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Calliandra selloi Macbride trypsin inhibitor: Isolation, characterization, stability, spectroscopic analyses

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Abstract

A trypsin inhibitor was purified from *Calliandra selloi Macbride* seeds (CSTI). SDS–PAGE under non-reducing conditions showed a single band of approximately 20,000 Da, while under reducing conditions two bands of 16,000 and 6000 Da were observed, indicating that CSTI consists of two polypeptide chains. Molecular masses of 20,078 and 20,279 were obtained by mass spectrometry, although only one pI of 4.0 was observed and one peak was obtained by reversed phase chromatography. Amino-terminal sequence analysis showed homology to Kunitz-type inhibitors. CSTI was able to inhibit trypsin (K_i 2.21×10^{-7} M), α-chymotrypsin (K_i 4.95×10^{-7} M) and kallikrein (K_i 4.20×10^{-7} M) but had no effect on elastase. Trypsin inhibitory activity was stable over a wide range of pH and temperature. CSTI was particularly susceptible to DTT treatment, followed by addition of iodoacetamide. Far-UV circular dichroism measurements revealed that CSTI is a β-II protein. Thermal unfolding showed a two-state transition with a midpoint at 68 °C. Far-UV CD spectra of CSTI at pH extremes showed little changes, while more pronounced differences in near-UV CD spectra were detected. Remarkably, treatment with 1 mM DTT caused very slight changes in the far-UV CD spectrum, and only after carbamidomethylation was there was a marked loss observed in secondary structure.

Keywords: Kunitz-type trypsin inhibitor; *Calliandra selloi* MacBride; Leguminosae; Protein purification; Characterization; Stability studies

1. Introduction

Plants have developed several defense mechanisms that offer protection from predators or from infection by pathogens. One of these defenses involves the production of several proteins such as lectins, enzyme inhibitors, chitinases, ureases and ribosome-inactivating proteins (Carlini and Grossi de Sa, 2002; Lajolo and Genovese, 2002; Murdock and Shade, 2002). Among these, protease inhibitors have been the subject of extensive research not only for their potential use as bioinsecticides (Casaretto and Corcuera, 1995; Haq et al., 2004; Abdeen et al., 2005) but also for their antifungal activity (Park et al., 2005). These findings have thus attracted interest in developing insect-resistant transgenic crops (Mandal et al., 2002; Abdeen et al., 2005) expressing a trypsin inhibitor gene. Other biotechnological application reported for protease inhibitors is that they can inhibit growth of transformed cells, i.e. thereby showing anticarcinogenic properties (Clawson, 1996; Das and Mukhopadhyay, 1994; Kennedy, 1998).

Protease inhibitors are widely distributed in the plant kingdom. Three main plant families are known to be rich in these inhibitors, the Leguminoseae, Solanaceae and

Abbreviations: CSTI, *Calliandra selloi* trypsin inhibitor; BAEE, *N*-α-benzoyl-L-arginine ethylester; BTEE, *N*-benzoyl-L-tyrosine ethylester; DTT, dithiothreitol; TFA, trifluoroacetic acid; CD, circular dichroism.

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Graminaceae families. Legume seeds contain various trypsin inhibitors of which the Kunitz-type (Richardson, 1977) and Bowman Birk type inhibitors (Birk, 1996) are the best characterized. Kunitz-type inhibitors are usually proteins of M_r 20 kDa, with four cysteine residues forming two disulfide bridges and a single reactive site, which is generally an arginine residue located in one of the protein loops. They are relatively abundant in the seeds of the three Leguminosae subfamilies: Mimosoidae, Caesalpinoidae and Papilionoidae. Some Kunitz-type trypsin inhibitors isolated from the Mimosoidae subfamily, are formed by two polypeptide chains, joined by a disulfide bridge (Lin et al., 1991; Negreiros et al., 1991; Batista et al., 1996; Oliva et al., 2000). Although the archetypical member of the Kunitz inhibitor family, soybean trypsin inhibitor, is not a glycoprotein, several glycosylated inhibitors have been described, such as those obtained from *Echinodorus paniculatus* (Paiva et al., 2003), *Peltophorum dubium* (Troncoso et al., 2003; Macedo et al., 2003), *Carica papaya* (Azarkan et al., 2006) and *Swatzia pickellii* seeds (do Socorro et al., 2002). Some inhibitors belonging to this family, such as those isolated from soybean and *P. dubium* seeds, show lectin-like properties in addition to their protease inhibitory activity (Troncoso et al., 2003) and trigger rat lymphoma cell apoptosis. In contrast, a protein from *Labramia bojeri* seeds, homologous to Kunitz-type trypsin inhibitors, had lectin-like properties despite being devoid of trypsin inhibitory activity (Macedo et al., 2004).

