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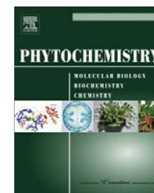

# Physico-chemical and antifungal properties of protease inhibitors from *Acacia plumosa*

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ABSTRACT

This study was aimed at investigating the purification, biological activity, and some structural properties of three serine protease inhibitors isoforms, denoted ApTIA, ApTIB, and ApTIC from *Acacia plumosa* Lowe seeds. They were purified from the saline extract of the seeds, using Superdex-75 gel filtration and Mono-S ion exchange chromatography. They were further investigated by mass spectrometry, spectroscopic measurements, surface plasmon resonance, and inhibition assays with proteases and phytopathogenic fungi. The molecular mass of each isoform was estimated at ca. 20 kDa. Each contained two polypeptide chains linked by a disulfide bridge, with different isoelectric points that are acidic in nature. The N-terminal sequences of both chains indicated that they were Kunitz-type inhibitors. Circular dichroism (CD) analyses suggested the predominance of both disordered and beta-strands on ApTI isoforms secondary structure, as expected for β-II proteins. In addition, it was observed that the proteins were very stable, even at either extreme pH values or at high temperature, with denaturation midpoints close to 75 °C. The isoinhibitors could delay, up to 10 times, the blood coagulation time in vitro and inhibited action of trypsin (Ki 1.8 nM), α-chymotrypsin (Ki 10.3 nM) and kallikrein (Ki 0.58 μM). The binding of ApTIA, ApTIB, and ApTIC to trypsin and α-chymotrypsin, was investigated by surface plasmon resonance (SPR), this giving dissociation constants of 0.39, 0.56 and 0.56 nM with trypsin and 7.5, 6.9 and 3.5 nM with α-chymotrypsin, respectively. The growth profiles of *Aspergillus niger*, *Thielaviopsis paradoxa* and *Colletotrichum* sp. P10 were also inhibited by each isoforms. These three potent inhibitors from *A. plumosa* may therefore be of great interest as specific inhibitors to regulate proteolytic processes.

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## 1. Introduction

Protease inhibitors (PIs) play essential roles in biological systems regulating proteolytic processes and participate in defense mechanisms against attack by a large number of insects (Carlini and Grossi-de-Sá, 2002), fungi (Kim et al., 2005) and other pathogenic microorganisms (Breiteneder and Radauer, 2004).

Several studies on PIs have been published with the aim of investigating: enzyme mechanisms (Shin et al., 2001; Tawa et al., 2006); control disease and pathological processes (Powers et al., 2002); and protection of crops from pests as bioinsecticides by using the genes encoding protease inhibitors' (Haq et al., 2004). More recent studies have employed protease inhibitors as new drugs in highly active antiretroviral combination therapy (HAART), increasing life expectancy in HIV-positive patients (Asztalos et al., 2006; Yeni, 2006).

Natural PIs are widely distributed in all living organisms, though they have been largely described in plants (Brady, 2003), especially in those from the Leguminosae, where there is a high content in seeds (Teles et al., 2004; Vargas et al., 2004; Bhattacharyya et al., 2006). The Leguminosae family comprises ca. 18,000 species distributed in more than 650 genera (Doyle et al., 1996), and they are classified into three different subfamilies: Caesalpinioideae, Papilionoideae and Mimosoideae. Although different biological activities have been demonstrated in the saline extract of members from the genus *Acacia* (Hung et al., 1993; Popoca et al., 1998; Arias et al., 2004), an important genus from Mimosoideae subfamily, the isolation and characterization of protease inhibitors were only described in a small number of its species, e.g. *Acacia elata* (Kortt and Jermyn, 1981), *Acacia sieberana* (Joubert, 1983) and *Acacia confusa* (Lin et al., 1991). Besides, few structural and physico-chemical studies have been performed with the protease inhibitors described in this genus.

Neither biochemical nor physico-chemical studies were previously performed with *Acacia plumosa*, which is a perennial thorny plant common in pastures of Centre-South region of Brazil and usefully employed, according to popular knowledge, in the treatment

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of arthritis, infections and muscle pain. In this paper, we have purified and investigated the biochemical and structural properties of three hitherto unknown serine protease isoinhibitors, that were isolated from the saline extract of *A. plumosa* seeds. These are denoted as ApTIA, ApTIB and ApTIC (*A. plumosa* Trypsin Inhibitor, isoform A, B and C). We have also explored the antifungal activity of these isoinhibitors, binding and inhibition of some serine proteases and action on blood coagulation cascade.

## 2. Results and discussion

### 2.1. Purification

From the crude extract (CE) of *A. plumosa* seeds, three protease inhibitors isoforms were purified by SEC (size exclusion chromatography) on Superdex-75, and by ionic exchange Mono-S CC, with each denoted as being an ApTI (*A. plumosa* Trypsin Inhibitor). Three peaks were eluted from SEC, but only the third peak (with $MM_r$ estimated at ca. 20 kDa) had anticoagulant and trypsin inhibitory activities, indicating the presence of protease inhibitors in this fraction. Afterwards, this fraction was loaded onto a Mono-S column the elution of which resulted in the separation of four major peaks (Fig. 1A). The activities mentioned above were detected in the last three peaks eluted with 0.18, 0.22 and 0.32 M NaCl solutions, and sequentially named as ApTIA, ApTIB and ApTIC. The total protein content of *A. plumosa* seeds was estimated at ca. 6.5% as the ApTI isoinhibitors represent 7.0% of this fraction. These data are in agreement with other protease inhibitors purified from Leguminosae seeds, like EcTI (12%) from *Enterolobium contortisiliquum*, (Batista et al., 1996); AeTI (8.0%) from *Archidendron ellipticum*, (Bhattacharyya et al., 2006); BvcTI (4.1%) and BvlTI (8.5%) from *Bauhinia variegata* candida and lilac (Ciero et al., 1996), respectively. The homogeneity of these

