

Purification of Trypsin Inhibitor from *Achyranthes aspera* seeds

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ABSTRACT

A trypsin inhibitor (AATI) from the seeds of prickly chaff flower (*Achyranthes aspera*) was purified to near homogeneity with about 15% recovery using ammonium sulphate fractionation, chromatography on DEAE-cellulose and Sephadex G-100. The inhibitor gave a single band on acrylamide gels when subjected to Substrate Reverse Zymography. A molecular mass of 20 kDa was determined for AATI by SDS-PAGE and gel filtration on Sephadex G-200.

Key words: *Achyranthes aspera* seeds, Purification, Trypsin inhibitor, Kunitz family, Substrate Reverse Zymography

INTRODUCTION

Protease inhibitors are widely distributed in nature and in plants they are abundant in storage organs such as seeds and tubers where they comprise up to 10% of the soluble proteins. Inhibitors active against the four mechanistic classes of proteases *ie.* serine, aspartic, thiol and metallo proteinases are known. Apart from regulating the endogenous protease activities, these inhibitors also serve as storage and defensive proteins. Much of the earlier work on them was focused on their anti-nutritional effects and the ability of protease inhibitors to strongly inhibit mid gut proteases of insects was explored in the agricultural front and the first transgenic tobacco plant was developed through genetic engineering approach (1,2).

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. The inhibitors have also been identified as

antimicrobial proteins affecting the growth of bacteria and fungi (3). The seeds of *Achyranthes aspera*, rich in protein and protease inhibitors, are used as food and seed extracts find applications in medicine. The influence of protease inhibitors on the nutritional quality of the seeds and their effect on gut proteases of insects are not yet known. The present work deals with the isolation and purification of trypsin inhibitor from the seeds of *Achyranthes aspera*.

MATERIAL AND METHODS

Materials: Seeds of *Achyranthes aspera*, removed from the ripened spikes, were used for the isolation and purification of trypsin inhibitor. Bovine pancreatic trypsin (1 x crystallized, DCC-treated, type xi), bovine pancreatic α -chymotrypsin (3 x crystallized, type ii), molecular markers, soybean trypsin inhibitor (type I-S), soybean Bowman-Birk inhibitor, N-acetyl-DL-phenylalanyl- β -naphthylester (APNE), N-acetyl-L-tyrosine ethyl ester

(ATEE), α -N-benzoyl-DL-arginine-p-nitroanilide HCl (BAPNA), blue dextran, DEAE-cellulose, N,N-dimethylformamide, N,N-dimethylsulfoxide, N,N'-methylene bis acrylamide, sodium dodecyl sulfate, diazoblu-B were purchased from Sigma Chemical company, St. Louis, Missouri, U.S.A. Sephadex G-100 and Sephadex G-200 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Acrylamide was purchased from J.T. Baker Chemical Company, Phillipsburg, N.J., U.S.A. N,N,N',N' – tetramethylene 1,2 diaminoethane (TEMED) was purchased from B.D.H. Chemical Ltd., Poole, England. Coomassie brilliant blue R250 was from Kochlight Laboratories Ltd. Colnbrook, Berks, England. All other chemicals used were of analytical grade.

Methods

Assay of trypsin and trypsin inhibitory activity.

Trypsin activity was assayed by the method of Kakade *et al.*(4) using BAPNA as the substrate. Trypsin (30 μ 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.

Protein estimation.

Protein was estimated by the method of Lowry *et al.*(5) using BSA as the standard. The protein content in the column effluents collected during chromatographic separation were determined by measuring the absorbance at 280 nm.

Purification of *Achyranthes aspera* trypsin inhibitor (AATI).

This was followed by the method of Annapurna *et al.* (6). Dehusked seeds (20g) of *Achyranthes aspera* were homogenized with 200 ml of 0.1M sodium phosphate buffer, pH 7.6 and the extract was then centrifuged at 10,000 g for 15 min at 5°C. The supernatant was dialyzed against the buffer for 24h in the cold, and rapidly heated to 60°C and maintained at this temperature for 10 min. The extract was quickly cooled in ice and then centrifuged at 10,000 g for 15 min at 4°C. To the supernatant, solid ammonium sulfate was added to 60% saturation with constant stirring at 4°C. The suspension was centrifuged at 10,000 g for 15 min and the precipitate was dissolved in 0.1 M sodium phosphate buffer, pH 7.6 and dialyzed against the same buffer for 48h.

