 M sodium phosphate buffer, pH 7.6 and dialyzed against the same buffer. The dialyzed sample was loaded on to a DEAE-cellulose column (2.2 x 34 cm) and the elution was performed with 0.1- 1.0 M NaCl in the 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 using BAPNA as the substrate. Unbound fractions and 0.1 M NaCl eluted fractions were used for further purification of AMTI-I and AMTI-II respectively.

Proteins from the previous step were loaded on Sephadex G-100 column (1.9 x 63 cm) and eluted with the same buffer. Fractions (2 ml) were collected at a flow rate of 12 ml/ h and the protein was monitored by measuring the absorbance at 280 nm. The trypsin inhibitory activities of the fractions were assayed using BAPNA as the substrate. Fractions containing the trypsin inhibitory activities were pooled, dialyzed against distilled water at 4-6°C and then lyophilized.

Protein estimation

Protein was estimated by the method of Lowry et al., (1951) using bovine serum albumin as the standard.

Determination of molecular weight

Molecular weight of AMTI-I was determined by SDS-PAGE using the method of Laemmli (1970) 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 et al., (1969) using BAPNA as the substrate. Trypsin (30 g) 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.

To determine the inhibitory activities, suitable aliquots of the inhibitor solutions were included in the assay medium to obtain 30-70% inhibition. 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 Elastase:

Elastase was assayed using elastin congo red as the substrate by the method of Naughton and Sanger (1961).

Hemagglutination assays

Erythrocyte agglutinating activity of AMTI-I and AMTI-II was determined according to the method of Paulova et al., (1971). Both normal and trypsin treated erythrocytes from sheep and human subjects were used in these assays.

Blood collected in Alsever´s solution (Bukantz et al., 1946) was centrifuged at 3,000 rpm for 10 min and the sedimented erythrocytes were washed 4 times with physiological saline (0.9% NaCl). A 4% (v/v) erythrocyte suspension made in 0.9% NaCl was used for the experiments. Erythrocytes were subjected to trypsin treatment on the day of the assay. The erythrocyte suspension (4%) was incubated with 0.1% trypsin for one hour at 37º C. The cells were then centrifuged and resuspended in saline to get a 4% suspension after washing several times with saline.

The hemagglutination assay was carried out in a plexi-plate with a concave wells using the two fold serial dilution technique. 0.2 ml of saline was added to each well followed by 0.2ml of each of the inhibitor solution (2 mg/ml in Phosphate buffered saline (PBS), pH 7.4) to the first well. The samples were mixed and was serially diluted each time transferring 0.2ml to each well. Finally 0.2 ml of 4% erythrocyte suspension was added to each of the wells, gently mixed and incubated for 90 min at 37ºC and the agglutination of the cells was examined visually. The control was without inhibitor in the system.

JSTI possessing lectin activity was selected for positive control. The highest dilution which showed positive hemagglutination was taken as the titre. The amount of protein present at this dilution represents the minimum quantity of the inhibitor necessary for agglutination under the experimental conditions and is defined as one hemagglutinating unit.

Sugar inhibition assays

These assays were carried out using 2- 4 hemagglutinating units of AMTI-I and AMTI-II. In a total volume of 0.2 ml, each of the inhibitor was preincubated with saline containing the sugars, D-galactose, D-mannose, D-glucose, lactose (0.05 - 0.5M concentration) for one hour at room temperature. Trypsin- treated rabbit erythrocytes (0.2 ml) were then added, gently mixed and incubated at 37ºC for 90 min. The extent of hemagglutination was examined and the minimum concentration of each sugar required to inhibit the agglutination of erythrocytes was recorded. The effect of D- galactosamine hydrochloride and methyl- β- D-thiogalactoside (5-50 mM concentration) were also investigated on the hemagglutinating activity of AMTI-I and AMTI-II by the same method.

