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ARTICLE

Purification and Characterization of a Kunitz-Type Trypsin Inhibitor from *Acacia victoriae* Bentham Seeds

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An *Acacia victoriae* trypsin inhibitor (AvTI) was purified from the seeds of prickly wattle (*A. victoriae* Bentham) by salt precipitation, ion exchange, and gel filtration chromatography and then characterized by electrophoresis and N-terminal amino acid sequencing. AvTI had a specific activity of 138.99 trypsin inhibitor units per milligram (TIU mg^{-1}), which was 21-fold higher than that of the salt precipitate. A molecular mass of 13 kDa was estimated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, which also indicated that AvTI may consist of two polypeptide chains linked by at least one disulfide bond. Although only a single peak was resolved by ion exchange and reverse phase high-performance liquid chromatography (RP-HPLC), native-PAGE and isoelectric focusing revealed the presence of three isoforms possessing acidic p*I* values of 5.13, 4.76, and 4.27, respectively. N-Terminal amino acid sequencing analysis of native and reduced AvTI showed two sequences with a high degree of homology with a typical Kunitz-type trypsin inhibitor. All isoforms had considerable trypsin inhibitory activity but showed relatively very low inhibition against α-chymotrypsin.

KEYWORDS: Wattle seed; purification; isoinhibitors; gel electrophoresis; RP-HPLC

INTRODUCTION

Naturally widespread Australian species of *Acacia* subgenus Phyllodineae are among the most common leguminous plants and most promising native plants in Australia (*1*, *2*). The seeds from several species of *Acacia*, which were used traditionally as a food source by Australian Aborigines, have been resurgent economically as foods or versatile food additives, such as flavoring agents and emulsifiers/stabilizers (*3*−*5*). The most common species, *Acacia victoriae* Bentham (prickly wattle), has been recognized to have significant functional properties in food systems due to its high amounts of water-soluble carbohydrates and proteins, including protease inhibitors (*6*, *7*).

Protease inhibitors (PIs) are small regulatory proteins generally present in high concentration; plant PI contents can vary from 1 to > 10% of their soluble proteins and are especially well spread in virtually all of the Leguminosae, particularly in soybeans, which contain Kunitz- and Bowman−Birk-type inhibitors (*8*−*10*). PIs exhibit strong inhibitory activities by intramolecular interactions such as disulfide bond, hydrogen bond, and hydrophobic interaction, forming stable complexes with cognate proteases in the digestive tract, thus reducing the ability of the body to utilize proteins in food (*11*−*14*). There are 10 families of plant PIs that are presently recognized on the basis of the protein primary structure, that is, the active amino acid in their reactive site, which are serine-, cysteine-, aspartic-, and metallo-PIs, with serine-PIs being the most widely studied (*15*−*18*). Plant serine-PIs are grouped into Kunitz, Bowman−Birk, potato I and II, and squash families of inhibitors. Kunitz and Bowman−Birk families of PIs are found abundantly in various leguminous plants (*19*); Kunitz inhibitors are usually 18−24 kDa heterogeneous proteins consisting of a number of isoinhibitors, with two disulfide linkages and a single trypsin reactive site (defined by an arginine residue) in one of the protein loops, whereas Bowman−Birk inhibitors are smaller in size (8−10 kDa), with seven disulfide linkages, a high cysteine content, and two independent reactive sites for trypsin and chymotrypsin (*13*, *17*).

The chemistry of wattle seed functional compounds, including protease inhibitors, is worthy of further investigation as they can have potential beneficial effects on human health (*18*). They may also have significant applications in the biological control of insects and fungal diseases in crops (*20*−*23*). Previously we have reported crude wattle seed extracts as possessing significantly high level of trypsin and α-chymotrypsin inhibitor activities, especially in the fraction precipitated with 25−50% (w/v) ammonium sulfate solution, the inhibitors being also susceptible to inactivation by moist heat treatment (*6*, *7*). Current studies on PIs, however, focus on the isolation and characterization of various potent trypsin and chymotrypsin inhibitors and their role in agriculture and human health (*14*, *17*, *20*). This paper reports

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the purification, characterization, and partial N-terminal sequence of the major trypsin inhibitor from the seeds of *A. victoriae* Bentham.

MATERIALS AND METHODS

Materials. Whole wattle seeds (*A. victoriae* Bentham) were supplied by Outback Bushfoods, Alice Springs, Australia. Chromatography media Fast Protein Q-Sepharose and Superdex 200 were obtained from GE Healthcare Life Sciences, Uppsala, Sweden. The Luna analytical C_{18} HPLC column was purchased from Phenomenex, Torrance, CA. All other analytical grade chemicals used were purchased from Sigma-Aldrich, Castle Hill, NSW, Australia, and Merck, Darmstadt, Germany.

Isolation and Purification of Wattle Seed PIs. Soluble proteins were extracted from milled whole wattle seeds in 10 volumes of deionized water with constant stirring at 4 °C for 2 h. After centrifugation (Beckman Coulter, Inc.) for 10 min at 10000*g* at 4 °C, the supernatant (crude extract) was precipitated with 25−50% (w/v) ammonium sulfate as this fraction, designated AS-2, has been shown to possess the highest protease inhibitor activity (*7*). This fraction was then dissolved in a minimal amount of deionized water, dialyzed against cold (4 °C) deionized water for 24 h, lyophilized (Christ-Alpha 1-2 LDplus freeze-dryer, Biotech International, Germany), and subjected to protease inhibitor assays or stored at −20 °C prior to further purification.