The dialyzed sample was loaded on a DEAE-cellulose column (2.2 x 34 cm) and the elution was performed with 0.1-0.3M NaCl in the buffer. Fractions of 8 ml were collected at a flow rate of 60 ml/h. Fractions containing trypsin inhibitory activities were pooled, concentrated, dialyzed against distilled water for 24h and lyophilized.

Protein from the previous step was loaded on Sephadex G-100 column (1.9 x 63 cm) and eluted with the same buffer. Fractions (2ml) were collected at a flow rate of 12ml/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, dialysed against distilled water at 4 - 6°C and then lyophilized. The purified inhibitor was stored as a dry powder at 0-4°C.

Polyacrylamide Gel-Electrophoresis (PAGE).

PAGE was performed in 12% slab gels at pH 8.3 according to the method of Davis (7). Gels were stained for protein with Coomassie brilliant solution (0.1 mg/ml) and then with the substrate/dye mixture (APNE/ Diazoblue-B; Filho and Moreira (8). The TIA appeared as a clear zone against pink background.

Determination of molecular weight by SDS-PAGE and Sephadex G-200.

Molecular weight of the inhibitor was determined by the method of Laemmli (9). The marker proteins in 0.1 ml of phosphate buffer, pH 7.6, were heated at 100°C for 2 min with 0.1 ml of sample buffer (0.5M Tris-HCL buffer, pH 6.8, containing 40 g/l SDS and 100 g/l 2-mercaptoethanol (2-ME)). The inhibitor protein in

RESULTS

Isolation and purification : AATI was purified by extraction in 100Mm phosphate buffer pH 7.6, thermal denaturation, ammonium sulphate fractionation and chromatography on DEAE-cellulose and Sephadex G-100. AATI active against trypsin was isolated from *Achyranthes aspera* seeds in an apparently homogeneous form. Proteins eluted with 0.1 M NaCl on DEAE-cellulose column (**Figure 1A**) showed

Polyacrylamide Gel Electrophoresis: AATI was found to be homogeneous by the criteria of native PAGE and gel filtration. PAGE was carried out under non denaturing conditions using slab gels and a sharp protein band was obtained in 12% slab gels at pH 8.3 (**Figure 2A**). Under acidic conditions, the mobility of the inhibitor was very low and it migrated as a broad band on 10% slab gel (Result not shown). Substrate reverse zymography (**Figure 2B**) and Specific staining for the visualization of the trypsin inhibitory activity (**Figure 2C**) also showed a single band corresponding to coomassie blue stainable band. AATI was positive towards PAS (Periodic acid Schiff's) staining suggesting the presence of carbohydrate moieties in the inhibitor (**Figure 2D**). Gel

blue. For visualization of trypsin inhibitory activity (TIA) in acrylamide gels, the gels after electrophoresis, were incubated first with trypsin the sample buffer with and without 2-ME was kept at 100°C for 2 min prior to electrophoresis. Molecular weight of the inhibitor on Sephadex G-200 was determined by the method of Andrews (10) using a column of dimensions 1.65 x 94 cm (bed volume 185 ml). The column was equilibrated and developed with the same buffer. The void volume (V_0) was determined using Blue Dextran. Fractions of 1 ml were collected. Elution volume (V_e) for the molecular weight standards was plotted against log molecular weight to obtain the calibration curve.

antitryptic activity. The other two protein peaks eluted with 0.2 M and 0.3M NaCl did not show any trypsin inhibitory activity. **Figure 1B** shows antitryptic activity associated with second protein peak eluted from Sephadex G-100. Recoveries and relative purification at each step for a typical purification from 20 g seeds are shown in **Table 1**. By this procedure about 32 mg of the inhibitor is obtained. The final yield of the inhibitor was about 15%.

filtration on Sephadex G-200 was carried out with 10 mg of the inhibitor. AATI eluted out as a single protein with a corresponding activity peak (**Figure 3A**). The plot of the (V_e/V_0) versus log molecular weight for calibrating proteins is shown (**Figure 3B**). The molecular weight of the inhibitor calculated from the plot was 19.5 kDa. AATI when treated with SDS and 2-mercaptoethanol for 24 h at room temperature showed a single coomassie blue stainable, protein band on 5-20% gradient gels. The presence or absence of 2-mercaptoethanol in the sample buffer did not show any difference in the band pattern as well as in the relative band intensities on SDS-PAGE (**Figure 4A**). From the plot of distance migrated in cm versus log molecular weight for standards proteins, a single

protein band of AATI with molecular weight 20

kDa was obtained (**Figure 4B**)