Trypsin inhibitory and hemagglutinating activities of AMTI-I and AMTI-II subjected to various treatments

Temperature

Two ml sample of each inhibitor (2 mg/ml) in 0.1M sodium phosphate buffer, pH 7.6 was incubated in a water bath at different temperatures (40- 100ºC) for 15 min. After the heat treatment, the solutions were rapidly cooled on ice and suitable aliquots were used for the assay of trypsin inhibitory and lectin activities.

pH

Each inhibitor (5 mg/ml) in an appropriate 10 mM buffer was kept at 4ºC for 24 h. The buffers used were glycine - HCl (pH 3), sodium citrate (pH 5), sodium phosphate (pH 7), Tris-HCl (pH 9) and glycine-NaOH (pH 12). The inhibitors were dialyzed against PBS and aliquots of both the inhibitors were assayed for trypsin inhibitory and hemagglutinating activities.

Denaturants

Each inhibitor (2mg/ml) was prepared in 0.1M sodium phosphate buffer, pH 7.6 containing 8M urea or 6M guanidine hydrochloride and incubated at 4ºC for 24 h. The inhibitor solutions in 8M urea were then dialyzed against PBS containing 1M urea and the inhibitor solutions in guanidine hydrochloride were dialyzed against PBS. Suitable aliquots were taken and used for the assay of trypsin inhibitory and hemagglutinating activities.

Sodium metaperiodate:

This was carried out as described by Chowdhury et al., (1987). Each inhibitor (4mg/ml) in 50 mM acetate buffer (pH 5.1), was mixed with an equal volume of 0.25 M sodium metaperiodate dissolved in the same buffer. The mixture was incubated at 0-4ºC in the dark. Aliquots were taken at hourly intervals and dialyzed against PBS. Proteinase inhibitory and hemagglutinating activities of each oxidized inhibitor were determined.

PNGase F (**Peptide -N-Glycosidase F)**

Each inhibitor (100 µg) in one ml of 0.25M sodium phosphate buffer, pH 8.6 containing 20 mM EDTA was incubated with 5 µl of PNGase F (2000 units) for 18 h at 37ºC. Enzyme treated inhibitors were dialyzed against PBS and then assayed for proteinase inhibitory and hemagglutinating activities. The inhibitor treated in the same way but without PNGase F served as control.

Results and Discussion

Isolation and purification

A trypsin inhibitor (AMTI-II) with an apparent molecular mass of 21.2 kDa was also isolated and purified from the seeds of *Abelmoschus moschatus* following the procedure described earlier (Muni Kumar and Siva Prasad, 2014).

AMTI-I from *Abelmoschus moschatus* seeds was isolated and purified following thermal denaturation, ammonium sulphate fractionation and chromatography on DEAE-cellulose and Sephadex G-100. When the ammonium sulphate 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. By employing DEAE- cellulose chromatography, four protein peaks have been resolved by linear NaCl gradient (0.1M 1M NaCl in buffer). AMTI-I eluted through flow through fractions on DEAE - cellulose column showed higher antitryptic activity and seems to be one of the major potent trypsin inhibitor obtained when compared to the other protein peaks. When the lyophilized active fractions were subjected to Sephadex G-100 column chromatography, the inhibitor eluted out as a single peak with corresponding trypsin inhibitory activity (Fig 1).

Recoveries and relative purification at each step for a typical purification from 25 g seeds are shown in Table - 1. By this procedure, about 40.8 mg of AMTI-I was obtained with a final yield of about 11%.

The molecular weight of AMTI-I as determined by SDS-PAGE (Fig 2) was found to be 22.4 kDa which was close to that obtained with gel filtration on Sephadex G-200 (Fig 3). The inhibitor gave a single sharp band on SDS-PAGE even in the presence of 2-mercaptoethanol indicating the monomeric nature of the protein.

Hemagglutinating activity

As majority of the lectins reported in literature agglutinated both human and rabbit erythrocytes, AMTI-I and AMTI-II were also tested for their ability to agglutinate erythrocytes from different species.

Table - 2 shows that AMTI-I and AMTI-II agglutinated both normal and trypsin- treated erythrocytes of rabbit, rat, human and sheep but with different efficiencies. Both the inhibitors agglutinated trypsin-treated rabbit and rat erythrocytes at a much lower concentration compared to those of human and sheep. As low as 12.5 µg AMTI-I and AMTI-II was sufficient to cause the visible agglutination reaction with trypsin-treated rabbit erythrocytes. Although the inhibitors agglutinated trypsin- treated rabbit and rat erythrocytes equally, the titre value obtained with native rabbit erythrocytes was two times higher than that obtained with untreated rat erythrocytes. AMTI-I and AMTI-II also agglutinated trypsin-treated human erythrocytes irrespective of the blood groups.