Fast-Performance Liquid Chromatography (FPLC). An aliquot (2 mg mL^{-1}) of AS-2 was applied to anion exchange column HiPrep 16/10 Q-Sepharose Fast Flow, pre-equilibrated with 50 mM Tris-HCl buffer at pH 7.0. Fractions (5 mL) were collected using a gradient 0.0−0.5 M NaCl at the flow rate of 2.0 mL min^{-1}. The peaks obtained, designated fractions I-1−I-6 were collected, dialyzed, lyophilized, and subjected to protease inhibitor assays individually.

The ion exchange fractions (I-4, I-5, and I-6) showing high protease inhibitor activity were pooled and applied (4 mg mL^{-1}) to a HiLoad 26/60 Superdex 200 preparatory grade column pre-equilibrated with 50 mM Tris-HCl buffer at pH 7.0, containing 50 mM NaCl. Fractions (5 mL) from the protein peak were collected at a flow rate of 2.5 mL min^{-1}. The peaks obtained, designated G-40m, G-50m, G-62m, G-75m, G-90m, G-105m, and G-118m based on elution time in minutes, were collected, dialyzed, lyophilized, and subjected to protease inhibitor assays individually.

The gel permeation fraction (G-90m) with maximum inhibitory activity was applied again to anion exchange column HiPrep 16/10 Q-Sepharose Fast Flow, pre-equilibrated with 50 mM bis-Tris-HCl buffer at pH 6.0. The protease inhibitors were eluted with a gradient of 0.0−0.5 M NaCl at the flow rate of 1.0 mL min^{-1}. All peaks, designated S-1−S-7, were collected, and the active fractions, S-5 and S-6, were dialyzed individually against deionized water at 4 °C for 24 h and lyophilized for further analyses. Peak S-5, possessing a higher trypsin inhibitor activity than S-6, was also rechromatographed by using the same anion exchange column to confirm purity.

Reverse Phase High-Performance Liquid Chromatography (RP-HPLC). RP-HPLC of protein fraction S-5 was also carried out on a Luna analytical C18 RP column (i.d. = 3.0 mm, length = 150 mm with a 10 μm particle size) using a Varian HPLC system equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA) in deionized water. A 0.5 mg aliquot of lyophilized protein was injected onto the column, and separation was achieved using an acetonitrile gradient (2−100%, 60 min) in 0.1% (v/v) TFA at a flow rate of 1 mL min^{-1}. The eluent was monitored at 214 nm.

Protease Inhibitor Activity Assays. Assays for bovine trypsin and α-chymotrypsin inhibitors were carried out, as described in a previous study (*7*), by estimating the remaining esterolytic activity of trypsin and α-chymotrypsin toward the substrate *p*-toluenesulfonyl-L-arginine methyl ester (TAME) and *N*-benzoyl-L-tyrosine ethyl ester (BTEE), respectively. Because the hydrolyzed product can be determined by using a spectrophotometric method, the inhibition can be observed from the decrease of the ultraviolet (UV) light absorbance if the enzymatic reaction is hindered. One trypsin unit (TU) or α-chymotrypsin unit (CU) is defined as 1 μmol of substrate hydrolyzed per minute of reaction. One inhibition unit is defined as a unit of enzyme inhibited. Trypsin and α-chymotrypsin inhibitor activity assays were carried out in every step of purification to identify the specific activity of inhibitors. Specific activity is defined as trypsin inhibition units (TIU) per milligram of protein.

Estimation of Proteins. Protein analysis for the purification test was carried out using a Biuret test kit (Sigma Diagnostics, Micro Protein Determination, procedure 690) and following the instruction in the catalog. A plot of absorbance values (550−750 nm) versus protein concentration generated the standard curve to determine protein concentration (mg dL^{-1} or μg mL^{-1}) of the 0.01 g mL^{-1} protein mixtures. Bovine serum albumin was used as the standard protein.

Polyacrylamide Gel Electrophoresis (PAGE). SDS-PAGE (PhastGel gradient 10−15) and native-PAGE (PhastGel homogeneous 20) of all stages of purified protease inhibitors were carried out using the Pharmacia Phast System (GE Healthcare Life Sciences). The protein samples were treated with Laemmli's buffer for both SDS-PAGE and native-PAGE. However, for native-PAGE, SDS and β-mercaptoethanol (ME) were excluded from Laemmli's buffer (*24*). Samples for SDS-PAGE were incubated for 10 min at 100 °C before resolving onto the gel along with a wide range of molecular mass markers (SigmaMarker) ranging between 6.0 and 205 kDa. Samples for native-PAGE were incubated at room temperature for 30 min before they were resolved on the gel. All gels were stained by PhastGel blue R and analyzed by using a Gel Doc 2000 Video Gel Documentation System (Bio-Rad Laboratories, Pty., Ltd., Sydney, NSW, Australia).

Trypsin and α-Chymotrypsin Inhibitor Activity Gels. Native-PAGE gels (PhastGel homogeneous 20) were stained for trypsin and α-chymotrypsin inhibitor activities as described in a previous study (*7*). The principle behind this method relied on the separation of the seed extract into protein bands using native-PAGE. The inhibitor bands, on exposure to trypsin or α-chymotrypsin, reacted with the respective enzyme and were depleted, showing up as clear bands on the gel after staining and comparison with gels unexposed to the enzymes. Immediately after separation, the gels were rinsed in deionized water to remove excess chemicals followed by incubation in assay buffer containing 2 mg mL^{-1} bovine trypsin or α-chymotrypsin for 20−30 min at room temperature. The gels were rinsed in deionized water before incubation in staining solution for 20−30 min without shaking. For each gel, the staining solution was prepared fresh by dissolving 17.5 mg of *N*-acetyl-DL-phenylalanine β-naphthyl ester in 5 mL of *N*,*N*-dimethylformamide and 25 mg of tetrazoitized (zinc chloride complex) *o*-dianisidine (Fast blue B salt) in 50 mL of assay buffer separately. These solutions were mixed immediately before they were poured on the gel. The stained gels were rinsed in deionized water and stored in 7.5% (w/v) acetic acid. The presence of trypsin or α-chymotrypsin inhibitors was visualized as clear bands in a dark violet or pink background.