Calliandra selloi MacBride is a leguminous bush belonging to the Mimosoidae subfamily, which grows in tropical and subtropical regions of America. Although it is used ornamentally and for forage, nothing is known about the content of its seeds. This paper describes the purification, biochemical and structural characterization of the first serine protease inhibitor described in the *Calliandra* genus. Furthermore, it is the first protein purified from *Calliandra selloi* MacBride seeds. The inhibitor was denoted CSTI (*Calliandra selloi* trypsin inhibitor).

2. Results and discussion

2.1. Purification and molecular weight determination of Calliandra selloi trypsin inhibitor (CSTI)

CSTI was purified by a combination of two chromatographic steps. First, the seed saline extract was submitted to affinity chromatography on a trypsin-agarose column and then the fraction with trypsin inhibitory activity was subjected to ion exchange chromatography on a DEAE column. Yield was approximately 0.5% w/w of CSTI/total protein of the seed extract.

The purified fraction of CSTI showed a single band in SDS–PAGE under non-reducing conditions (Fig. 1a), corresponding to an apparent M_r of 20,000. After reduction with β-mercaptoethanol, two bands of 16,000 and 6000 apparent M_r, respectively were obtained, showing that

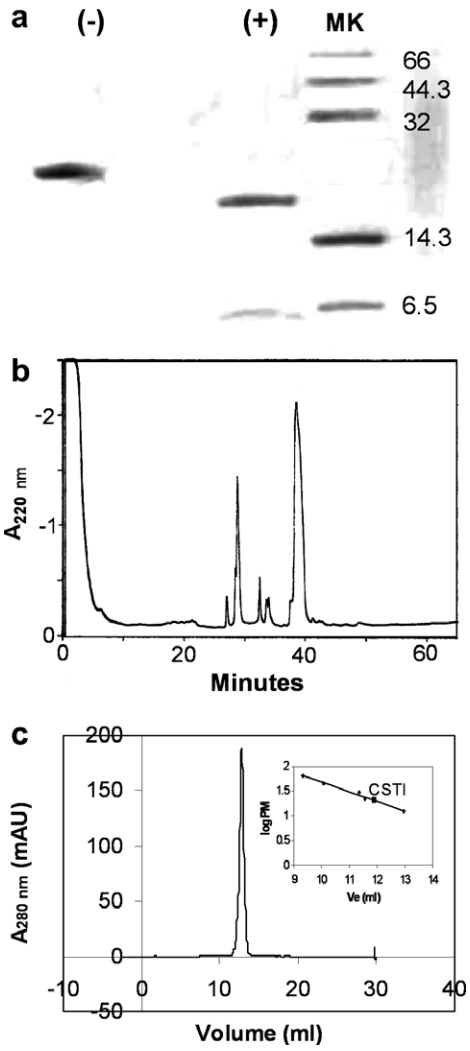

Fig. 1. Characterization of purified CSTI. (a) SDS–PAGE of CSTI. Lane 1, unreduced CSTI; lane 2, CSTI under reducing conditions, lane 3, molecular mass markers. The proteins were detected by Coomassie brilliant blue staining; (b). Reversed phase HPLC isolation of CSTI chains after reduction and modification with 4-vinylpyridine, as described in Section 4. Separation was performed on a C_8 column, with a 2–96% CH_3CN gradient in 2% acetic acid. (c) Assessment of native protein M_r by gel filtration on a Superdex S75 column. Inset shows the calibration curve using bovine serum albumin, ovoalbumin, bovine erythrocyte carbonic anhydrase, soybean trypsin inhibitor and bovine heart cytochrome *c* as standards.

