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Trypsin inhibitor from *Dimorphandra mollis* seeds: purification and properties

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Abstract

A trypsin inhibitor from *Dimorphandra mollis* seeds was isolated to apparent homogeneity by a combination of ammonium sulfate precipitation, gel filtration, ion-exchange and affinity chromatographic techniques. SDS-PAGE analysis gave an apparent molecular weight of 20 kDa, and isoelectric focusing analysis demonstrated the presence of three isoforms. The partial N-terminal amino acid sequence of the purified protein showed a high degree of homology with various members of the Kunitz family of inhibitors. This inhibitor, which inhibited trypsin activity with a K_i of 5.3×10^{-10} M, is formed by a single polypeptide chain with an arginine residue in the reactive site.

Keywords: *Dimorphandra mollis*; Leguminosae; Trypsin inhibitor; Kunitz inhibitor; N-terminal sequence

1. Introduction

Protease inhibitors are proteins or peptides capable of inhibiting catalytic activities of proteolytic enzymes. They are grouped primarily as either serine, cysteine, aspartic or metallo-proteinase inhibitors. Protease inhibitors have been known since the end of the last century in nematodes and human blood serum, and their ubiquitous distribution in microorganisms, animals and plants has been widely documented (Weder, 1985; Richardson, 1991; Birk, 1994). A large number of those characterized are serine proteinase inhibitors, which are found in animal tissues, microorganisms, as well as in plant tissues (Weder, 1985). Proteins that inhibit serine proteases have been classified in families according to their amino and acid sequences (Weder, 1985; Laskowski and Kato, 1980).

Two of the families of serine proteinases, the Kunitz- and Bowman–Birk-type inhibitors, have been the subject of much research, especially in the Graminaceae, Leguminosae and Solanaceae. These families differ from each other in mass, cysteine content, and number of reactive sites (Richardson, 1977). Kunitz-type inhibitors are proteins of $M_r \sim 20$ kDa, with low cysteine content and a single reactive site, whereas the Bowman–Birk-type inhibitors have $M_r \sim 8$–10 kDa, as well as high cysteine content and two reactive sites (Richardson, 1991).

Results of a previous study clearly indicated that there is a relationship between the families of inhibitors found in leguminous seeds and leguminous plant evolution. In tropical trees, for example, Kunitz inhibitors are found mainly in the seeds of the very primitive Caesalpinoideae and primitive Mimosoideae, whereas Bowman–Birk inhibitors are more frequent in the

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seeds of the highly evolved Papilionoideae (Norioka et al., 1988).

Interest in the study of plant protease inhibitors, especially from seeds, began when a trypsin protein from soybean seeds (Kunitz, 1945), which formed the Kunitz family of inhibitors (Kalume et al., 1995), was purified. Since then, several studies have been conducted involving various aspects of their properties. These proteins are now thought to exert defensive functions against pathogens (Geoffrey et al., 1990; Lorito et al., 1993; Tamir et al., 1996). Moreover, they are able to reduce the growth and development of herbivorous insects (Richardson, 1977; Janzen et al., 1986; Ceciliani et al., 1997).

Dimorphandra mollis (Leguminosae–Mimosoideae) is commonly found in the cerrado (savannah-like) ecosystem in central Brazil, where it is known as 'faveirodoce'. Initial studies carried out in our laboratory showed that *D. mollis* seed extract had antitryptic activity. In the present paper, we describe the purification, characterization and partial NH_2-terminal sequence of a *D. mollis* trypsin inhibitor (DMTI).