Isoelectric Focusing (IEF). Isoelectric points of purified inhibitors were determined by performing isoelectric focusing using a Phast System apparatus from GE Healthcare Life Sciences. PhastGel IEF 3−9, which operates in the pH range of 3−9, was applied for analysis. An Amersham Biosciences broad-range p*I* calibration kit containing various proteins with known isoelectric points ranging from 3 to 10 was used. The protein bands were developed using the silver staining method according to the manufacturer's instructions (GE Healthcare Life Sciences). Gel was analyzed by using Gel Doc 2000 Video Gel Documentation System (Bio-Rad Laboratories, Pty., Ltd.).

N-Terminal Sequencing. Partially purified fraction S-5 was electrophoresed on PhastGel homogeneous 20 (native-PAGE) as well as on PhastGel gradient 10−15 (SDS-PAGE). The separated protein bands from both native- and SDS-PAGE gels were passively eluted to polyvinylidene fluoride (PVDF) membrane overnight before the eluent was applied to the sequencer. The first 10 amino acids from the N-terminal end of each protein were sequenced by the Edman degradation method on an Applied Biosystems Procise HT Protein Sequencer (Life Technologies Corp., Carlsbad, CA). Amino acid sequences of AvTI were analyzed with the sequences of other related proteins in the SWISS-PROT/Protein Knowledgebase (UniProtKB) database (http://www.uniprot.org).

Statistical Analysis. All extractions and analyses were carried out at least in triplicates and the means (with standard deviations) reported. Data collected were subjected to analysis of variance, and when applicable, means of treatments were subjected to Fisher's least significant difference test. Significant difference was reported at $p \leq 0.05$.