CSTI is a protein composed of two chains named α and β linked by a disulfide bridge. Consistent with these results, several trypsin inhibitors composed of two chains linked by a disulfide bridge have been reported, all of them belonging to the Mimosoidae subfamily of the Leguminosae family (Lin et al., 1991; Negreiros et al., 1991; Batista et al., 1996; Oliva et al., 2000). The isoelectric focusing showed only one band corresponding to a pI value of 4.0 (data not shown). This value is in agreement with the acidic nature of most inhibitors in the Kunitz family (Macedo et al., 2000). Besides, only one peak was obtained by reversed phase chromatography on a C_8 column (data not shown). Nevertheless, when analyzed by electrospray

mass spectrometry, approximately equal amount of two molecular species taken as isoforms of 20,078 Da and 20,279 Da were observed. This heterogeneity was only detected by mass spectrometry and the difference of molecular mass is consistent with the presence of *N*-acetyl glucosamine or *N*-acetyl galactosamine, although other sequence heterogeneity cannot be excluded. Mass spectrometry analysis of the two polypeptide chains was also performed after previous reduction, 4-vinylpyridine modification of the inhibitor and subsequent HPLC separation on a C_8 column (Fig. 1b). The large α chain showed the presence of two molecular species of 16,052 Da and 16,253 Da while the β chain corresponded to a molecular mass of 4450 Da. The sum of the masses of the modified polypeptide chains, 16,052 Da and 4450 Da, when compared with that of the un-modified inhibitor, 20,078 Da, showed a difference of 424 Da which is compatible with modification of four cysteines, three cysteine residues in the α chain and one in the β chain. The presence of four cysteine residues is in agreement with most of the Kunitz-type trypsin inhibitors described. However, an unusual inhibitor isolated from *Copaifera langsdorffii* tree (*Leguminoseae* – Caesalpinoidae) with two non-covalently bound polypeptide chains and a single disulfide bridge has been reported (Krauchenco et al., 2004).

In an attempt to determine native molecular mass, CSTI was submitted to gel filtration (Fig. 1c) and only one peak, corresponding to an estimated molecular mass of 20.3 kDa was observed.

2.2. Protease inhibitory properties

CSTI was found to specifically inhibit trypsin, α-chymotrypsin and kallikrein but no inhibitory activity towards elastase was detected. Fig. 2 shows the titration curves for the three proteases with CSTI. The K_i values were calculated using the equation for slow tight binding inhibition (Morrison, 1982) and were 2.21×10^{-7} M for trypsin, 4.95×10^{-7} M for α-chymotrypsin and 4.20×10^{-7} for kallikrein.

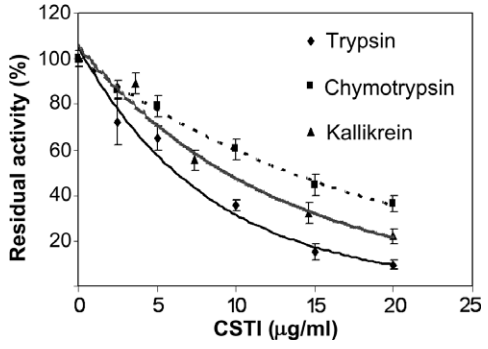

Fig. 2. Inhibitory activity of CSTI on bovine trypsin, α-chymotrypsin and kallikrein. Inhibitory activities, represented as % of residual enzymatic activity, were determined by measuring the remaining hydrolytic activity compared to the activity of the enzyme alone. Each point represents the mean ± SE of three independent assays.

2.3. Biological activities

Due to the fact that soybean trypsin inhibitor, as well as the Kunitz-type trypsin inhibitor from *P. dubium*, have been shown to have lectin-like properties, i.e. being able to hemagglutinate rabbit erythrocytes and to induce Nb2 lymphoma cell line apoptosis (Troncoso et al., 2003), CSTI was tested at different concentrations for these activities. However, the results obtained were negative (data not shown), thus indicating that not all Kunitz-type protease inhibitors share these properties. Similarly, no antifungal activity was detected on the fungal strains tested (indicated in Section 4).