2. Results and discussion

DMTI was purified by saline extraction, ammonium sulfate precipitation (30–60% saturation), gel filtration on Sephadex G-75, anion exchange chromatography on DEAE–Sepharose, affinity chromatography on Sepharose–Trypsin and HPLC on a Bondapak C_{18} column. Only one peak from the gel filtration step showed antitryptic activity (peak 2), while the DEAE–Sepharose step yielded two active peaks. The second of these was chosen for additional purification in Sepharose–Trypsin affinity chromatography, due to its higher protein content, and a single peak with antitryptic activity (DMTI) was isolated. Affinity chromatography proved to be a very convenient way of isolating this group of inhibitors, even though the possibility of a limited digestion of these inhibitors by the immobilized trypsin during the course of purification cannot be excluded. However, the high yield of inhibitory activity even after affinity chromatography and the presence of only one protein band by SDS-PAGE (Fig. 2) suggest that DMTI did not undergo hydrolysis, and that its functional properties were preserved. The ultimate step for purification of DMTI by HPLC is shown in Fig. 1 Peaks 1 and 2 both showed antitrypsin activity, but the larger amount of protein represented by peak 2 led to its choice for further experiments. The results for a typical purification protocol for DMTI are shown in Table 1.

The pl values of 5.6, 5.8 and 5.9 found for DMTI (data not shown), suggest the presence of different isoforms. An acidic nature and the presence of isoforms are common characteristics of Kunitz inhibitors (Richardson, 1991; Kalume et al., 1995), although the physiological role for the existence of isoforms in plants is not clearly understood.

The inhibitory activity against trypsin and chymotrypsin was measured as the increase in the concentration of the inhibitor, using BAPNA and casein as substrates (Fig. 3), respectively. The K_i value was calculated using the equation for slow–tight binding inhibition (Morrison, 1982) and was found to be 2.8×10^{-10} M for trypsin. The K_i value thus establishes a high affinity between the enzyme and the inhibitor, in agreement with data reported for other plant trypsin inhibitors (Birk, 1994; Laskowski and Sealock, 1971). DMTI, however, showed no activity against chymotrypsin.

The apparent homogeneity of the purified DMTI was revealed by SDS-PAGE (Fig. 2); an apparent mol-

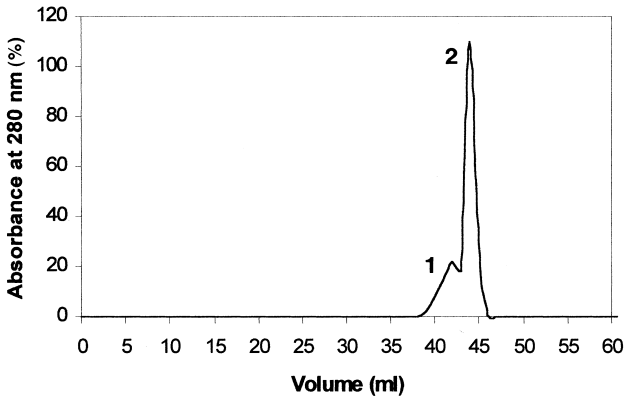

Fig. 1. μ-Bondapak C-18 reversed phase chromatography (HPLC). Elution: acetonitrile gradient (0–80%) in 0.1% TFA, flow rate: 1 ml/min.

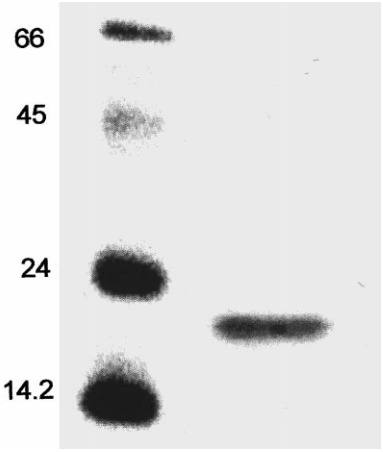

Fig. 2. Electrophoretic (12.5% SDS-PAGE) analysis of fractions from different stages of purification DMTI. MW, M_r standards from Sigma; lane 1, fraction HPLC (20 μg protein). The gel was stained with Coomassie brilliant blue.