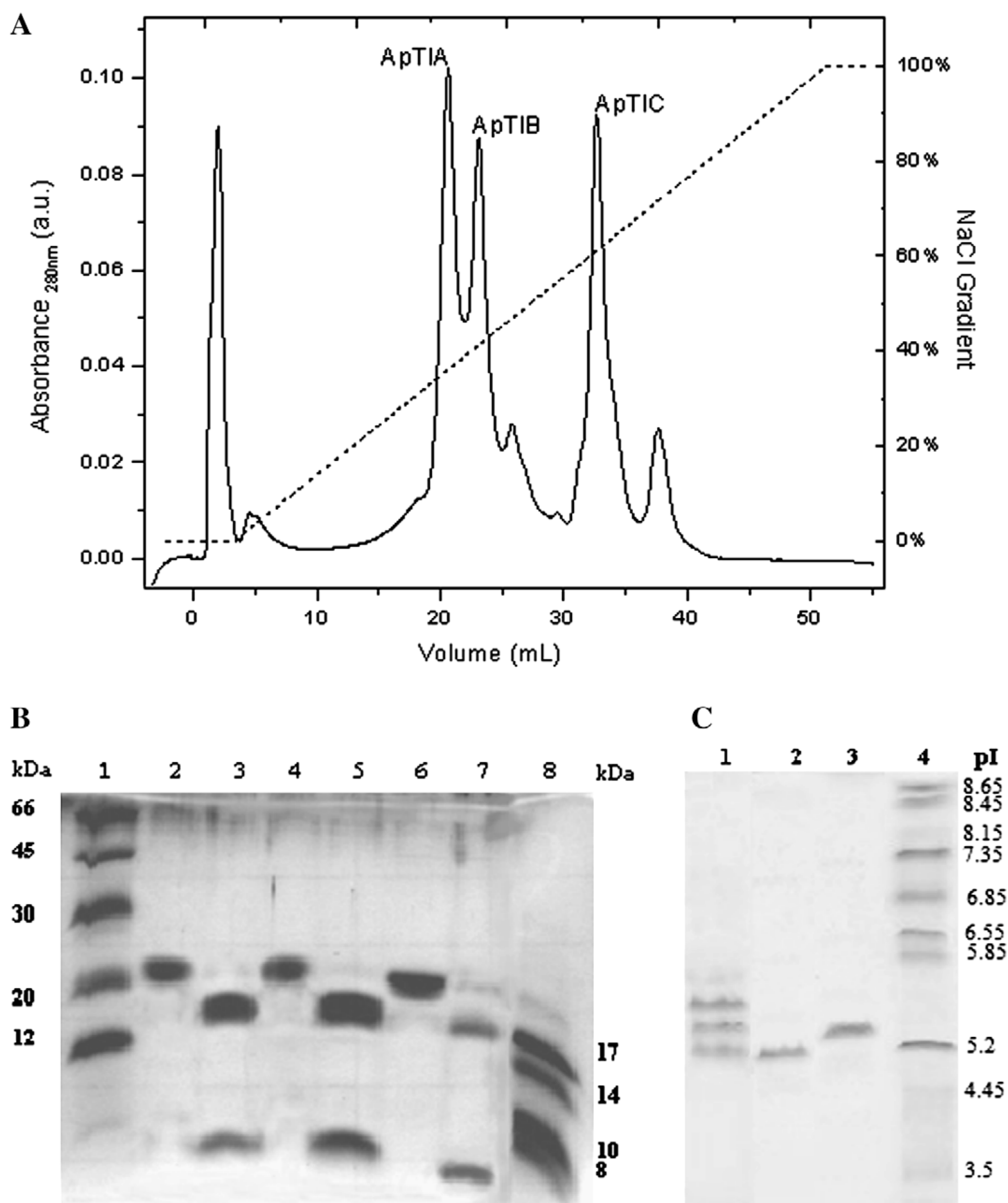


**Fig. 1.** (A) Ion exchange chromatography of ApTI isoforms on Mono-S HR 5/5 equilibrated with NaOAc 50 mM (pH 5.0) and eluted in a linear gradient with the same buffer containing 0.5 M NaCl in 50 min, flow rate of 1 mL/min. (B) Tricine–SDS–PAGE of ApTI isoforms. Lanes: 1-$MM_r$, 2-ApTIA, 3-ApTIA under reducing conditions, 4-ApTIB, 5-ApTIB under reducing conditions, 6-ApTIC, 7-ApTIC under reducing conditions, 8-$MM_r$; (C) Isoelectric focalization gel of ApTI isoforms obtained in a PhastSystem using pH gradient from 3 to 9.0. Lanes: 1-Fraction from SEC with anticoagulating activity (ApTI isoforms), 2-ApTIA; 3-ApTIB; 4-pI standards.