Sugar inhibition studies

As lectins are sugar specific, the hemagglutination inhibition test with various haptenic sugars was carried out with AMTI-I and AMTI-II and the results are presented in Table-3. Agglutinating activity was specifically inhibited by D-galactose at 0.3M concentration. On the other hand, sugars such as D-glucose, D-mannose and lactose were found to be non-inhibitory even at 0.5M concentration. Derivatives of D-galactose such as D-galactosamine hydrochloride and methyl β-D-thiogalactoside, even at a concentration of 50 mM, were not effective in inhibiting hemagglutination by AMTI-I and AMTI-II.

Effect of temperature and pH on the stability of AMTI-I and AMTI-II

One of the characteristic features of trypsin inhibitors is their stability towards extremes of pH and high temperatures. In order to ascertain the stability of lectin activity of AMTI-I and AMTI-II at different pH and temperatures, the following experiments were done.

AMTI-I and AMTI-II were subjected to variations of pH in the range of 3-12. The hemagglutinating activity of AMTI-I and AMTI-II was not affected either at acidic or alkaline conditions (Table-4). Trypsin inhibitory activity of both inhibitors was also not affected under these conditions.

The effect of temperature on the lectin activity of AMTI-I and AMTI-II is given in Table-5. Lectin activity is quite stable up to 70^{o}C for 15 min. Heating both the inhibitors in a boiling water bath for 15 min resulted in a total loss of their lectin activity but with a fall of 85% in its proteinase inhibitory activity. AMTI-I and AMTI-II had completely lost their trypsin inhibitory activity after heating them for one hour in a boiling water bath.

Effect of denaturants on AMTI-I and AMTI-II

The hemagglutinating activity of AMTI-I and AMTI-II was not affected when both the inhibitors were exposed to 8M urea or 6M guanidine hydrochloride for 24 h (Table-4).The trypsin inhibitory activity of both inhibitors was also not affected when exposed to 8 M urea. However, there was a loss of about 45% trypsin inhibitory activity of the inhibitors when exposed to 6M guanidine hydrochloride.

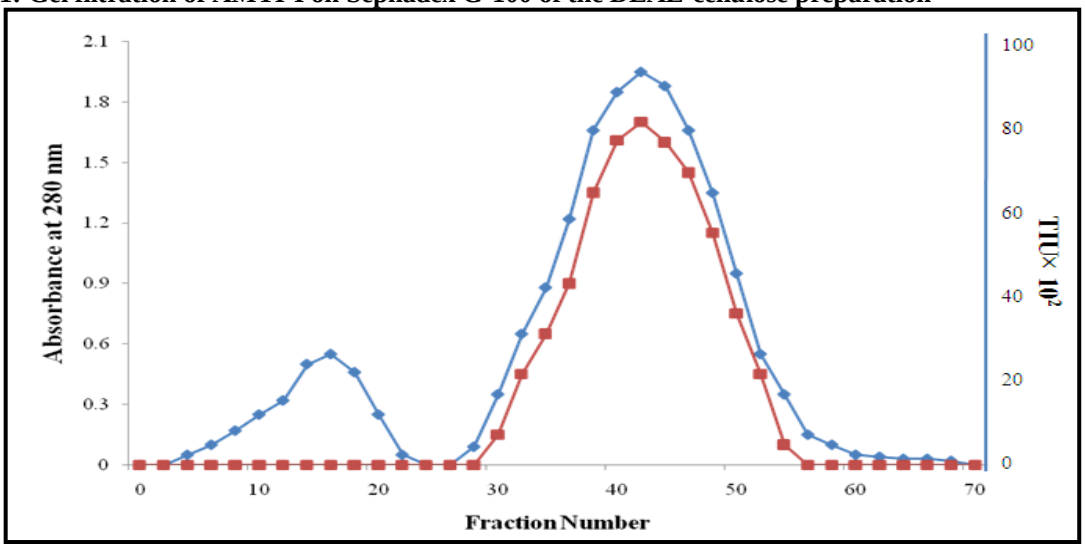
Oxidation of AMTI-I and AMTI-II by sodium metaperiodate

Oxidation of the hydroxyl groups of the carbohydrate moieties of AMTI-I and AMTI-II by sodium metaperiodate resulted in a 50% decrease in the hemagglutinating activity of both the inhibitors. This treatment, however, did not show much effect on trypsin inhibitory activity of the inhibitors (Table-4).