Table 1
Results of a typical purification of DMTI from *D. mollis* seeds

	Total protein (mg)	Total activity (UI)	Specific activity (UI/mg)	Purification (fold)	Yield (%)
Saline extraction	220350	4200	0.019	1.0	100.0
Fraction 30–60	125000	3020	0.024	1.3	71.9
Sephadex G-75	5217	1980	0.38	20	47.1
DEAE-Sepharose	2520	980	0.64	33.7	23.3
Sepharose-Trypsin	450	450	1.0	52.6	10.7
C_{18} (HPLC)	145	320	2.21	116.2	7.6

ecular mass of ca. 20 kDa was determined by Sephacryl S-100 column chromatography (data not shown) and SDS-PAGE. The approximate molecular masses are in general agreement with those found for other Kunitz inhibitors (Richardson, 1991; Macedo and Xavier-Filho, 1992).

The reactive site of a protease inhibitor is defined as that part of the molecule that enters into direct contact with the active centre of the enzyme to form an enzyme-inhibitor complex (Tscheche, 1974). When DMTI was incubated with trypsin at a 1:1 molar ratio, a complex with a molecular mass of approximately 40 kDa was found by gel filtration, indicating the presence of a single reactive site of trypsin (Tanaka et al., 1997). For trypsin inhibitors, reactive sites are almost always formed with arginine or lysine residues linked to another amino acid (Joubert et al., 1987). By employing lysine and arginine modifying reagents such as trinitrobenzene-sulphonic acid (TNBS) (Habeeb, 1966) and cyclohexanedione (CHD) (Liu et al., 1968), respectively, the inhibitor was inactivated. Table 2 shows that modification of the arginine residue of inhibitor DMTI with CHD caused a great loss of inhibitory activity, which suggests the involvement of arginine in the reactive site.

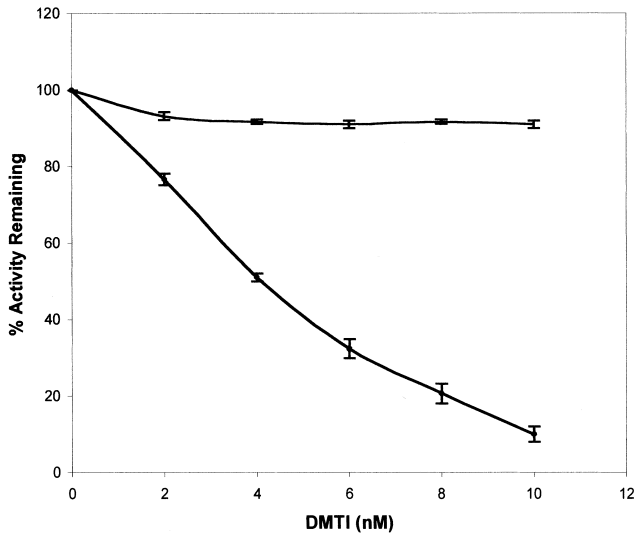

Fig. 3. Titration curves of trypsin and chymotrypsin inhibition by DMTI. Increasing quantities of inhibitor were added to a constant quantity of enzyme (10 μg). Remaining activity of each enzyme was determined using BAPNA and casein as substrates. Each point is the average of three assays. (A) Inhibition of chymotrypsin by DMTI using casein as a substrate. (B) Inhibition of trypsin by DMTI using BAPNA as a substrate.

The partial NH_2-terminal sequence of 26 amino acids of DMTI indicates a high degree of homology with other Kunitz family inhibitors from several sources (Fig. 4). The fact that the inhibitor found in *D. Mollis* belongs to the Kunitz family provides support for the theory that there is a relationship between the families of inhibitors found in leguminous seeds and the evolution of those leguminous plants, since only Kunitz family inhibitors are found in the relatively primitive plants of Mimosoideae. This conclusion was reached by Norioka et al. (1988), who investigated the presence of both Kunitz and Bowman–Birk inhibitors in seeds of 34 legumes by gel filtration. The results found were compared with the morphological classification of Leguminosae; the seeds of the more primitive Leguminosae (Caesalpinoideae and Mimosoideae) contained mainly Kunitz family inhibitors, whereas those of a more advanced family (Papilinoideae) revealed the presence of only Bowman–Birk inhibitors.