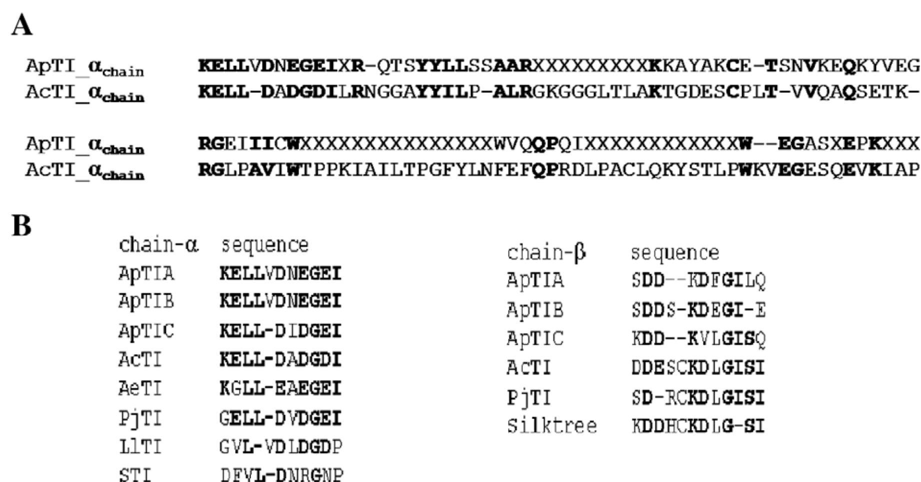


**Fig. 2.** (A) Primary structure sequence alignment between α and β-chains of ApTIC and AcTI; (B) Sequential N-terminal alignment among ApTIA, ApTIB, ApTIC chains and some representative Kunitz-type inhibitors using the program ClustalX. AcTI: *Acacia confusa* trypsin inhibitor (Lin et al., 1991), AeTI: *Acacia elata* trypsin inhibitor (Kortt and Jermyn, 1981), PjTI: *Prosopsis juliflora* trypsin inhibitor (Negreiros et al., 1991), LlTI: *Leucaena leucocephala* trypsin inhibitor (Oliva et al., 2000), STI: soybean trypsin inhibitor (Kunitz, 1945), silktree: silktree trypsin inhibitor (Odani et al., 1979). The conserved amino acids residues are highlighted in bold.

fractions was checked by SDS-electrophoresis, as showed in Fig. 1B for ApTIA, and reversed phase HPLC (similar chromatography data were obtained for the other isoforms).

The presence of protease inhibitors isoforms in plant seeds is commonly reported, but the characterization of all of them is rarely performed (Bhattacharyya et al., 2006; Batista et al., 1996; Morrison et al., 2007).

### 2.2. Structural characterization

Tricine–SDS–PAGE of *A. plumosa* isoforms gave a single band around 20 kDa under non-reducing conditions, characteristic of Kunitz type inhibitors, while under reducing conditions (β-mercaptoethanol) two polypeptide chains around 15 kDa (α-chain) and 5 kDa (β-chain), were obtained, i.e. as for other inhibitors described from the Mimosoideae (Batista et al., 1996; Oliva et al., 2000; Vargas et al., 2004). In agreement with these data, mass spectrometry analyses were measured from the SEC samples, as they indicated the presence of three isoforms with the following molecular mass: 19,709 Da, 19,869 and 20,378 Da (data not shown). Isoelectric focusing gel of ApTIA, ApTIB and ApTIC gave isoelectric points of an acidic nature: 5.05, 5.25 and 5.55, respectively (Fig. 1C). The acidic nature of the isoelectric point of ApTI isoforms is another feature generally found in the Kunitz type inhibitors, and can be compared to the isoelectric points of the isoforms *B. variegata:* 4.85, 5.0 and 5.15 (Ciero et al., 1996) and from *A. ellipticum:* 4.1, 4.55, 5.27 and 5.65 (Bhattacharyya et al., 2006), all members of this group. These results prove that ApTI are isoforms and not the same chain with a different degree glycosylation.

Partial protein primary structure was obtained by N-terminal analyses and sequencing of peptides derived from trypsin digestion. A high structural identity between *A. plumosa* inhibitor and *A. confusa* was observed (Fig. 2A). The sequential alignment of ApTI isoinhibitors established identity with Kunitz type inhibitors from *Acacia* (Kortt and Jermyn, 1981; Lin et al., 1991) and other Leguminosae (Oliva et al., 2000; Negreiros et al., 1991; Odani et al., 1979).

For the other isoforms, after reduction reactions, the α and β-chains from each were separated by reversed phase chromatography, and submitted to N-terminal analyses (Fig. 2B). The first 10 amino acid residues of both chains of each isoform indicated sequential identity among them, especially in the α-chain where only two differences were noted for the ApTIC α-chain, in comparison to the ApTIA and ApTIB sequences. The differences are an absence of a Val residue at position 5 and an Asn/Ile exchange. In the β-chain, the three isoforms were of higher sequential variability.

Circular dichroism spectra of the three ApTI isoforms gave similar results (Fig. 3A), where each exhibited a weak positive maximum at 230 nm, assigned to disulfide bridge and aromatic side contributions (Woody, 1978), and a minimum at 202 nm, due to contribution of the β-sheet and disordered elements typical of βII class proteins (Sreerama and Woody, 2003).

Deconvolution of the CD spectra, performed using the CDPro package (Sreerama and Woody, 2000) and a reference set of 43 proteins, gave as an average for the three isoforms: 42% β-strand, 21% turns and 37% of disordered structures, with root mean square deviation (RMSD) around 1%. Individual values of each isoform were very close to this average. This confirms classification of these isoforms as βII class proteins (Sreerama and Woody, 2003), as these results are in agreement with the structural characteristics of other plant Kunitz-type inhibitors described in the literature (Araújo et al., 2005; Haq and Khan, 2003). In addition, static fluorescence spectra of the three isoforms measured after excitation at 280 and 295 nm, gave the same $\lambda_{max}$ emission value at 341 nm; this value corresponds to the position of spectra of class II chromophores, in which tryptophan residues are exposed to the surface of the compact native protein molecule (Burstein et al., 1973). Only a difference in the intensity between the spectra with excitation at 280 and 295 nm was observed, suggesting the preservation of aromatic residues in the inhibitor's structure.

Thermal denaturation experiment of the ApTI isoforms was followed by elipticity loss of the 202 and 230 nm bands, i.e. when increasing the temperature for ApTIA, ApTIB and ApTIC (Fig. 3B). These data (typical native CD spectra) demonstrated that the inhibitors were thermally stable up to 65 °C but underwent abrupt denaturation with a midpoint at 75 °C for ApTIA and ApTIB, and 72 °C for ApTIC, suggesting a two-state model for the thermal denaturation in these isoforms. The internal disulfide bridges that are present in all three isoforms structures supports this high thermal stability.