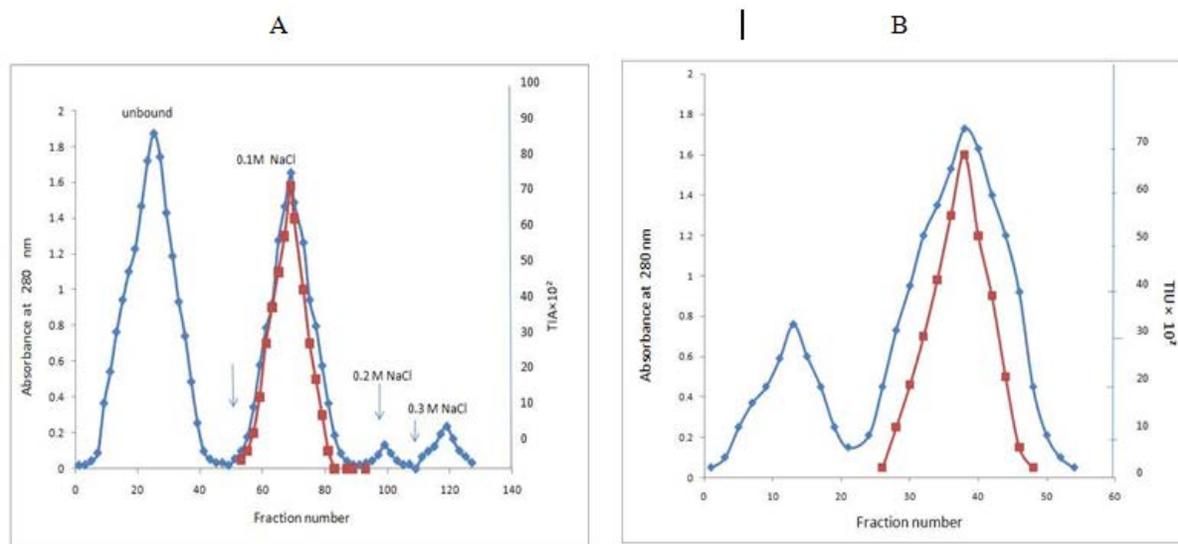


Figure 1 (A) Ion exchange chromatography on DEAE- cellulose. Ammonium sulfate fraction (60%) was loaded on to the column (2.2 x 34 x cm) in 0.1 M phosphate buffer, pH 7.6 and the adsorbed proteins were eluted with 0.1-0.3 M NaCl in the buffer. Fractions, each 8.0ml, were collected at a flow rate of 60 ml/h. (**B**) Gel filtration on Sephadex G- 100 of the DEAE-cellulose preparation.62mg of the lyophilized preparation was applied 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 (◆---◆). TIA (■---■)

Table 1.Summary of purification of proteinase inhibitor from seeds of *Achyranthes aspera*

Preparation	Volume (ml)	Total Protein (mg)	Total activity (units) TIU x10 ³	Specific activity (units/mg of protein) TIA x10 ²	Yield %	Fold* Purification
Crude extract	200	1040.0	300.55	2.89	100.0	1.00
Heat treated Extract	160	456.5	271.40	5.94	90.30	2.05
60% ammonium Sulfate	50	135.6	192.11	14.16	63.91	4.898
DEAE – cellulose	100	62.0	154.31	24.88	51.33	8.592
Sephadex G-100(ml)	60	32.5	140.31	43.17	46.67	14.90