3. Experimental

3.1. Materials

Seeds of *D. mollis* were obtained locally and were provided by the Chamflora of Três Lagoas, Mato Grosso do Sul, Brazil. Bovine serum albumin (BSA), bovine pancreatic trypsin, bovine pancreatic α-chymotrypsin, soybean (SBTI) and bovine pancreatic (BPTI) trypsin inhibitors, casein and *N*-α-benzoyl-DL-arginyl-*p*-nitroanilide (BAPNA) were purchased from Sigma (St. Louis, MO, USA), as were SDS-PAGE molecular weight markers, acrylamide, bis-acrylamide and other electrophoresis reagents. TNBS, 1,2-cyclohexanedione (CHD), acetonitrile and trifluroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). Ampholines and chromatography supports were from

Table 2
Residual inhibitory activity (%) of trypsin inhibitors treated with TNBS and CHD[a]

	DMTI	Bovine pancreatic trypsin inhibitor	Soybean trypsin inhibitor
−TNBS	100.0	100.0	ND[b]
+TNBS	98.0	5.5	ND[b]
−CHD	100.0	ND[b]	100.0
+CHD	50.6	ND[b]	59.8

[a] Values are percentages of the controls; 1 μg of each inhibitor was utilized.
[b] ND = not detected.

Pharmacia (Uppsala, Sweden), All other chemicals and reagents used were of analytical grade.

3.2. Purification procedure of DMTI

D. mollis seeds free of integument and defatted with hexane were ground in a coffee mill. A crude inhibitor preparation was obtained by extraction of this meal with 0.1 M phosphate buffer, ph 7.6 (1:10, w/v) for 1 h at 25°C with subsequent centrifugation at 7500 $\times g$ for 30 min. The supernatant was then fractionated by ammonium sulfate precipitation into three fractions, corresponding to 30%, 60% and 80% ammonium sulfate saturation. All three fractions contained trypsin inhibitor activity.

Trypsin inhibitors were purified by chromatography of pooled ammonium sulfate precipitated fractions 30–60% on Sephadex G-75 (columns of 100×2.7 cm, eluted with 0.1 M phosphate buffer, pH 7.6). The column fraction with DMTI activity was then subjected to ion exchange chromatography on DEAE–Sepharose (columns of 20×2.0 cm, equilibrated with 0.01 M Tris–HCI buffer, pH 8.0) and eluted with the same buffer containing NaCl in a gradient of 0–1.0 M NaCl. The fraction eluted with 0.5 M NaCl was submitted to Sepharose–Trypsin affinity chromatography (columns of 10×2.0 cm, equilibrated with 0.1 M phosphate, pH 7.6, 0.1 M NaCl) (Macedo and Xavier-Filho, 1992) and the DMTI active fraction was finally purified by reversed phase HPLC (μBondapak C_{18} column) with a flow rate of 1.0 ml/min with 100% solvent A (0.1% TFA in water) for 10 min, 100% solvent B (0.08% TFA in 80% acetonitrile) over 55 min. Proteins were detected by monitoring the absorbance at 280 nm.

Apparent molecular weights were obtained by Sephacryl S-100 (60×2.5 cm in 0.1 M Tris buffer, pH 8.0) gel filtration calibrated with proteins of known molecular weight.

3.3. Protein determination

Protein contents were determined by Coomassie blue staining (dye-binding method) (Bradford, 1970) or from the absorbance at 280 nm. BSA (1 mg/ml) was used as standard.