2.4. Structural characterization

The N-terminal region sequence of both α and β chains exhibited a high degree of identity to known Kunitz-type protease inhibitors. The internal sequence stemmed from modified chains which were cleaved by specific enzymes as described in Section 4. Partial sequence data was determined successfully for 52% of the α and 79% of the β chain. Because of the high sequence identity of CSTI with other members of the Kunitz type inhibitor family, all fragments obtained could be aligned with them (Fig. 3). For comparison, the sequences obtained were aligned with two chain trypsin inhibitors that showed highest identity, isolated from *Acacia confusa* (ACTI) (Wu and Lin, 1993), *Adenanthera pavonina* (APTI) (Macedo et al., 2004), *Prosopsis juliflora* (PJTI) (Negreiros et al., 1991), *Leucaena leucocephala* (LITI) (Oliva et al., 2000), *Copaifera langsdorffii* (CTI) (Silva et al., 2001; Krauchenco et al., 2004) as well as one chain trypsin inhibitors isolated from *Bauhinia variegata* (BvTI) (Di Ciero et al., 1998) and soybean (SBTI) (Koide and Ikenaka, 1973). The amino terminal sequence of the α chain had the highest identity with ACTI (79%), followed by PJTI (74%), APTI (68%), BvTI (68%), LITI (58%) CTI (53%) and SBTI (53%). The same order of identity was observed for the β chain except that CTI had the least identity (36%). Due to the overall sequence similarity between all members of the Leguminosae family, it has been strongly suggested that the origin of the two chains is a post-transcriptional proteolytic cleavage (Creighton and Darby, 1989). The alignment shows that CSTI has the highest identity with other two chain inhibitors when the cleavage occurs around residue 160. However, the cleavage does not always take place at the same location in the molecule since in the case of CTI it occurs around residue 102; furthermore, the polypeptide chains are non-covalently bound (Silva et al., 2001; Krauchenco et al., 2004) and the identity with CSTI is the lowest (see Fig. 3).

To gain insight into the secondary conformation of CSTI, far-UV CD was determined in the wavelength range of 190–250 nm (Fig. 4a). CSTI has a CD spectrum characteristic of the class of β-II proteins or "disordered" class of proteins, with a minimum ellipticity at about 203 nm and a slight shoulder close to 230 nm (Wu et al., 1992). The