Additionally, for the experiments on the effect of pH, the CD data showed clearly that for all isoforms, the incubation in a pH range from 2.0 to 12.0 did not affect the shape of the native CD spectra. This in turn suggested that there were no significant alterations in the secondary structure of the inhibitors. The fluorescence experiments are also in agreement with these data, as there was no alteration in the $\lambda_{max}$ of the emission in the whole pH range (even at extreme alkaline or acid values). Fluorescence quenching

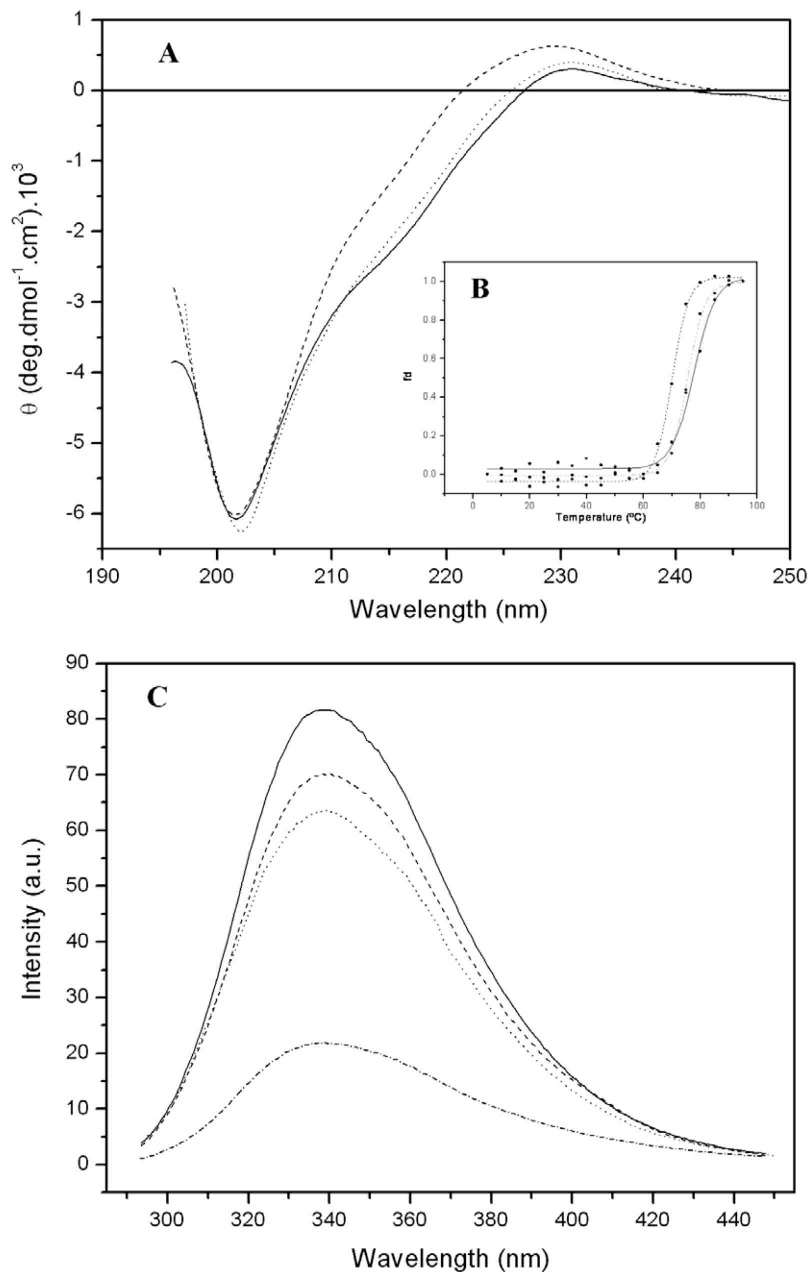


**Fig. 3.** Circular dichroism spectra and fluorescence emission of ApTI isoforms (A) Far UV CD spectra of ApTIA (straight line), ApTIB (dotted line) and ApTIC (dashed line) in PBS pH 7.4, using a 0.1 cm pathlength cylindrical quartz cuvette. The spectra were recorded from 195 to 250 nm as an average of 16 scans. (B) Thermal denaturing curves of ApTIA (straight line), ApTIB (dotted line) and ApTIC (dashed line) plotted using CD values of the 202 nm bands of each inhibitor as a function of temperature. The midpoint for ApTIA and ApTIB was 75 °C and for ApTIC was 72 °C. (C) Fluorescence emission spectra of ApTIB (0.07 mg/mL) in sodium acetate borate-phosphate 50 mM in pH 12.0 (dash dot), pH 8.0 (straight), pH. 6.0 (dash), pH 2.0 (dot), excited at 295 nm and recorded from 305 to 450 nm.

under both acid and alkaline conditions was noted (Fig. 3C), which can be explained by nonradiative processes including photoionization of neighboring protonated acidic groups such as Asp or Glu. Lys and Arg are also dynamic quenchers, but are more effective when charged (Ladoklhin, 2000).

### 2.3. Binding constants

Surface Plasmon Resonance (SPR) measurements were carried out to determine association ($K_A$) and dissociation constants ($K_D$) for each ApTI isoform using trypsin and α-chymotrypsin immobilized on a CM5 sensorchip. $K_A$ and $K_D$ values were measured after injection of different concentrations of ApTIA, ApTIB and ApTIC onto both enzymes (Fig. 4), with kinetic parameters shown in Table 1. The binding of the three isoforms to both enzymes established the formation of stable complexes; however, the α-chymotrypsin-ApTI complex presented a higher decay rate than the trypsin-ApTI complex (about 20 times higher, according to the kinetic parameters). These data show a strong inhibition of this serine protease by these ApTI isoforms.

SPR measurements were also carried out with the ApTI (sample from SEC, containing all three isoforms) on the trypsin sensorchip, but the BIAcore system was not able to detect analyte heterogeneity in these measurements since the data could not be fitted by this model. Therefore, a plain 1:1 Langmuir binding model was used, instead. The false homogeneity in ApTI can be attributed to the affinity to trypsin that is almost the same for the three isoforms, besides all the others similarities they share.