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

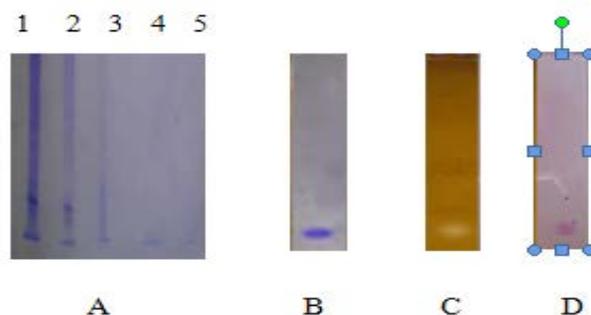


Figure 2. (A)Native-PAGE pattern of *Achrynanthes aspera* seeds . 1 . Crude fraction; 2. Heat treated extract; 3. Ammonium sulphate fraction; 4. DEAE cellulose fraction; 5.Sephadex G-100 fraction. (B) Substrate reverse zymography .(C) specific staining of the inhibitor.(D) Periodic acid staining

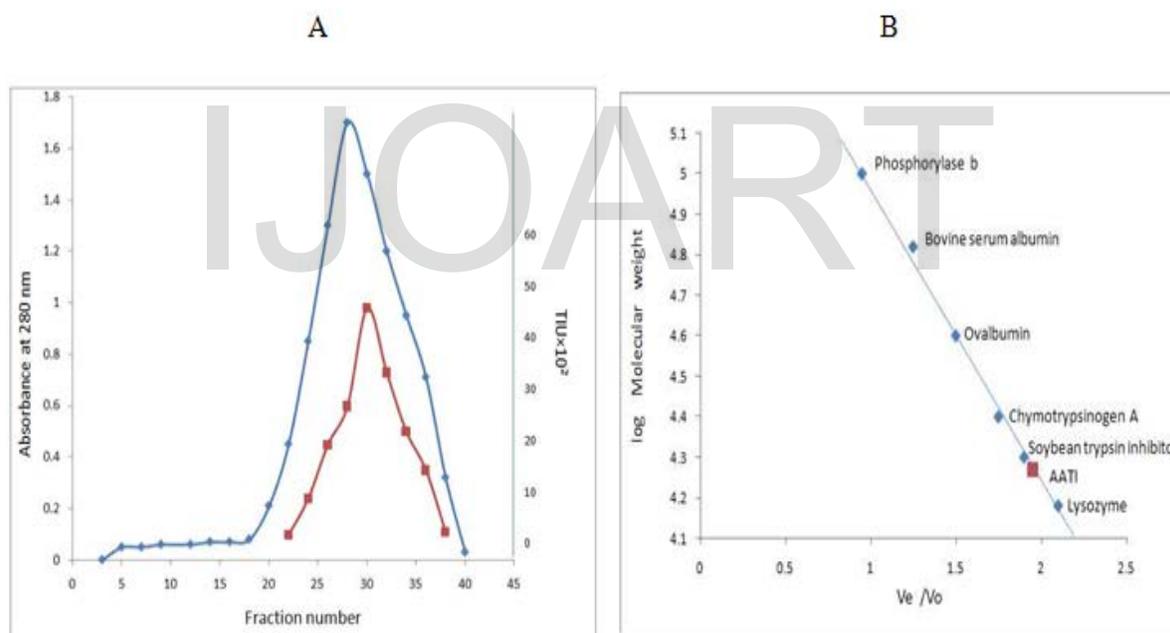


Figure 3. (A) Gel filtration on Sephadex G- 200 of AATI.10mg AATI in 1 ml 0.1 M phosphate buffer, pH 7.6 was loaded on Sephadex G-200 column (1.6 x 94 cm). Elution was done with the same buffer and fractions of 1.5 ml were collected at a flow rate of 10 ml/h. Protein was monitored by measuring the absorbance at 280 nm (◆---◆).(■----■) Trypsin inhibitory activity. (B) Molecular weight determination of AATI by gel filtration Sephadex G-200.Plot of elution volume against log molecular weight of standard proteins (◆) and AATI (■).