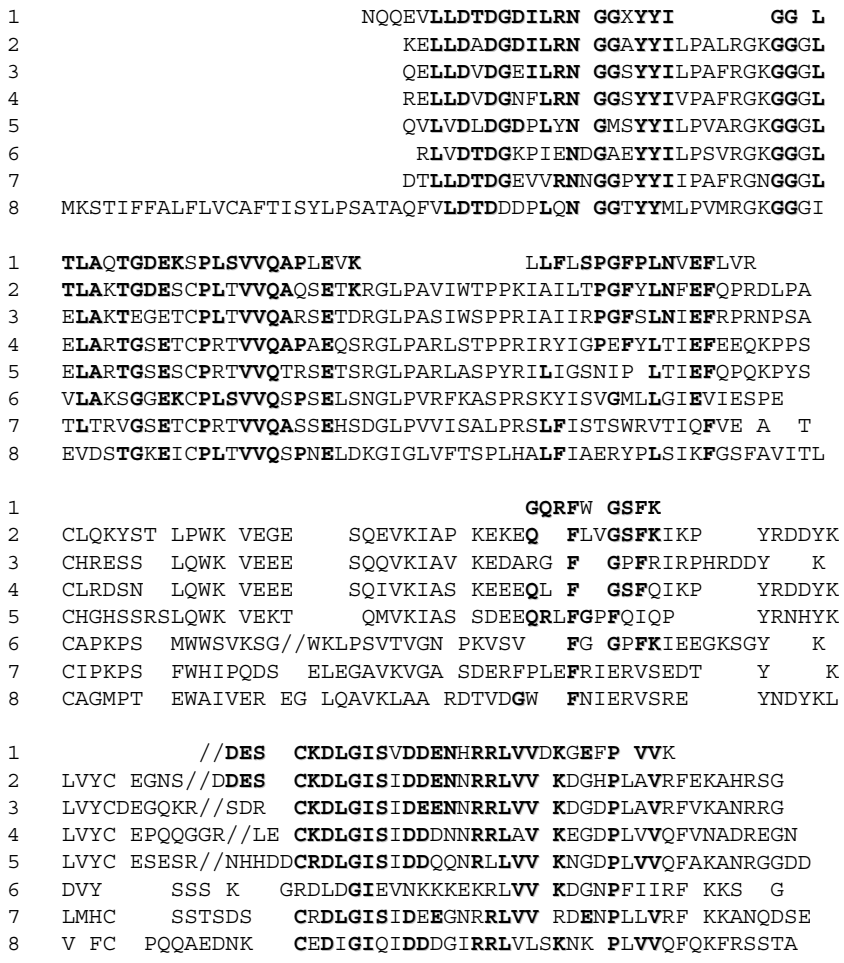

Fig. 3. Comparative sequences of related Kunitz trypsin inhibitors. (1) CSTI, *Calliandra selloi*; (2) ACTI, *Acacia confusa* (Wu and Lin, 1993); (3) PJTI, *Prosopsis juliflora* (Negreiros et al., 1991); (4) APTI, *Adenanthera pavonina* (Macedo et al., 2004); (5) LITI, *Leucaena leucocephala* (Oliva et al., 2000); (6) CTI, *Copaifera langsdorffii* (Silva et al., 2001); (7) SBTI, soybean (Koide and Ikenaka, 1973); (8) BvTI, *Bauhinia vairegata* (Di Ciero et al., 1998). The beginning of the β chain is indicated (//).

deconvolution with CONTINLL showed that CSTI consists of 5% α-helix, 40% β strand, 22% turns and 33% of unordered structure. This spectral pattern closely resembles that of SBTI (Roychaudhuri et al., 2004), a protein that has been shown to belong to the β-II class. The β-II is a subclass of all β proteins in which most amino acid residues are involved in irregular β sheets.

Near-UV CD spectra of proteins arise from the environments of each aromatic amino acid side-chain and therefore provide information about the tertiary structure of the folded protein (Kelly and Price, 2000). The near-UV CD spectrum of CSTI (250–300 nm) (Fig. 4b) is characterized by sharp peaks at 290 nm and at 285 nm which could be assigned to tryptophan and tyrosine residues, respectively. These peaks distinguish β-II-type from unfolded proteins (Wu et al., 1992).

Fluorescence spectroscopy provides information about the polarity of the environment of tryptophan and tyrosine residues. Since the near-UV spectra revealed the presence of Trp residues, Trp fluorescence emission spectra were recorded. The amino acid sequence showed the presence of at least one Trp residue and, taking into account the sequence similarity with other trypsin inhibitors, it is probable that there are two. The emission spectra of CSTI showed a maximum at 347 nm (data not shown), suggesting that the Trp residues are in a non-polar environment but it cannot be excluded that one of them is completely buried while the other is solvent exposed.

2.5. Stability studies on inhibitory activity and protein conformation

2.5.1. Effect of temperature

Proteins involved in defense mechanisms are known to be heat stable (Azarkan et al., 2006; Macedo et al., 2003; Deshimaru et al., 2002; Haq et al., 2004). Regarding the stability of CSTI, the inhibitory activity was resistant to increasing temperatures since the results of thermal denaturation of CSTI showed that the inhibitor remained quite stable at increasing temperatures, retaining more than 75% of its inhibitory activity after incubation at 80 °C for 30 min (Fig. 5a). At 100 °C the inhibitor lost 50% of its