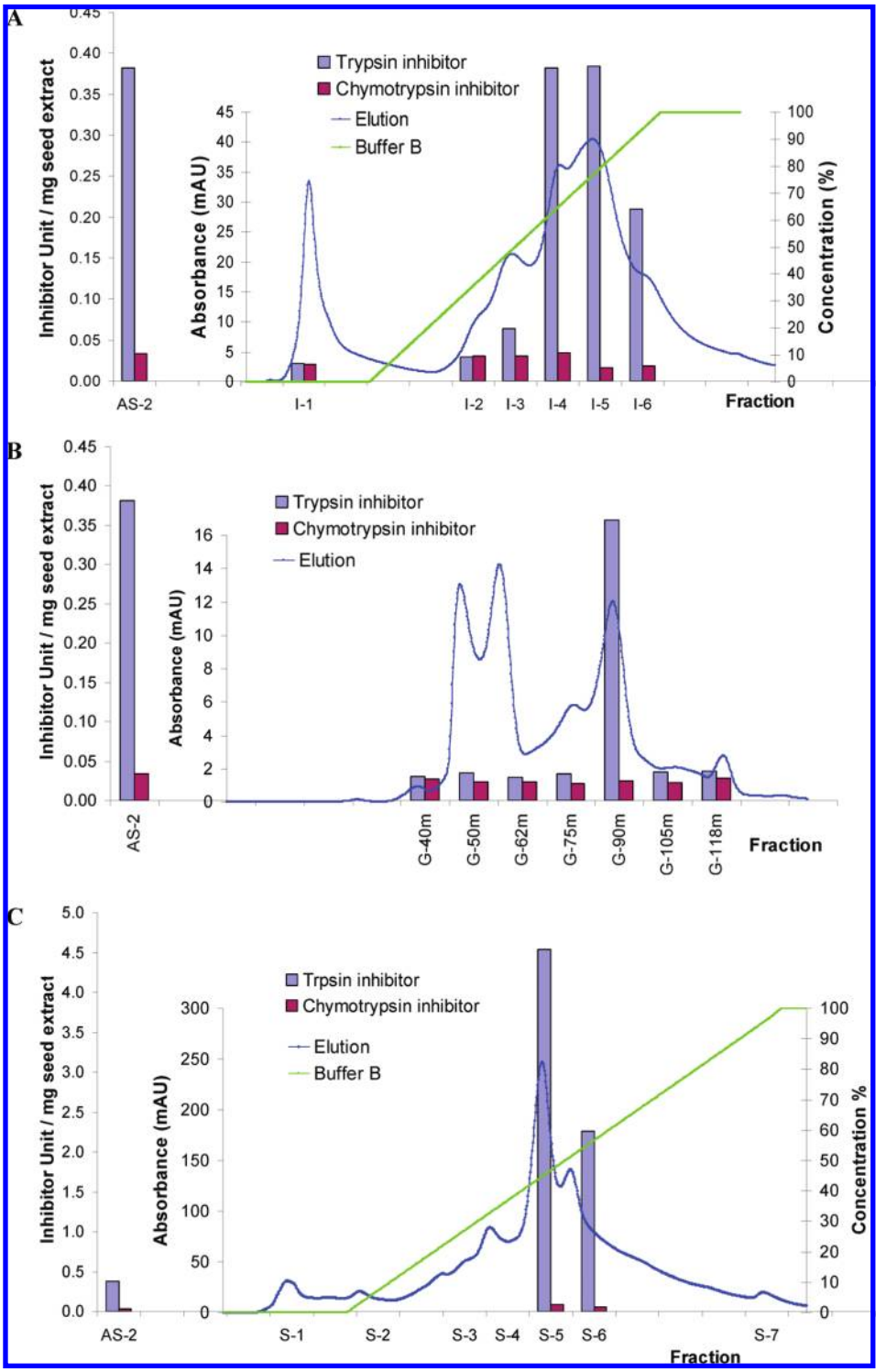

Figure 1. Elution profiles for the FPLC fractionation of wattle seed extracts (line chart) and protease inhibition analysis of the peaks (inset bar charts). Ammonium sulfate precipitate (AS-2) was fractionated by ion exchange (**A**) from where the most active peaks were separated by gel filtration (**B**) before the main active peak was rechromatographed by ion exchange (**C**). See Materials and Methods for more details of FPLC and inhibitor activity assays.

RESULTS

Initial Extraction and Purification. Figure 1 shows the results of FPLC analysis of extracts obtained from ammonium sulfate precipitate (AS-2) by ion exchange (**Figure 1A**) and after active ion exchange fractions (I-4, I-5, and I-6) were pooled and loaded onto gel filtration chromatography (**Figure 1B**). Additionally, the active gel filtration fraction (G-90m) was rechromatographed on ion exchange chromatography (**Figure 1C**) for further purification. The bar chart insets in **Figure 1** show protease inhibitor activity assays of all protein fractions, and the data in **Table 1** complement these results by tracking the purification steps from crude wattle extracts through purified fractions. The crude water extract showed a specific activity of 6.62 TIU mg^{-1} (**Table 1**), whereas the salt-precipitated fraction (AS-2) had a markedly increased specific activity of 11.72 TIU mg^{-1}, although the protein recovery was only about 55.44%. Three major protein peaks, I-4 (0.28−0.34 M NaCl), I-5 (0.35−0.46 M NaCl), and I-6 (0.46−0.50 M NaCl), were eluted when AS-2 was subjected to

Table 1. Purification of *Acacia victoriae* Trypsin Inhibitor (AvTI) from *Acacia victoriae* Bentham Seeds

step/characteristics	total protein,[a] mg	specific activity,[b] TIU mg^{-1}	% recovery[c]	purification factor[d]
crude extract	1016.63 ± 2.77	6.62 ± 0.19	100.00	1.00
ammonium sulfate precipitate (AS-2)	563.61 ± 0.82	11.72 ± 0.86	55.44	1.77
pooled ion exchange fractions I-4−I-6	292.40 ± 0.43	11.80 ± 0.83	28.76	1.78
gel filtration fraction (G-90m)	243.10 ± 0.57	13.77 ± 0.07	23.91	2.08
rechromatographed ion exchange fractions				
S-5 (AvTI)	4.87 ± 0.14	138.99 ± 1.39	0.48	21.00
S-6	5.45 ± 0.18	69.45 ± 0.69	0.54	10.50

[a] Based on extraction of 10 g of milled whole wattle seed. Total protein content was measured by using Biuret test kit (Sigma Diagnostics, Micro Protein Determination, procedure 690). [b] One trypsin unit (TU) was defined as 1 μmol of substrate hydrolyzed per minute of reaction, whereas one trypsin inhibitor unit (TIU) was defined as unit of enzyme inhibited. Specific activity was defined as inhibition units per milligram of protein. [c] The (protein) recovery was expressed as percentage of isolated inhibitor. [d] The purification factor was determined by using specific activity values.

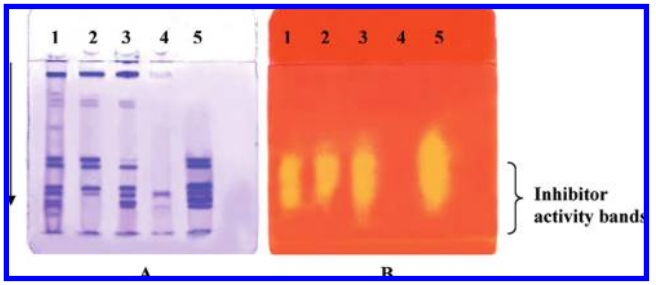

Figure 2. (**A**) Native-PAGE profile of wattle seed extracts and (**B**) equivalent native-PAGE gel showing trypsin inhibitor activity. Lanes: 1, 25−50% (w/v) ammonium sulfate precipitated fraction (AS-2); 2−4, anion exchanged fractions I-4 (0.2−0.34 M NaCl), I-5 (0.35−0.46 M NaCl), and I-6 (0.46−0.50 M NaCl), respectively; 5, gel filtration fraction G-90m. Arrow indicates direction of protein migration.