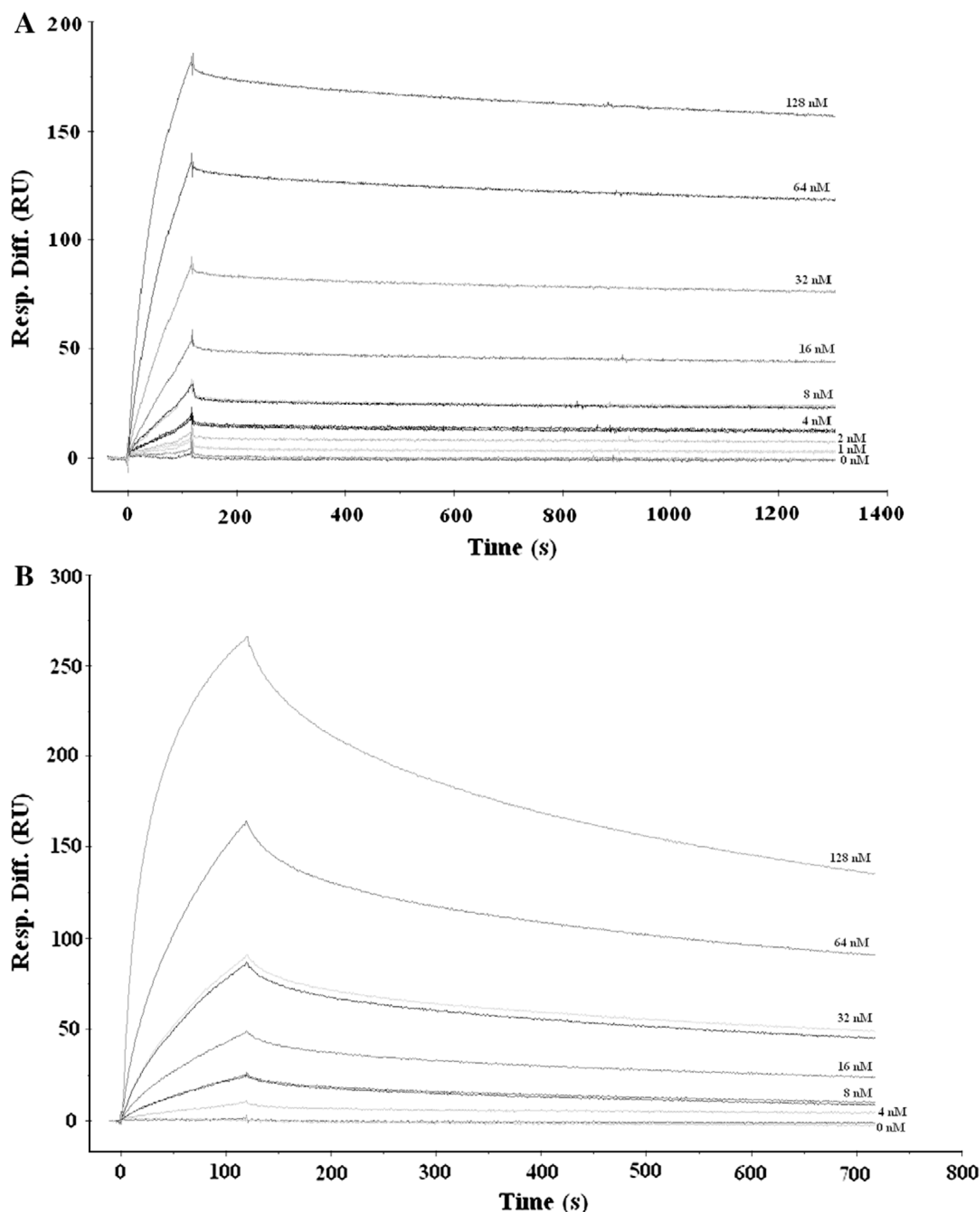


**Fig. 4.** Overlay of the sensorgrams for kinetic study of ApTI isoforms binding to trypsin/chymotrypsin immobilized on a CM5 sensorchip measured at 25 °C by a Biacore X system, at a flow rate of 30 μL/min. (A) Sensorgrams of the interaction between trypsin and ApTIC from 1 to 128 nM. (B) Sensorgrams of the interaction between αchymotrypsin and ApTIB from 4 to 128 nM. Raw binding data were analyzed by BIAevaluation Version 4.1 Software and fit to a 1:1 Langmuir binding model.

**Table 1**
Kinetic parameters for binding of ApTI isoforms to trypsin and a-chymotrypsin.

| Enzyme | ApTI isoform | $k_{on}$[a] ($10^5$ $M^{-1}$ $s^{-1}$) | $k_{off}$[a] ($10^{-4}$ $s^{-1}$) | $K_D$[b] (nM) | $K_i$[c](nM) |
|---|---|---|---|---|---|
| Trypsin | ApTIA | 1.68 | 0.653 | 0.389 | 2.31 |
| | ApTIB | 1.32 | 0.737 | 0.558 | 1.67 |
| | ApTIC | 1.54 | 0.858 | 0.557 | 1.52 |
| α-Chymotrypsin | ApTIA | 0.896 | 6.68 | 7.46 | 10.0 |
| | ApTIB | 1.03 | 7.11 | 6.90 | 10.2 |
| | ApTIC | 0.763 | 2.65 | 3.47 | 10.9 |

[a] Values for ApTI isoforms binding to trypsin and a-chymotrypsin were derived from simultaneous fit of association and dissociation sensorgrams of SPR, shown in Fig. 3.
[b] $K_D$ values were calculated as $k_{off}/k_{on}$.
[c] $K_i$ values were calculated by Morrison's procedure.

For ApTIA, ApTIB, and ApTIC, respectively, the isoform specificity for serine proteases like trypsin, α-chymotrypsin, human plasma kallikrein, and porcine pancreatic elastase using synthetic substrates was considered as strong inhibition. For trypsin: $K_i$

**Table 2**
Delay on the coagulation time[a] by ApTI isoinhibitors.

| Inhibitor | Coagulation time (min) |
|---|---|
| Control | 3.9 ± 0.4 |
| ApTIA | 38 ± 2 |
| ApTIB | 40 ± 1 |
| ApTIC | 27 ± 2 |

[a] Measured right after the addition of 20 mM $CaCl_2$.