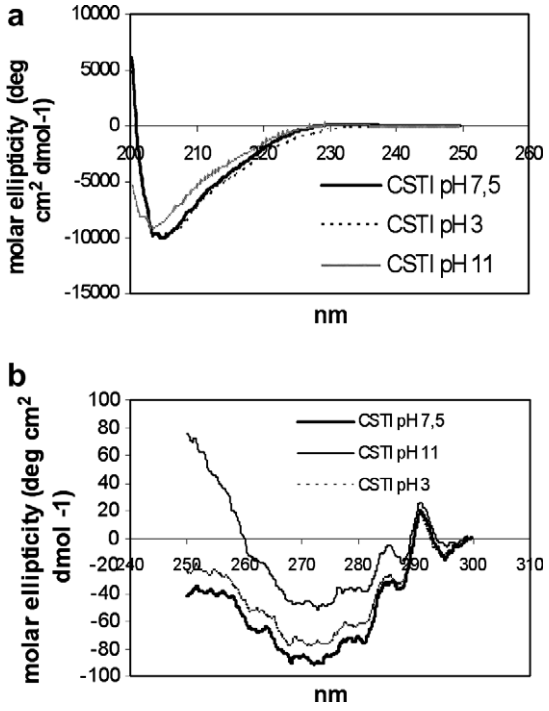

Fig. 4. Circular dichroism measurements of CSTI. (a) Far UV-CD spectra of CSTI in the following buffers: 20 mM sodium acetate buffer, pH 3.0, 5 mM $CaCl_2$/20 mM Tris–HCl buffer, pH 7.5, 5 mM $CaCl_2$/20 mM sodium borate buffer, pH 11.0 (b) Near UV-CD in the same buffers. Protein concentration was 1 mg/ml and the data shown is the average of three protein spectra corrected by the buffer blank.

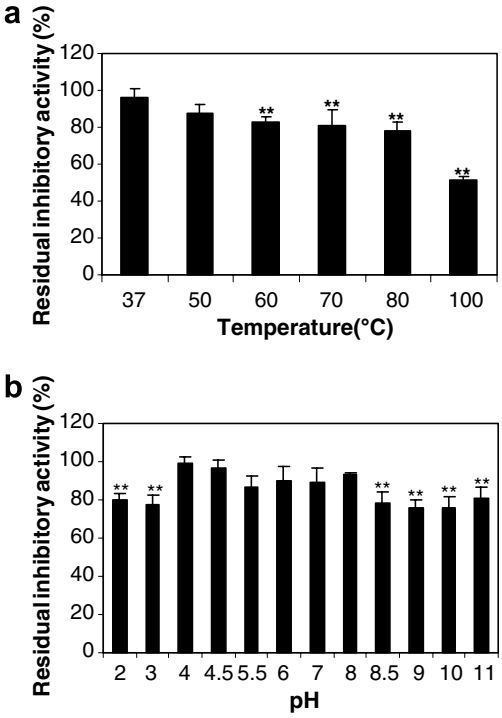

Fig. 5. Effect of temperature and pH on trypsin inhibitory activity of CSTI. (a) CSTI was submitted to various temperatures (37–100 °C) for 30 min and then cooled on ice. (b) CSTI was incubated with different pH buffers for 1 h at 37 °C. Residual anti-tryptic activity was measured using BAEE as a substrate and represented as the % of the inhibitory activity of untreated CSTI. Each point is the mean ± SE of three independent assays. Statistical analysis was done by one-way analysis with ANOVA post test Dunnet. $^{**}p < 0.01$.

activity due to protein denaturation. CSTI trypsin inhibitory activity seemed to be more stable than that of papaya inhibitor (PPI), where only 10% of the residual antitryptic activity remained after incubation for 10 min at 90 °C (Azarkan et al., 2006) but showed a similar stability to that of the trypsin inhibitor isolated from *P. dubium* (PDTI) seeds (Macedo et al., 2003). Even though these Kunitz-type trypsin inhibitors displayed a significant heat stability, Bowman-Birk inhibitors are usually more stable probably due to the fact that they consist of a cysteine-rich polypeptide chain (Richardson, 1977) bridged by seven conserved disulfide bonds (Singh and Appu Rao, 2002). These inhibitors have been reported to retain their full inhibitory activity after heat treatment at 90 °C for 10 min (Deshimaru et al., 2002; Haq et al., 2004).

Far-UV circular dichroism measurements (CD) were performed to evaluate changes in the inhibitor conformation due to the heat treatment. Far-UV CD at various temperatures and $f_{\text{denaturated}}$ as a function of temperature are shown in Fig. 6a and b, respectively. The thermal unfolding showed a transition midpoint at 68 °C and followed a two-state mechanism. The relatively sharp transition around 68 °C is another important feature that allowed CSTI classification into β-II proteins since compact and rigid proteins usually show a sharp transition upon thermal denaturation, whereas those of an unordered form change linearly when the temperature is increased (Wu et al., 1992). It should be noted that even though significant conformational changes were observed at temperatures above 68 °C, the trypsin inhibitory activity was quite stable up to 80 °C.