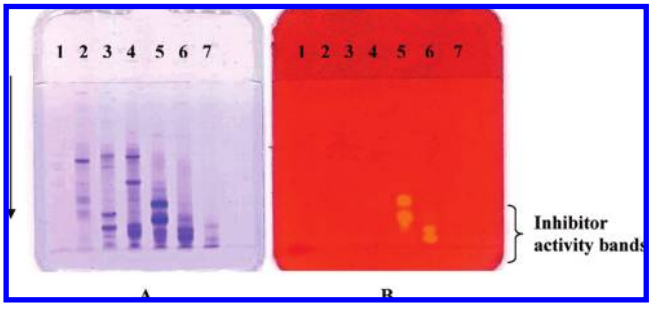

Figure 3. (**A**) Native-PAGE profile of rechromatographed anion exchanged fractions from gel filtration fraction G-90m and (**B**) equivalent native-PAGE gel showing trypsin inhibitor activity. Lanes: 1−7, S-1−S-7, respectively. Arrow indicates direction of protein migration.

anion exchange chromatography, exhibiting trypsin inhibitory activities of 0.382, 0.384, and 0.209 TIU mg^{-1} of seed extract, respectively (**Figure 1A**). Low or no significant trypsin or α-chymotrypsin inhibitor activity was observed in the other ion exchange factions. These active fractions, when pooled, dialyzed, and lyophilized for further purification, yielded a purification factor of 1.78% corresponding to a specific activity of 11.80 TIU mg^{-1}, similar to results obtained for AS-2, albeit with a reduced quantity of its total protein (**Table 1**).

The pooled ion exchange fractions, when applied to gel filtration, showed three major protein peaks (G-50m, G-62m, and G-90m) accompanied by four smaller ones (G-40m, G-75m, G-105m, and G-118m), all designated by their elution time in minutes (**Figure 1B**). All of the protein peaks exhibited measurable trypsin inhibitory activity, but G-90m (the fraction eluted at 90 min) was most active (and most abundant) at 0.357 TIU mg^{-1} of seed extract. However, proteins from G-90m (constituting 23.91% of the total proteins with specific activity of 13.77 TIU mg^{-1}) yielded a purification factor of only 2.08%. Therefore, G-90m was dialyzed, lyophilized, and reapplied on an anion exchange column (**Figure 1C**). Seven peaks were observed (S-1−S-7), but only two major peaks (S-5 and S-6) were active as mainly trypsin inhibitors, even though measurable α-chymotrypsin inhibitor activity was also recorded. Peak S-5 corresponded to a 0.48% recovery with a specific activity of 138.99 TIU mg^{-1} and a 21-fold purification, whereas S-6 constituted 0.54% recovery with a specific activity of 69.45 TIU mg^{-1} and a 10.5-fold purification (**Table 1**).

Molecular Properties of Wattle Extract Inhibitors. A native-PAGE gel obtained from AS-2, ion exchange fractions (I4, I-5, and I-6), and gel filtration fraction G-90m is shown in **Figure 2A**, whereas its activity gel counterpart is shown in **Figure 2B**. Electrophoresis was performed on the two gels at the same time to obtain protein bands, which aligned with each other. The trypsin inhibitor activity gel (**Figure 2B**) revealed the presence of several inhibitor bands with various intensities of clear bands against dark background in all of the fractions examined. Although all fractions exhibited trypsin inhibitor activity corresponding to the results of protease inhibitor activity assays (**Figure 1A**,**B** insets), fraction I-6 was negative on the inhibitor activity gel (**Figure 2B**, lane 4). Fraction G-90m (lane 5) showed up to six intensive trypsin inhibitory active bands, which were aligned with many protein bands of similar migration in AS-2.

Figure 3A shows the native-PAGE profile of ion exchange rechromatographed fractions (S-1−S-7), whereas **Figure 3B** shows their activity gel. All fractions show a significant number of protein bands, but only fractions S-5 (three active bands) and S-6 (two active bands) showed any activity (**Figure 3B**), corresponding to earlier results of trypsin activity assay (**Figure 1C** inset). As a result of its very high specific activity and the strong intensity of its protein band, fraction S-5 was rechromatographed by ion exchange FPLC to enhance its purity. This fraction, designated *Acacia victoriae* trypsin inhibitor (AvTI), was thus selected for further characterization studies. It was also analyzed by reverse phase HPLC (**Figure 4**) to confirm its purity. Both HPLC and FPLC (results not shown) analyses showed a single peak.

SDS-PAGE profiles of all fractions under reducing and nonreducing conditions are shown in panels **A** and **B**, respectively, of **Figure 5**. The total number of bands generally reduced in all fractions under reducing conditions. The protein bands also migrated farther and large molecular mass moieties were broken down owing to the disappearance of peaks greater than 60 kDa and the concomitant appearance of more peaks in the molecular mass range of < 10 kDa. It was also confirmed that the AS-2 lanes (2 and 7) were made up of the ion exchange fractions (lanes 3−5), whereas the sample obtained by gel chromatography (G-90m,

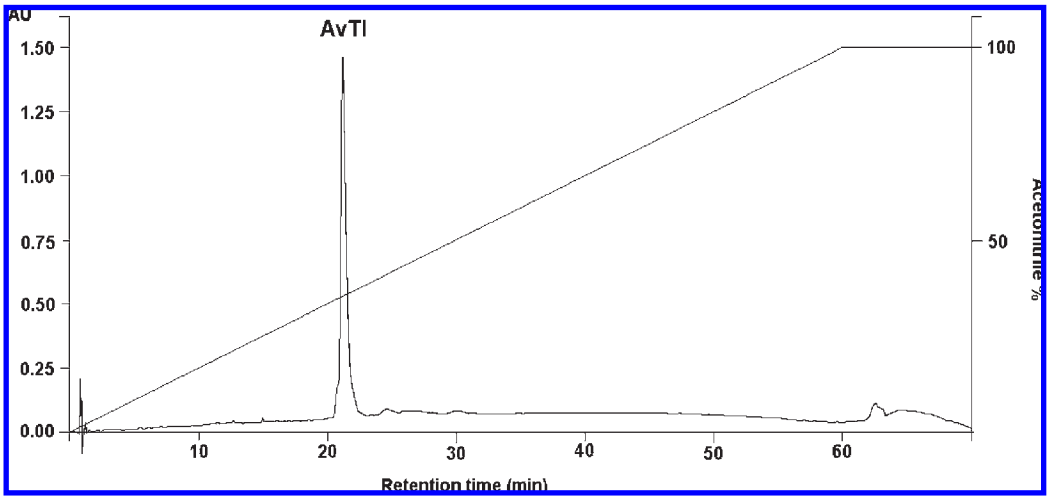

Figure 4. Elution profiles for RP-HPLC analysis of *A. victoriae* trypsin inhibitor (AvTI). Absorbance (vertical axis) was measured at 214 nm.