Deglycosylation of AMTI-I and AMTI-II

AMTI-I and AMTI-II were glycoproteins with a carbohydrate content of 2.8 and 4 % respectively. In order to ascertain the role of sugars on lectin activity, AMTI-I and AMTI-II were treated with PNGase F for 18 h and then assayed for hemagglutinating and trypsin inhibitory activities. The deglycosylated inhibitors have retained their trypsin inhibitory activity but lost their hemagglutinating activity (Table-4). The results obtained indicate that sugars may be needed for the lectin activity of both the inhibitors.

Figure 1: Gel filtration of AMTI-I on Sephadex G-100 of the DEAE-cellulose preparation

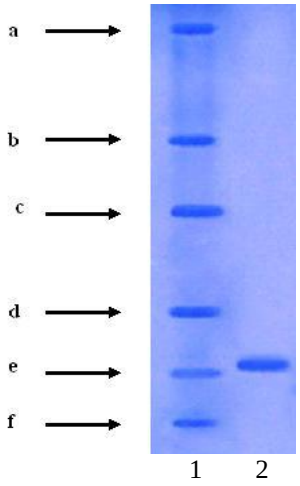


55.2 mg of AMTI-I, was applied on to the column (1.9 x 63) in 0.1 M phosphate buffer, pH 7.6 and eluted with the same buffer. Fractions, each 2 ml, were collected at a flow rate of 12 ml/h.

(♦ ---------♦) Protein was monitored by absorbance at 280 nm

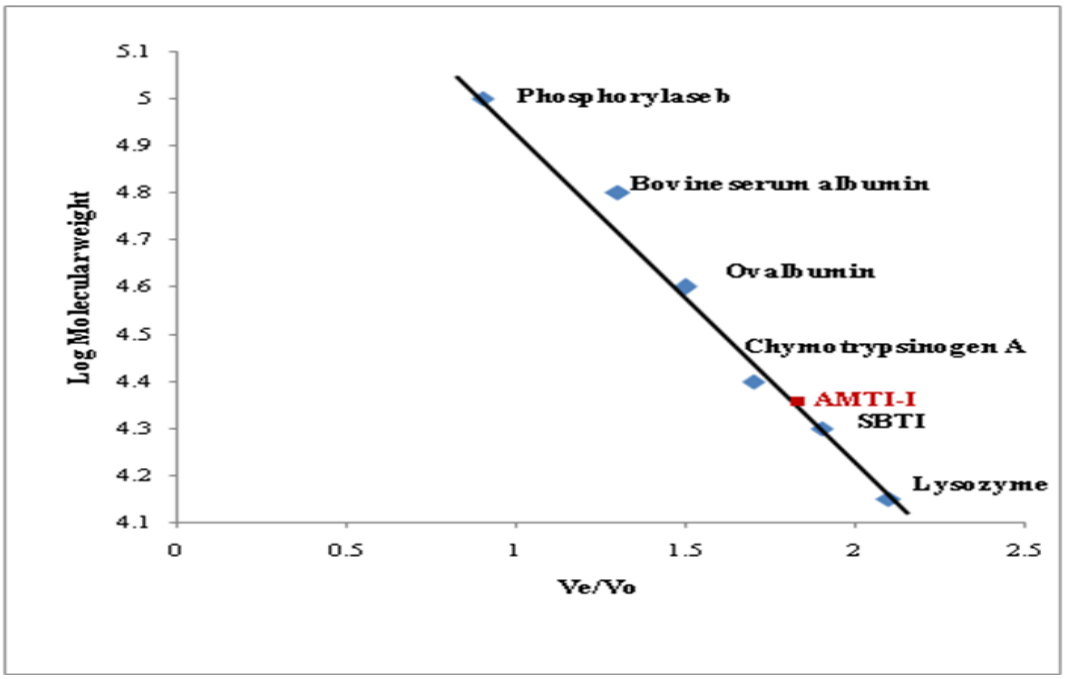
(■---------■) Trypsin inhibitory activity