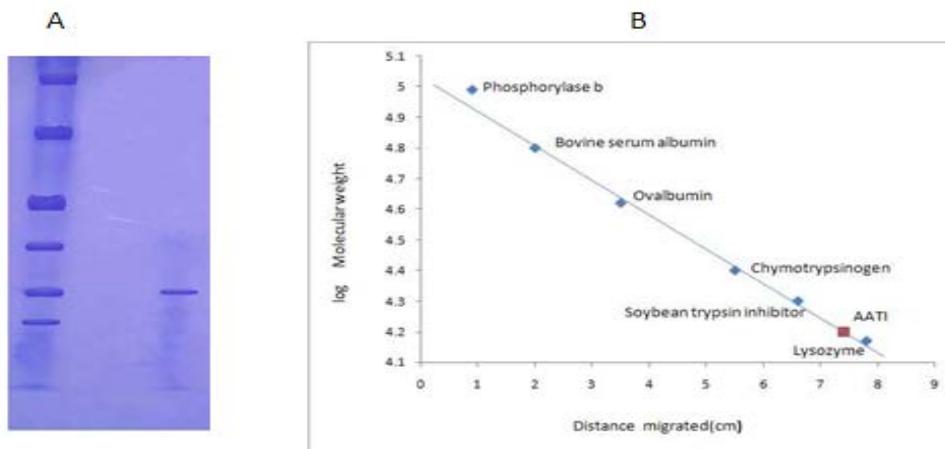


Figure 4. (A) SDE-PAGE on a 5 - 20% gradient slab gel. Standard proteins(A) Phosphorylase b, 97kDa , (B) Bovine serum albumin, 67kDa (C) Ovalbumin, 44kDa,(D) Chymotrypsinogen A, 25kDa,(E) Soybean trypsin inhibitor, 20.1 kDa (F) Lysozyme, 14kDa, (2) AATI kept at 100°C for 2 min with SDS and 2-mercaptoethanol. (B) Molecular weight determination of AATI by SDS-PAGE. Plot of distance migrated against log molecular weight of standard proteins (◆) and AATI (■).

DISCUSSION

Trypsin inhibitor has been obtained from *Achyranthes aspera* seeds in an apparently pure state. The observation that trypsin inhibitory activity in the crude extracts of the seeds is stable at 60°C for 10 min has led to the use of this treatment as the first step in the purification of the inhibitor. About 55% of proteins present in the crude extract were removed by this step. When the ammonium sulphate fraction was subjected to DEAE-cellulose column chromatography, protease inhibitory activity was found to be associated with the protein eluted from the column by 0.1M NaCl. The inhibitor also eluted out as a single protein with corresponding trypsin inhibitory activity when subjected to gel filtration on Sephadex G-100. The final yield of the inhibitor was about 15%. AATI has been found to be homogeneous by the criteria of native PAGE and gel filtration.

Several methods are available for separation of protease inhibitors by gel

electrophoresis - anionic inhibitors by the method of Davis (7), cationic inhibitors by the method of Reisfeld(11) and neutral inhibitors by the methods of Weber and Osborn (12) and Laemmli(9). While these methods are useful for establishing the purity and determining the molecular weight of proteins, they cannot be used to distinguish isoforms or compare the activity of particular inhibitor against different proteolytic enzymes. AATI, when subjected to native PAGE, moved at a faster rate on the gels probably due to its small size and having more negative charges on the protein. The inhibitor showed a single band on gelatin PAGE corresponding to coomassie blue stainable protein band signifying the absence of isoforms in the preparation as observed in many plant sources. (13-16)

The molecular weight of AATI was found to be 20.0 kDa as determined by SDS-PAGE. This is close to the value 19.5 kDa obtained for the inhibitor by gel filtration on Sephadex G-200. Even in the presence of 2-mercaptoethanol, the inhibitor gave a single

sharp band on SDS-PAGE supporting the monomeric nature of the protein. Some protease inhibitors have exhibited anomalous behavior on Sephadex gel columns due to the existence of oligomeric forms of inhibitors arising from monomer-dimer-trimer equilibrium (17-19) or due to the presence of carbohydrate moieties. Although AATI is a glycoprotein with 2.5% carbohydrate content, it is unlikely to show anomalous behavior on Sephadex beads since it moved out of them symmetrically with a narrow peak.

CONCLUSION

Our results demonstrate that AATI is a monomeric protein with a molecular mass of 20-kDa. Its ability to inhibit trypsin only indicate that it belongs to kunitz type of inhibitors.

ABBREVIATIONS USED.

BSA, bovine serum albumin; GuHCl, guanidine hydrochloride; TIU, trypsin inhibitory units; SDS sodium dodecyl sulphate; APNE, N-acetyl-DL-phenylalanyl- β -naphthylester; BAPNA, α -N-benzoyl-DL-arginine-p-nitroanilide HCl; AATI, *Achyranthes aspera* trypsin inhibitor; DEAE, diethylaminoethyl.

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