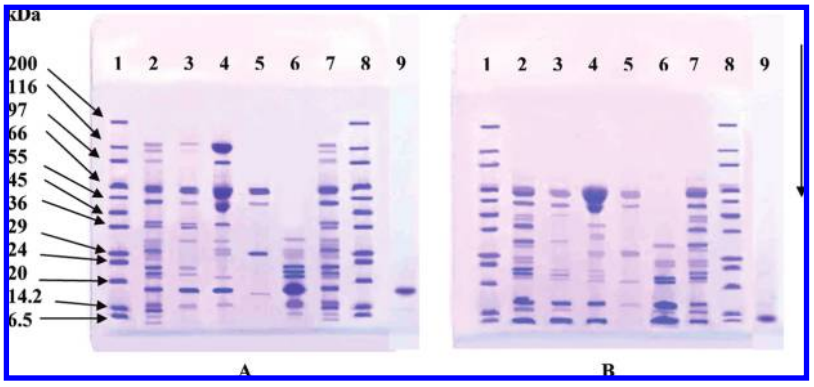

Figure 5. SDS-PAGE profiles of FPLC fractions of wattle seed trypsin inhibitors obtained under (**A**) nonreducing condition and (**B**) reducing condition with β-mercaptoethanol (ME). Lanes: 1 and 8, Sigma wide-range molecular mass markers from 6.5 to 200 kDa as labeled; 2 and 7, 25−50% (w/v) ammonium sulfate precipitated fraction (AS-2); 3, I-4; 4, I-5; 5, I-6; 6, G90m; 9, S-5. Arrow indicates direction of protein migration.

lane 6) was largely based on a concentration of some hitherto faint component of the salt-precipitated fraction. This lane showed four faint inhibitor bands with molecular masses of 33.23, 28.98, 22.86, and 22.11 kDa, and two very high intensity inhibitor bands with molecular masses of 16.42 and 13.30 kDa. The purified fraction S-5 exhibited only one protein band in SDS-PAGE under nonreducing conditions (**Figure 5A**, lane 9), corresponding to an apparent molecular mass of 18.30 kDa. After reduction with β-mercaptoethanol, also one protein band with a molecular mass of 13 kDa was obtained (**Figure 5B**, lane 9).

Although, as shown in **Figure 4**, a single peak was obtained with RP-HPLC analysis of AvTI, native-PAGE and activity gels (**Figure 6A**) showed three very intense protein bands with very strong trypsin inhibitory activity. The protein bands compared very well with the S-5 fraction (**Figure 3B**, lane 5) but, as would be expected from the extra purification step, were much clearer. Similarly, the activity bands did not merge into each other as in the S-5 fraction, being better separated. These three bands corresponded to the three p*I* values of 5.13, 4.76, and 4.27 as demonstrated by isoelectric focusing gel (**Figure 6B**), designated AvTI-1, AvTI-2, and AvTI-3, respectively.

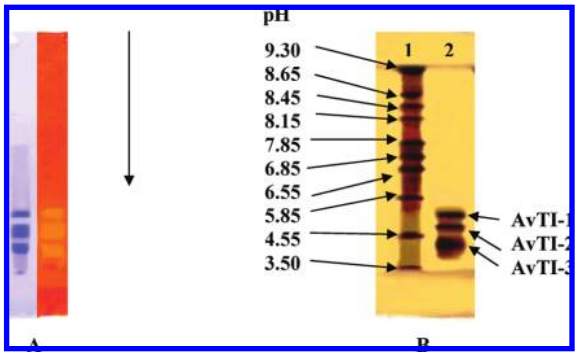

Figure 6. (**A**) Native-PAGE (and equivalent activity gel) profile and (**B**) isoelectric focusing gel of rechromatographed S-5 (AvTI). IEF was carried out on a PhastGel IEF 3−9 (pH range 3−9, Pharmacia) over a broad-range p*I* calibration kit containing proteins with various isoelectric points ranging from 3 to 10. Lanes: 1, p*I* markers; 2, AvTI isoinhibitors with p*I* at 5.13, 4.76, and 4.27, respectively. Arrow indicates direction of protein migration.