Figure 2: Molecular weight determination of AMTI-I by SDE-PAGE



1. Standard proteins
(a) Phosphorylase b, 97kDa
(b) Bovine serum albumin, 67kDa
(c) Ovalbumin, 45kDa
(d) Chymotrypsinogen A, 25kDa
(e) Soybean trypsin inhibitor, 20.1 kDa
(f) Lysozyme, 14kDa
2. Purified AMTI-I

Figure 3: Molecular weight determination of AMTI-I by gel filtration on Sephadex G-200.



Plot of elution volume against log molecular weight of standard proteins (♦) and AMTI-I (■).

Table-1: Summary of purification of AMTI-I from seeds of *Abelmoschus moschatus*

Preparation	Volume (ml)	Total protein (mg)	Total activity units	Specific activity Units/mg protein	Yield (%)	Fold purification
			TIU×10^3	TIA×10^2		
Crude extract	250	2087.5	788.4	3.77	100	1.00
Heat treatment	215	1016.4	626.4	6.16	79.45	1.63
Ammonium sulphate (60%) Fractionation	60	424.8	482.8	11.36	61.24	3.01
DEAE-Cellulose flow through fraction	248	55.2	104.4	18.91	13.24	5.01
Sephadex-G-100 fraction	46	40.8	88.4	21.67	11.21	5.75

*Yield and fold purification were calculated on the basis of TIU and TIA respectively.
TIU- Trypsin inhibitory units TIA-Trypsin inhibitory activity

Table-2: Agglutination of human and animal erythrocytes by AMTI-I and AMTI-II

Origin of erythrocytes		Minimal hemagglutinating dose (µg)					
		AMTI-I		AMTI-II		JSTI	
		Normal	Trypsin - treated	Normal	Trypsin - treated	Normal	Trypsin - treated
Rabbit		50	12.5	50	12.5	50	6.25
Rat		100	12.5	100	12.5	100	6.25
Sheep		200	100	200	100	200	100
Human	A	100	50	100	50	100	50
	B	100	50	100	50	100	50
	AB	100	50	100	50	100	50
	O	100	50	100	50	100	50

0.2 ml of AMTI-I and AMTI-II each (2 mg/ml) was serially diluted with 0.9% NaCl and 0.2 ml of untreated or trypsin-treated erythrocytes suspension (4%) was added and hemagglutination assays were carried according to the method of Paulova et al., (1971). JSTI - Jack fruit seed trypsin inhibitor was used as positive control.

Table-3: Effect of various sugars on the hemagglutinating activity of the AMTI-I and AMTI-II

Sugar	Sugar concentration(M)	Inhibitory effect	
		AMTI-I	AMTI-II
D-galactose	0.30	+	+
D-glucose	0.50	-	-
D-mannose	0.50	-	-
Lactose	0.50	-	-
Galactosamine- HCl	0.05	-	-
Methyl-β-D-thiogalactoside	0.05	-	-

\+ : Inhibition observed

\- : Inhibition not observed at the concentration of the sugar indicated.

Inhibitory activity of the sugar is expressed as the minimum concentration (M) required for complete inhibition of four hemagglutinating units of each of the inhibitor.