2.3, 1.7 and 1.5 nM; for α-chymotrypsin: $K_i$ 10, 10 and 11 nM; and for human plasma kallikrein: $K_i$ 0.55, 0.53, and 0.65 μM. The three isoforms did not show any inhibitory action to porcine pancreatic elastase.

The potent inhibition of the serine proteases suggests a high stability of the resulting enzyme-inhibitor complex. Our results clearly show that the interaction between the binding site of the ApTI isoforms and the reactive site of trypsin was more specific, so that the $K_i$ value was higher for this enzyme. These values are close to the $K_i$ values of AcTI (*A. confusa* trypsin inhibitor) for trypsin (0.294 nM) and for α-chymotrypsin (0.564 nM), and of EcTI (*E. contortisiliquum* trypsin inhibitor) (Batista et al., 1996) for trypsin (1.56 nM) and α-chymotrypsin (120 nM).

### 2.4. Biotechnological applications

In order to carry out our proposed studies, we investigated some applications of the *A. plumosa* inhibitors as both an anticoagulant and an inhibitor of phytopathogenic fungi growth. It was noted that ApTIA and ApTIB could delay up to 10 times the blood coagulation in vitro, while ApTIC promoted a seven times delay, according to Table 2. This delay can be explained by the inhibition (or different specificity) of enzymes that are implicated in the blood coagulation cascade.

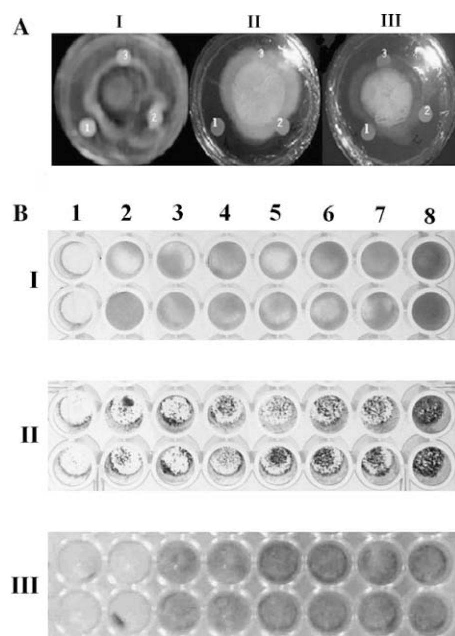


**Fig. 5.** (A) Antifungal activity of ApTI isoforms against *A. niger* (I), *Colletotrichum* sp. P10 (II) and *F. moniliforme* (III) on Petri dishes. Disk 1: 100 μg of ApTI, disk 50 μg of ApTI; disk 3: PBS buffer. (B) Inhibition assay on 96-well microplate using twofold serially dilution. Antifungal activity was noted against *A. niger* (I), *Colletotrichum* sp. P10 (II) and *T. paradoxa* (III). The amount of ApTI was 140, 70, 35, 17, 8, 4, 2, and 0 μg in the wells numbered from 1 to 8, respectively.

The antifungal activity of ApTI isoforms was tested on 23 different phytopathogenic fungi. In the assay performed on agar plates, the ApTI isoforms hindered normal development of *Aspergillus niger*, *Colletotrichum* sp. P.10 and *Fusarium moniliforme* hyphae (Fig. 5A) by generating an inhibition halo around the disk containing the higher concentration of the sample. The antifungal action of *A. plumosa* can be associated with inhibition of some serine proteases liberated in the medium by the fungi for starting their nutritional mechanism. The formation of the enzyme-inhibitor complexes is also responsible for the stopping of the fungi growth. The ApTI isoforms did not show any alteration in the growth of the other fungi. In the quantitative assay (Fig. 5B), ApTI was incubed with $10^7$ spores/mL of *A. niger*, *Colletotrichum* sp. P.10 and *Thielaviopsis paradoxa*, and inhibited their development at 140, 140 and 70 μg/well, respectively, i.e. thereby occasioning no germination in the wells where the inhibition was detected.

## 3. Concluding remarks

Several intrinsic physico-chemical features of Kunitz type inhibitors were found in the three novel protease isoinhibitors purified from the seeds of *A. plumosa*, a Leguminosae–Mimosoideae plant: a molecular mass close to 20 kDa comprising two polypeptide chains of 15 and 5 kDa linked by disulfide bridge(s) was found, isoelectric points with an acidic nature, sequential identity with STI (Kunitz, 1945), and a secondary structure mainly formed of β-strands and disordered elements were also determined. By examining the stability experiments, one can conclude that the isoinhibitors present highly stable secondary structures, which are able to resist to pH variation and to thermal heat up to 65 °C.

ApTIA and ApTIB presented slightly differences to ApTIC. These differences could be detected in the CD spectra, the denaturation midpoints and the anticoagulating activities, reflecting their minor differences in specificity against serine proteases. These results prompted us to continue the characterization of ApTI isoforms, since small structural differences can provide useful information on the specificity and mechanism of action of serine proteases inhibitors. Not only is the structure of the reactive site important, but also the aspects of the molecular structure should be considered; this includes the global architecture and the structural features responsible for inhibitor stability and efficiency.

The interaction between ApTI isoforms and serine proteases (trypsin and α-chymotrypsin) was studied using two different techniques: absorbance spectroscopy (employed to study the enzymatic activity inhibition of serine proteases using chromogenic substrates), and biosensor surface plasmon resonance detection (used to investigate the binding of inhibitors to the enzymes). Both SPR and the inhibition assay identified ApTI isoforms as potent trypsin inhibitors. By analyzing the $K_D$ values, obtained from SPR measurements, and inhibition constants ($K_i$), we can conclude that the binding and the enzyme inhibition are closely correlated, indicating that immobilization of the enzymes by amine coupling did not significantly alter the binding properties of the active site of the serine proteases.