Determination of Amino Acid Sequence of AvTI. The N-terminal amino acid sequence analyses of native (**Figure 3A**, lane 5) and reduced (**Figure 5B**, lane 9) bands of AvTI are summarized in **Table 2**. Each band gave three different sequences irrespective of whether they were obtained from native or reduced polyacrylamide gels. Two major sequences were reported here as the third sequence appeared to be a fragment of the second sequence. By comparison to the sequences of other proteins in the database and literature (see references cited in **Table 2**), AvTI amino acid sequences were matched with Kunitz-type trypsin inhibitors from positions 2 to 10 and from positions 65 to 75. Leucine, aspartate, glycine, isoleucine, proline, and phenylalanine were the identical

Table 2. Comparison of the Amino Acid Sequences of *Acacia victoriae* Trypsin Inhibitor (AvTI) with Other Plant Trypsin Inhibitors[a]

Source *(reference)*[c]	Amino acid residue number[d]									
	1	2	3	4	5	6	7	8	9	10
AvTI sequence 1[b]	Y	**E**	**L**	**L**	**D**	**A**	**D**	**G**	E	**I**
Acacia confusa (36)	K	**E**	**L**	**L**	**D**	**A**	**D**	**G**	D	**I**
Albizzia julibrissin (34)	K	**E**	**L**	**L**	**D**	**A**	**D**	**G**	D	**I**
Acacia elata (24)	K	G	**L**	**L**	**D**	**A**	**D**	**G**	N	**I**
Prosopis juliflora (37)	G	**E**	**L**	**L**	**D**	V	**D**	**G**	**E**	**I**
Adenanthera pavonina (38)	R	**E**	**L**	**L**	**D**	V	**D**	**G**	N	F
Archidendron ellipticum (10)	K	**E**	**L**	**L**	**D**	S	**D**	**G**	I	**I**
Enterolobium contortisiliquum (26)	K	**E**	**L**	**L**	**D**	S	**D**	**G**	D	**I**
Glycine max (19)	D	F	V	**L**	**D**	N	E	**G**	N	P

	63	64	65	66	67	68	69	70	71	72	73	74	75
AvTI sequence 2[b]	-	-	**I**	**A**	**I**	**L**	**T**	**P**	**G**	**F**	**Y**	**L**	**N**
Acacia confusa (36)	P	K	**I**	**A**	**I**	**L**	**T**	**P**	**G**	**F**	**Y**	**L**	**N**
Prosopis juliflora (37)	P	R	**I**	**A**	**I**	I	R	**P**	**G**	**F**	S	**L**	**N**
Adenanthera pavonina (38)	P	R	**I**	R	Y	I	G	**P**	E	**F**	**Y**	**L**	T
Glycine max (19)	R	**I**	R	F	I	A	E	**G**	N	P	**L**	R	L

[a] Residues identical to those of AvTI are shown in the corresponding sequences in bold letters; —, residue not determined. [b] The amino-terminal sequences were determined from elution of native and SDS-PAGE bands. The dotted lines indicate possible reactive sites. [c] See literature cited list of this publication. [d] Residue symbols: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; I, Ile; K, Lys; L, Leu; N, Asn; P, Pro; R, Arg; S, Ser; T, Thr; V, Val; Y, Tyr.

amino acids in all of the sequences. Results indicated that these two sequences were principally from a single polypeptide chain with a high degree of homology to the α-chain of a Kunitz-type trypsin inhibitor.

DISCUSSION

As previously reported (*7*), all wattle seed extracts exhibited trypsin inhibitor activity with the exception of the alkali-soluble extract. Furthermore, salt-soluble extract showed bands of similar migration but lower inhibitor activity compared to the water-soluble extract. Most of the trypsin inhibitory activity from the crude seed extract was thus obtained by precipitating the water extract with 25−50% (w/v) $(NH_4)_2SO_4$ to yield AS-2. AS-2 was then purified by anion exchange chromatography mainly to eliminate carbohydrate residues and other contaminants that would not bind to the media. Active fractions eluted as different peaks from ion exchange chromatography are probably related to the degree of glycosylation, which is typical of storage proteins from leguminous plants and cereals (*25*). There is a very large overlap among the peaks I-4, I-5, and I-6 (**Figure 2A**). In fact, electrophoretic analysis of these fractions shows that their profiles shared several bands and that most of these intense bands have strong antitryptic activity (**Figure 5**). Comparatively, the protein peaks from gel filtration were quite well-defined and, apparently, only one peak, which eluted at 90 min (G-90m), accounted for the major active fraction in the wattle seed extract. This would suggest that the majority of active protease inhibitors have a similar molecular mass range, typical of Kunitz-type serine protease inhibitors (*19*).

The monomeric form of S-5 from anion exchange chromatography analyzed by SDS-PAGE without β-mercaptoethanol yielded a single protein band with an apparent molecular mass of 18.30 kDa. In the presence of reducing reagent, however, S-5 was reduced to 13 kDa, suggesting that the purified fraction probably comprised two polypeptide chains, linked by one or more disulfide bridges. The other chain of polypeptide (about 5.3 kDa) was presumed to have run off the gel or was too faint to be detected as it was not observed from SDS-PAGE analysis. Although both identified sequences in this study have been shown to be part of a single (major) polypeptide chain, the absence of another N-terminal sequence (for the minor chain) could be due to the fact that the covalently (disulfide) bonded second chain was not properly separated during preparation for sequencing the native protein.

The homology of the amino-terminal sequences of trypsin inhibitors of *A. victoriae* compared with those of others (**Table 2**) suggests that they originally evolved as single-chain precursors, before being proteolytically cleaved at a susceptible bond during synthesis (*34*). Some Kunitz-type inhibitors, such as those from soybean (*19*), *Pithecellobium dumosum* seeds (*22*),and *Inga laurina* seeds (*32*), are, however, single polypeptide chains, confirming the heterogeneous nature of this subclass of protease inhibitors. Conversely, two polypeptide chains (one major, one minor), linked by disulfide bonds, have been reported for other

Kunitz-type trypsin inhibitors found in the Mimosoidae subfamily of the Leguminosae, such as seeds of *Archidendron ellipticum* (*10*), *Enterolobium contortisiliquum* (*26*), *Acacia elata* (*27*), *Acacia confusa* (*28*), *Dimorphandra mollis* (*29*), *Pithecellobium dulce* (*30*), and *Callian selloi* Macbride (*31*). It has been suggested that the presence of cysteine and the attendant intramolecular disulfide bridges may play important functional roles during seed development. These include regulation of endogenous enzymes and the stabilization of Kunitz-type inhibitors as defensive agents against attack by pathogens and pests (*17*, *21*, *25*).