Table-4: Effect of pH, denaturants, sodium metaperiodate and PNGase F on AMTI-I and AMTI-II

Treatment		% Residual trypsin inhibitory activity		% Residual hemagglutinating activity	
		AMTI-I	AMTI-II	AMTI-I	AMTI-II
AMTI exposed to [a]	pH – 3.0	96.50	97.50	100	100
	pH – 5.0	98.30	98.70	100	100
	pH – 7.0	99.20	98.80	100	100
	pH – 9.0	98.80	98.40	100	100
	pH – 12.0	97.40	98.10	100	100
AMTI treated with [b]	6Mguanidine hydrochloride	53.20	59.20	100	100
	8M urea	98.60	98.80	100	100
	Sodium metaperiodate	98.40	98.60	50	50
	PNGase F	94.80	96.80	0	0

a. AMTI- and AMTI-II (5 mg/ml) each was incubated separately for 24 h at 4^{o}C in the respective buffers, then dialyzed against PBS and assayed for the residual trypsin inhibitory and lectin activities. The activity of the inhibitors exposed to pH 7.6 was taken as 100%.
b. AMTI-I and AMTI-II were subjected to various treatments and then assayed for residual trypsin inhibitory and lectin activities. The activity of untreated inhibitor was taken as 100%.

Table-5: Effect of temperature on trypsin inhibitory and hemagglutinating activities of AMTI-I and AMTI-II

Temperature (^{o}C)	Time (min)	% Residual trypsin inhibitory activity		% Residual hemagglutinating activity	
		AMTI-I	AMTI-II	AMTI-I	AMTI-II
40	15	100	100	100	100
50	15	100	100	100	100
60	15	100	100	100	100
70	15	98.80	99.20	50	50
80	15	98.50	98.80	5	7
90	15	84.20	86.50	0	0
100	15	15.10	17.70	0	0
100	30	2.50	3.52	0	0
100	60	0	0	0	0

Inhibitory activity at 28^{o}C was taken as 100%.
Each inhibitor (2 mg/ml) in PBS was incubated separately in water-bath at different temperatures. After the heat treatment, the solutions were quickly cooled in ice and appropriate aliquots were used for the assay of trypsin inhibitory and lectin activities.

Discussion

Protease inhibitors are ubiquitous in plants generally acting as storage proteins and wound-induced defensive agents against herbivores and pathogens (Basir et al., 2000). Their role in plant protection against insects is studied relatively well. On the other hand, data pertaining to protease inhibitors exhibiting lectin activities are very few and need to be provided more.

In the present study, a novel trypsin inhibitor from *Abelmoschus moschatus* seeds have been isolated and purified to homogeneity following conventional methods of protein purification. The observation that trypsin inhibitory activity in the crude extracts of the seeds is stable at 70^{o}C for 10 min has led to the use of this treatment as the first step in the purification of the inhibitor. About 52% of proteins present in the crude extract were removed by this step. When the ammonium sulphate 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. One of the major trypsin inhibitor that obtained through void volume was also eluted out as a single protein with corresponding trypsin inhibitory activity when subjected to Sephadex G-100 gel filtration.

The final yield of the inhibitor was about 11%. AMTI-I was found to be homogenous by native PAGE and gel filtration on Sephadex G-200 column. The coomassie blue stainable protein band corresponded to the specific staining band for the visualization of the trypsin inhibitory activity**.** The molecular mass of AMTI-I was found to be 22.4 kDa as determined by SDS-PAGE and was close to the mass that obtained with gel filtration of Sephadex G-200 column.

The results presented in this paper clearly show that the inhibitors, AMTI-I and AMTI-II possess hemagglutinating activity, a property exhibited by a class of plant proteins called phytohemagglutinins (Goldstein and Hayes, 1978). Since reports are available in the literature on protease inhibitors exhibiting lectin activity, (Maia, et al., 2000; Troncoso et al., 2003, 2007), AMTI-I and AMTI-II have been examined for their hemagglutinating activity using erythrocytes from different animals and human beings. AMTI-I and AMTI-II agglutinated both normal and trypsin- treated erythrocytes of rabbit, rat, human and sheep but with different efficiencies. The high titre values of the inhibitors obtained with trypsin-treated erythrocytes compared to the untreated cells could be due to better exposure of receptors on the surface of cells by trypsin. AMTI-I and AMTI-II were human blood group nonspecific lectins as they agglutinated all the four groups of human erythrocytes with more or less similar efficiency. In this aspect, AMTI-I and AMTI-II resembles JSTI (Shakuntala, 1996) and other lectins such as Champedak (lectin c) (Hashim et al., 1991), KM^{+} lectin (Santos-de-Oliveira et al., 1994), Labramia bojeri lectin (Macedo et al., 2004) and Bauhinia variegata lectin (Pinto et al., 2008). On the contrary, a novel trypsin inhibitor form *Peltophorum dubium* seeds exhibited hemagglutination activity against rabbit erythrocytes but not native human erythrocytes (Troncoso et al., 2003) and Artocarpin and Delonix regia lectin showed no specificity for human erythrocytes of ABO blood groups (Miranda- Santos et al., 1991; Pando et al., 2002).