The study of biomolecular interactions on sensor chips can be used to investigate other enzyme inhibition processes, and also processes of molecular recognition and biological function. The binding assay performed with biosensor technology may also be useful for characterization of other macromolecular interactions besides proteinaceous inhibitors and serine proteases.

Apart from these findings, the action on the blood coagulation cascade and especially the antifungal activity of ApTI isoforms gave additional reasons to continue investigating these molecules, since their inhibition property was efficient against fungi that attack economically important plants.

## 4. Experimental

### 4.1. Purification

Mature *A. plumosa* Lowe seeds were ground and stirred into PBS (Phosphate Buffer Saline 0.15 M, pH 7.4, 0.15 M NaCl), 1:10 (w/v) for 2 h at 4 °C. After centrifugation at 12,000×g for 30 min at 4 °C, insoluble materials were removed by filtration and the supernatant was submitted to dialysis against PBS. The dialyzed crude extract was used for further purification, this being first subjected to size exclusion chromatography using a Superdex-75 HR 10/30 column coupled to an ÄKTA purifier system (Amersham Pharmacia Biotech); the column was pre-equilibrated and eluted with PBS, using a flow rate of 0.5 mL/min and collecting 1 mL fractions, monitored by absorbance at 280 nm. Fractions with inhibitory and anticoagulant activities were pooled and concentrated by diafiltration on a Centriprep-3. The concentrated fraction was next applied to a Mono-S HR 5/5 column coupled with the ÄKTA purifier system. It was equilibrated with NaOAc buffer (50 mM, pH 5.0) and eluted with the same buffer in a linear gradient containing 0–0.5 M NaCl, for 50 min with a flow rate of 1 mL/min. Fractions (1 mL) were collected and monitored by their absorbance at 280 nm. Protein concentrations and total protein contents from the CE were determined by the Lowry protein assay (Lowry et al., 1951), using bovine serum albumin (BSA) as standard. The purified inhibitors were denoted ApTIA, ApTIB, and ApTIC, respectively.

### 4.2. Characterization and structural methodology studies

Molecular mass determinations: The *molecular mass* (MM) of each inhibitor was determined by Tricine–SDS–PAGE (Schagger and Jagow, 1987) in a 16% separating gel, with and without β-mercaptoethanol. BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20 kDa), cytochrome C (12.4 kDa), and globin fragments (17; 14; 10.7; 8; 6 and 2.5 kDa) were used as molecular mass markers. Further experiments of mass spectrometry were carried out using electrospray ionization with a Quattro II electrospray triple quadrupole MS (Micromass, Manchester, UK).

*Isoelectric focusing*: This was performed with fractions having inhibitory activity from the two chromatographic processes using a PhastSystem – PhastGel IEF 3.0-9.0 (Pharmacia LKB Biotechnology). Electrophoretic analyses were carried under standard conditions and the gel was dyed with Coomassie Blue 250.

*Friedman reactions*: The method of Friedman et al. (1970) was used, with some modifications, to reduce the inhibitors as follows: inhibitors (2 nM) were dissolved in 0.25 M Tris–HCl (300 μL pH 8.0), 6 M guanidine-HCl, 2 mM EDTA, 5 μL β-mercaptoethanol and incubated for 4 h at 56 °C, in the dark, before $N_2$ purging. The free sulphydryl groups of each chain were exposed to 4-vinyl pyridine (5 μL) with the reaction continued for 2 more hours under the same initial conditions. The reaction was stopped with AcOH:$H_2O$ (1:9, v/v), with chains desalted and separated on a reversed-phase $C_{18}$ column performed in an HPLC system (monitoring at 220 nm). The column was equilibrated with 0.1% TFA in $H_2O$ and eluted using a linear gradient of this solution from 0 to 100% of $CH_3CN$:$H_2O$ (9:1, v/v) in acetonitrile 90%, 0.1% TFA in $H_2O$. Two chains, from each inhibitor, were obtained and submitted to amino acid sequencing.

*Primary structural sequencing*: This analyses were performed using an automatic protein sequencer PPSQ-23A Shimadzu (Kyoto, Japan), following the manufacturer's recommendations, as follows: 300 pmol of each chain purified in $H_2O$ and Sequa-Brene (hexadimethrine bromide, Sigma–Aldrich) was applied to a special fiberglass disk (Wako, Osaka, Japan), previously treated with Sequa-Brene.

Purified ApTIC was thermally denaturated (100 °C, 60 min) and submitted to trypsin digestion (1:20, mol/mol). Peptides derived from this reaction were analyzed for mass spectrometric and automated protein sequencer analyses.

The percentage of sequence identity with trypsin inhibitors from other members of the Mimosoideae sub-family was determined using the NCBI-BLAST (Altschul et al., 1990) databank and multiple sequence alignment, performed with CLUSTAL X (Higgins and Sharp, 1988).

*Far-UV circular dichroism (CD) and secondary structural contents*: The ApTI isoforms were studied in their native conditions, with a protein concentration of 0.3 mg/mL in PBS (pH 7.4) being recorded from 195 to 250 nm, using a JASCO J-715 spectropolarimeter (Jasco Instruments, Tokyo, Japan). An average of 16 scans using a 0.1 cm path length cylindrical quartz cuvette were made. The contributions of the secondary structure elements of each isoform inhibitor were determined by CD spectrum deconvolution analyses using a CDPro package (Sreerama and Woody, 2000), containing ContinLL, Selcon3 and CDSSTR programs.

*Steady-state fluorescence*: Emission spectra of ApTI isoinhibitors were obtained using an ISS K2 spectrofluorimeter (ISS Fluorescence, Analytical and Biomedical Instruments-Illinois, USA), kept at 18 °C with a circulating water bath (Fisher Scientific) around a 1 cm path length rectangular quartz cuvette. Samples (0.07 mg/mL in PBS) were excited at 280 and 295 nm with the emission spectra recorded from 295 to 450 and from 305 to 450 nm, respectively. Reference spectra were recorded and subtracted after each measurement.