Although chromatography showed only one clean peak for AvTI, native-PAGE (**Figure 6A**) and isoelectric focusing (**Figure 6B**) exhibited three protein bands, indicating the presence of three isoforms with separate p*I* values of 5.13, 4.76, and 4.27, respectively. An acidic nature and the presence of isoinhibitors are common characteristics of Kunitz-type inhibitors (*17*). For example, four isoinhibitors (p*I* 4.1, 4.55, 5.27, 5.65) were found in *Archidendron ellipticum* seeds (*10*), two (p*I* 4.6, 5.35) in *Caesalpinia bonduc* seeds (*20*), three (p*I* 6.4, 6.5, 6.7) in *Dimorphandra mollis* seeds (*28*), and three (p*I* 4.85, 5.00, 5.15) in *Bauhinia variegate* var. *candida* seeds (*33*). However, the physiological reasons for evolving two or more isoforms remains to be determined. It has been suggested that this may be adopted by the host plant to ensure synergism among isoforms to ensure its survival (*17*).

The inhibitory activity of Kunitz-type protease inhibitors is varied. Some inhibitors of this family are potent inhibitors for trypsin and do not inhibit chymotrypsin, but some inhibitors inhibit chymotrypsin to various degrees (*33*). The AvTI, which strongly binds trypsin, also reacts with α-chymotrypsin, but the inhibition is not significant compared with trypsin. Inhibitors that stoichiometrically inhibited trypsin and α-chymotrypsin in a 1:1 molar ratio have been reported in a number of studies (*10*, *20*, *27*, *29*, *32*). However, the binding of α-chymotrypsin by the AvTI was weak; thus, the inhibitor−chymotrypsin complex probably dissociated during the incubation period and showed insignificant activity compared with trypsin assay. In addition, this indicated that both enzymes were probably inhibited at an identical reactive site. Similar degrees of inhibition of chymotrypsin have been reported for a number of other trypsin inhibitors that have a single reactive site, such as seeds of *Acacia elata* (*27*), *Callian selloi* Macbride (*31*), *Albizzia julibrissin* (*34*), *Amaranthus hypochondriacus* (*35*), and *Acacia confusa* (*36*). Further studies on the kinetic properties of AvTI will be carried out to elucidate possible mechanisms of trypsin inhibition and to determine the relative affinity of the enzyme and inhibitor to the potential substrates.

Comparison of the amino acid composition of the trypsin inhibitors of *Acacia victoriae* with those of *Acacia confusa* (*36*), *Albizzia julibrissin* (*34*), *Acacia elata* (*27*), *Prosopis juliflora* (*37*), *Adenanthera pavonina* (*38*), *Archidendron ellipticum* (*10*), and *Enterolobium contortisiliquum* (*26*) shows the similarity of the proteins (**Table 2**). It is clear that the Mimosoideae trypsin inhibitors are homologous proteins which show considerable homology with *Glycine max* (soybean) Kunitz-type trypsin inhibitor (*34*). Although the complete amino acid sequence and the location of the reactive region of AvTI are yet to be determined, research has shown that the active site of soybean Kunitz trypsin inhibitor (Arg 63−Ile64) can be cleaved and resynthesized under acidic conditions without loss of inhibitory activity and that specificity toward enzyme is determined by the nature of amino acid located at position 63 (*19*). On the basis of AvTI's band 2 sequence alignment with *Acacia confusa* (*36*), *Prosopis juliflora* (*37*), and *Adenanthera pavonina* (*38*), its reactive site is most likely located at positions 64 and 65 (**Table 2**).

Our results demonstrate that AvTI is most likely composed of a two-chain protein belonging to the Kunitz-type trypsin inhibitor family. AvTI was purified in a group of three isoforms, which were not isolated individually due to the extremely narrow range of molecular masses, owing probably to different degrees of glycosylation. Although the archetypical member of the Kunitz inhibitor family, soybean trypsin inhibitor, is not a glycoprotein, several glycosylated inhibitors have been reported, such as those obtained from *Echinodorus paniculatus* (*39*), *Peltophorum dubium* (*40*), *Carica papaya* (*41*), and *Swatzia pickellii* seeds (*42*). Research continues on the characterization of these protease inhibitors for their glycosylation properties and inhibition kinetics, as well as possible health-functional properties.

ABBREVIATIONS USED

AvTI, *Acacia victoriae* trypsin inhibitor; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; kDa, kilodaltons; RP-HPLC, reverse phase high-performance liquid chromatography; PI, protease inhibitor; p*I*, isoelectric point; TU, trypsin unit; TIU, trypsin inhibitor unit; CU, chymotrypsin unit; CIU, chymotrypsin inhibitor unit; FPLC, fast-performance liquid chromatography; TFA, trifluoroacetic acid; TAME, *p*-toluenesulfonyl-L-arginine methyl ester; BTEE, *N*-benzoyl-L-tyrosine ethyl ester; ME, β-mercaptoethanol; IEF, isoelectric focusing.

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