2.5.2. Effect of pH

Preincubation of the inhibitor in the pH range from 2.0 to 11.0 for 60 min at 37 °C showed a decrease of activity (25%) only at extremes pH (Fig. 5b). Although CSTI displayed a significant pH stability, it was less stable than other inhibitors such as PPI whose residual antitryptic activity was still higher than 95% despite an incubation period of 16 h at 25 °C in a pH range from 1.5 to 11.0 (Azarkan et al., 2006).

Although the inhibitory activity can be taken as indicative of the folding of the native protein, it was of interest to evaluate the possible changes in conformation of CSTI due to exposure of the protein to different pH environment, and with this objective far-UV CD, near UV-CD and fluorescence spectra measurements were used.

Analysis of the far-UV CD region with variable pH showed little changes in the secondary structure, since the spectra were practically undistinguishable from each other (Fig. 4a). This finding is remarkable in view of the two-chain nature of CSTI, which could have rendered it more susceptible to conformational changes than the one chain

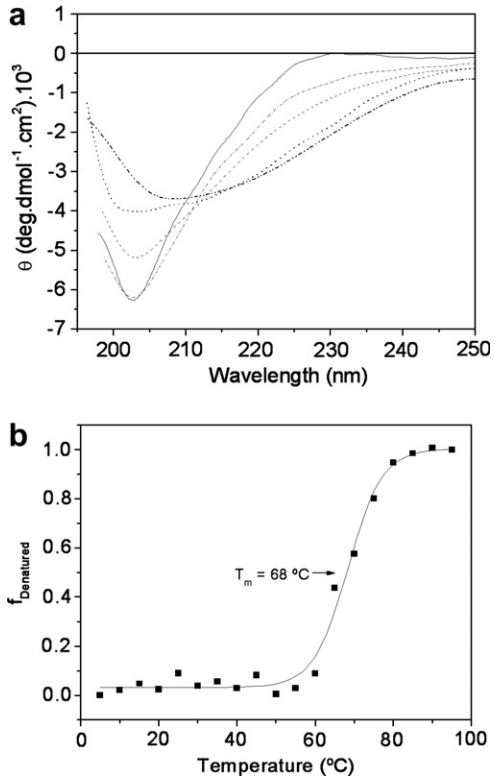

Fig. 6. Effect of temperature on CSTI conformation. (a) Far-UV circular dichroism spectra of CSTI at various temperatures and (b) $f_{\text{denaturated}}$ as a function of temperature.

Kunitz-type inhibitors. Near-UV spectra showed that the spectral pattern as well as the intensity of the shoulder at 293 nm remained almost unaltered at all pH values tested, although there was a decrease in the molar ellipticity in the region from 288 to 250 nm when CSTI was exposed to extreme pH (Fig. 4b).

The position of the maximum in the fluorescence emission spectra showed no difference associated to pH changes, although there was a noticeable quenching of the fluorescence at pH 3.0 and 11.0, suggesting a local change in the environment of the tryptophan residue due to the proximity of a quencher group (data not shown). Regarding the quenching observed at pH 11.0, it should be noted that the quenching of tryptophan emission above pH 8.5 is generally attributed to energy transfer implicating tyrosine residues and does not imply that denaturation occurs.

These differences are consistent with the small decrease of inhibitory activity observed after these treatments and could be indicative of a slight change in conformation.

2.5.3. Effect of dithiothreitol (DTT)

Disulfide bonds are believed to increase the stability of the native state of a protein leading to high resistance towards pH and heat treatment of trypsin inhibitors, which is more outstanding in Bowman-Birk type inhibitors than in Kunitz-type inhibitors. Thus, it was of interest to assess the effect of DTT reduction on the trypsin inhibitory activity and on the conformational features of CSTI.