3.4. Assay of trypsin and chymotrypsin inhibitory activity

Trypsin inhibitory activity was determined by measuring the remaining hydrolytic activity of trypsin towards the substrate BAPNA at pH 8.0 after pre-incubation with inhibitor (Erlanger et al., 1961). The caseinolytic activity of chymotrypsin and its inhibition

	Initial Position	Sequence/Homology	Identities (%)	Positives (%)
DMTI	2	Q V F **D** T E **G** N **G** I **R** **N** **G** **G** T **Y** y I **L** **P** D R W G **K** **G**		
KTI	28	V F **D** T E **G** N P I **R** **N** **G** T **Y** **Y** V **L** **P** V I R G **K** **G**	76	80
DE5	2	E L L **D** V D **G** N F L **R** **N** **G** **G** S **Y** **Y** I **V** **P** A F R G **K** **G**	50	73
ITRY	2	E L L **D** A D **G** D I L **R** **N** **G** **G** A **Y** **Y** I **L** **P** A L R G **K** **G**	50	69
ID5A	2	E L L **D** V D **G** E I L **R** **N** **G** **G** S **Y** **Y** I **L** **P** A F R G **K** **G**	50	69
IT1A	3	L L **D** S E **G** E L V **R** **N** **G** **G** T **Y** **Y** L **L** **P** D R W A L **G**	60	76

Fig. 4. Partial sequence of the trypsin inhibitor (DMTI) aligned with different regions of known Kunitz trypsin inhibitors. KTI/SOYBEAN: Kunitz trypsin inhibitor precursor-soybean — pir || S49196; DE5, *Adenanthera pavonina* trypsin inhibitor — prf || 1208243A; ITRY, *Acacia confusa* trypsin inhibitor — sp | P24924 | ; ID5A, *Prosopsis Juliflora trypsin* inhibitor — sp | P32733 | ; IT1A, Winged bean trypsin inhibitor — sp | P10821 |. Letters in bold represent completely conserved residues and letters in italics represent incompletely conserved residues.

were assayed at pH 7.6, with one inhibitory unit calculated as described by Xavier-Filho (1974).

3.5. Chemical modification of arginine

Free amino groups in the inhibitors were modified by TNBS (Habeeb, 1966). whereas 1,2-cyclohexanedione (CHD) was employed to modify arginine residues (Liu et al., 1968). In both cases, residual inhibitory activity was measured as described by Kunitz (1945).

3.6. Polyacrylamide gel electrophoresis

Relative molecular weights estimates were obtained by polyacrylamide gel electrophoresis (Laemmli, 1970). The protein utilized as molecular weight standards for SDS-PAGE were bovine serum albumin (66 kDa), ovalbumin (43 kDa) trypsinogen (24 kDa) and α-lactoglobulin (14.2 kDa).

3.7. Complex formation of DMTI with trypsin

DMTI (0.25 mg) was individually incubated with trypsin (0.5 mg) and chymotrypsin (0.5 mg), in 50 mM Tris–HCI buffer, pH 7.5 for 30 min at 22°C. Gel filtration of the protein mixture was performed on Sephadex G-150 using 50 mM sodium phosphate buffer, 100 mM NaCl, pH 7.5 as eluent.

3.8. K_i determination assay

The trypsin was pre-incubated with different concentrations of the purified inhibitors and its remaining activity was measured with the enzymatic hydrolyzate of BAPNA (Oliva et al., 1987), Inhibitor concentration was determined by absorbance at 280 nm. The value of the dissociation constant (K_i) of the complex enzyme-inhibitor was determined following Morrison's procedure (Knight, 1986).

3.9. Isoelectric focusing

Isoelectric focusing was performed on a flat bed apparatus (LKB). Ampholine solutions (40% v/v) in the pH range 3.5–9.5 were utilized, with Coomassie Brilliant Blue-R staining according to Westermeier (1993).

3.10. Protein sequencing

The N-terminal sequence was analysed on a Shimadzu PPSQ-10 automated protein sequencer performing Edman degradation. Phenylthiohydantoin amino acids (PTH-AA) were detected at 269 nm after separation on a reverse phase C_{18} Wakopack Wakosil HPLC column (4.6 mm × 25 cm) from Shimadzu, under isocratic conditions, using 40% acetonitrile, 20 mM acetic acid, and 0.014% sodium dodecyl sulfate as the mobile phase at a flow rate of 1.0 ml/min at 40°C. The sequence was submitted to automatic alignment, which was performed using the NCBI-Blast search system.

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