*Inhibitors stability*: These analyses examine inhibitors stability effects, after exposure to different pH and temperatures, using CD and fluorescence monitoring. For pH experiments, the inhibitors were concentrated to 2 mg/mL and then diluted to 0.2 mg/mL (for CD experiments) and then to 0.07 mg/mL (for fluorescence emissions) in each buffer, namely 50 mM sodium acetate-phosphate-borate adjusted to different pH values (2.0, 4.0, 6.0, 8.0, 10.0 and 12.0). Samples were incubated for 30 min and each CD spectrum was then subsequently recorded. The reference spectrum of each measurement was recorded and subtracted. For thermal denaturation assays, samples of each isoinhibitor (0.2 mg/mL in PBS, pH 7.4) were heated gradually (5 °C steps) from 5 to 95 °C using a circulating water bath TC-100 (Jasco). At each temperature, the protein was incubated for 30 min and the spectrum was recorded from 195 to 250 nm.

### 4.3. Association, dissociation and inhibition constants ($K_A$, $K_D$, and $K_i$) determination

*Surface plasmon resonance* experiments were employed to determine the $K_A$ and $K_D$ of ApTI isoforms with proteolytic enzymes (trypsin and α-chymotrypsin) on a BIAcore X instrument (BIAcore AB, Uppsala, Sweden). Each enzyme was coupled to a carboxymethyldextran CM5 sensor chip using an amine coupling kit containing N-ethyl-N-(3-dimethylaminopropyl) carbodiimide HCl, ethanolamine HCl, and N-hydroxysuccinimide, according to the manufacturer's recommendations. Trypsin (1.5 μM) in 10 mM NaOAc (pH 5.0), and α-chymotrypsin (1.2 μM) in 10 mM NaOAc (pH 5.5) were coupled to the CM5 sensor surface using 20 μL of each enzyme, followed by washing in the BIAcore running buffer: HBS-EP (10 mM HEPES, pH 7.4, 150 mM NaCl, and 3 mM EDTA containing 0.005% surfactant $P_2O$), at 25 °C. Each binding assay was performed with a constant flow rate of 30 μl/min at 25 °C with ApTI isoforms concentrations in the range of 1–128 nM. The association was monitored during the injection time (2 min), and dissociation was monitored for 10 min after the end of each injection. Before the next cycle started the surfaces were regenerated with 1 min injections of 4 M $MgCl_2$ in 0.1 M NaOAc sodium acetate

(pH 4.5) followed by the running buffer for 3 min. For fitting of binding kinetics, BIAevaluation Version 4.1 software (BIAcore AB) was applied, and the 1:1 Langmuir binding model was chosen.

*Morrison's procedure* was employed to determine $K_i$ values of the ApTI isoforms on bovine trypsin, α-chymotrypsin, human plasma kallikrein, and porcine pancreatic elastase. The $K_i$ values were determined by measuring the remaining hydrolytic activity of the enzymes towards specific *N*-α-Benzoyl-DL-arginine *p*-nitroanilide (BAPNA) synthetic substrates after pre-incubation of enzyme and inhibitor for 10 min. Mixtures containing different concentrations of enzyme and inhibitor (50 μL) were added to a final volume of 250 μL of substrate in PBS (pH 7.4) and incubated at 37 °C for 30 min. The reactions were stopped by adding AcOH–$H_2O$ (40 μL, 4:6, v/v). Substrate hydrolysis was followed by absorbance of p-nitroaniline at 405 nm using a Hitachi U-2801 spectrometer. $K_i$ values were determined following Morrison's procedure, with the calculations of this model performed with the enzymatic kinetics program Grafit 3.0 (Knight, 1986).

### 4.4. Biological activities

*Anticoagulant activity* assays were employed throughout the purification procedure in order to check the presence and the activity of the inhibitors after each chromatographic step. In these assays, human plasma (50 μL) together with 20 mM sodium citrate (1:10 v/v) were incubated with the same volume of inhibitors (0.5 mg/mL in PBS) for 30 min at 37 °C. Afterwards, 20 mM $CaCl_2$ (50 μL) were added to the mixture. A solution containing plasma diluted in PBS was used as control. The coagulation time of each inhibitor was measured from that moment on.

*Antifungal activity* of ApTI from size exclusion chromatography, that had inhibitory activity, was employed in two different assays with the following phytopathogenic fungi: *Aspergillus chevalier, Aspergillus furmigatus, A. niger, Botrytis cinerea, Colletotrichum gloeosporioides, Colletotrichum lindemuthianum, Colletotrichum musae, Colletotrichum* sp. F37, Colletotrichum sp. P10, *Colletotrichum truncatum*, *Fusarium graminearum, F. moniliforme, Fusarium oxysporum, Fusarium pollidoroseum, Hendersonina* sp., *Lasiodiplodia theobromae, Mucor* sp., *Phomopsis* sp., *Pyricularia* sp., *Pythium oligandrum, Rhizoctonia solani, T. paradoxa*, and *Tricoderma viridae.* First, a hyphal-extension inhibition assay on PDA plates, where fungal mycelia were placed over the solid PDA (Potato-Dextrose-Agar-Acumedia Manufacturers, Michigan), was carried out. In these experiments, three disks of sterile filter paper were distributed over the plate: on the first disk, ApTI (100 μg) samples were deposited, on the second disk 50 μg, and on the third disk only PBS was deposited. The plates were incubated at 28 °C for 5 days and evaluated visually after this period. The second assay was performed by incubation of ApTI isoinhibitors with different fungal spores. Growth inhibition bioassays were carried out by adding 140 μg of the sample to 10 μL of a solution with $10^7$ spores/mL in a 96-well microplate, containing 50 μL of PD medium, followed by a twofold serial dilution. Each microplate was incubated for 6 days at 28 °C and growth inhibition was observed visually and compared with control microcultures containing only spores, PBS, and culture medium.

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