AMTI-I and AMTI-II agglutinated trypsin-treated rabbit and rat erythrocytes at a much lower concentration compared to those of human and sheep. As low as 12.5 µg AMTI-I and AMTI-II was sufficient to cause the visible agglutination reaction with trypsin-treated rabbit erythrocytes. This was similar to JSTI which was reported to exhibit strong hemagglutinating activity towards erythrocytes from different animals and human beings. This double-headed inhibitor gave a titre value corresponding to an amount of 6.25µg.

Of a number of sugars that were tested for their ability to inhibit the hemagglutinating activity by AMTI-I and AMTI-II, only D-galactose was found to be inhibitory. Derivatives of galactose which are considered to be more potent inhibitors of hemagglutinating activity of D-galactose specific lectins (Chowdhury et al., 1987; Sikdar et al., 1990) were ineffective on AMTI-I and AMTI-II even at 50 mM concentration. Lactose, a known inhibitor of hemagglutinating activity of a number of specific lectins (Mazumder et al., 1981; Yeasmin et al., 2001; Sarkar et al., 2007; Pinto et al., 2008), did not exert any effect on the lectin activity of AMTI-I and AMTI-II.

The hemagglutinating activity of AMTI-I and AMTI-II was found to be stable over a broad pH range (3-12) and when heated up to 60^{o}C for 15 min. The trypsin inhibitory activity of both the inhibitors was, however, not altered even when exposed to extremes of pH or temperatures up to 80^{o}C for 15 min. The stable nature of inhibitors to these conditions could be due to hydrophobic interactions in their protein molecules. Thus by carefully regulating the temperature, the lectin activity of the inhibitors can be substantially reduced with only a minor alteration in their trypsin inhibitory activity. It is noteworthy that the hemagglutinating activity of AMTI-I and AMTI-II were resistant to denaturants such as 8 M urea or 6 M guanidine hydrochloride.

Oxidation of AMTI-I and AMTI-II by sodium metaperiodate while having not much effect on trypsin inhibitory activity resulted in a decrease in their lectin activity by 50%. Periodate is known to cause oxidation of carbohydrates or amino acid residues such as tryptophan and cysteine of proteins. AMTI-I and AMTI-II did not

depend on tryptophan residues for their hemagglutinating activity and the decrease observed in its lectin activity could be due to the oxidation of carbohydrate moieties only. Further evidence that sugars are required for hemagglutinating activity of the inhibitors came from the deglycosylated experiment using PNGase F. The deglycosylated inhibitors while retaining their trypsin inhibitory activity lost their hemagglutinating activity completely. This supports that sugar residues might be essential for the hemagglutinating activity of the inhibitors. Sugars may be involved in the binding of inhibitors to their receptors on the surface of erythrocyte membranes. Bauhinia purpurea lectin (Rao and Balasubramaniyam, 1994), in contrast to AMTI-I and AMTI-II, retained its hemagglutinating activity even after endoglycosidae treatment.

The hemagglutination assays confirm that the AMTI-I and AMTI-II have an associated lectin activity. Specific tests such as precipitation of polysaccharides or glycoconjugates and mitogenic transformation of cells with inhibitors need to be carried out for a detailed understanding of lectin activity of the inhibitors.

Conclusion

In conclusion, the purified trypsin inhibitors, AMTI-I and AMTI-II, from the seeds of *Abelmoschus moschatus*, were found to exhibit hemagglutinating property against human and animal erythrocytes. AMTI-I and AMTI-II may be useful in the agricultural front for developing transgenics after carrying out extensive in vitro studies against midgut proteases of insect pests.

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