Residual trypsin inhibitory activity was evaluated after incubation at two different DTT concentrations followed by carbamidomethylation. At 10 mM DTT, a rapid decrease of inhibitory activity was observed after 20 min of incubation at 37 °C since only 22.7% activity remained while at 1 mM DTT the activity observed was of 53.6%. Nevertheless the residual antitryptic activity reached a minimum of close to 12% for both DTT concentrations after 3 h incubation at 37 °C (Fig. 7a), showing the marked susceptibility of CSTI towards DTT treatment in contrast with the heat and pH treatment. This susceptibility could be due to the fact that the two polypeptide chains of CSTI should be linked by a disulfide bridge in order to retain the inhibitory activity. In fact, mass spectrometry analyses confirmed that both disulfide bridges were reduced and carbamidomethylated after 3 h incubation with 1 mM DTT followed by iodoacetamide treatment. These results show that CSTI is less stable than others Kunitz type inhibitors composed of a single chain such as PPI and PDTI, which conserved, respectively 67.5% and 40% of the residual inhibitory activity after 120 min incubation with 2.5 mM and 10 mM of DTT and iodoacetamide treatment, respectively (Macedo et al., 2003; Azarkan et al., 2006). In addition, *Erythrina caffra* trypsin inhibitor retains its native

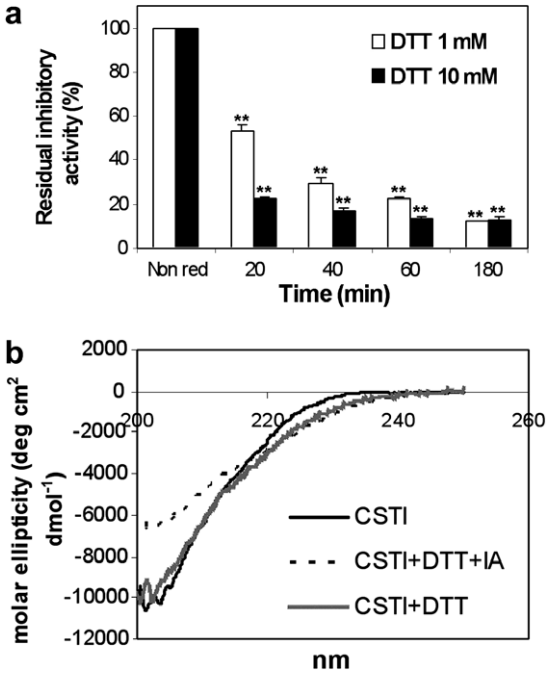

Fig. 7. Effect of disulfide bridge reduction on trypsin inhibitory activity and far-UV CD spectra of CSTI. (a) CSTI was incubated with 1 mM or 10 mM DTT for 20–180 min at 37 °C. Residual anti-tryptic activity was measured using BAEE as a substrate and represented as the % of the inhibitory activity of untreated CSTI. Each point is the mean ± SE of three independent assays. Statistical analysis was done by one-way analysis with ANOVA post test Dunnet. $^{**}p < 0.01$. (b) Spectra of native CSTI, CSTI in 1 mM DTT, 20 mM Tris–HCl buffer, pH 7.5, after 180 min incubation at 37 °C and CSTI after the same treatment followed by addition of 2 mM iodoacetamide and dialysis against 20 mM Tris–HCl buffer, pH 7.5.

structure and function after reduction of its disulfide bonds (Lehle et al., 1994). However, the loss of activity is more striking in the soybean trypsin inhibitor (SBTI) which, despite the fact of being an one chain inhibitor, is even more labile than CSTI (Azarkan et al., 2006). In SBTI, the disulfide bonds are known to be solvent exposed (Steiner, 1966; Tetenbaum and Miller, 2001). Using a new spectroscopic approach, it can be demonstrated that, although they are not located near the active site, disulfide bridges play a crucial role in stabilizing the protein structure, thus indicating that the accessibility of the disulfide bridges is another important factor. Hung et al. (1994) showed, by site-specific mutagenesis of *Acacia confusa* trypsin inhibitor (two-polypeptide chains), that the intrachain disulfide bridge located in the α chain is non-essential for inhibitory activity but the interchain disulfide bridge is essential.

To evaluate the conformational changes associated with reduction of disulfide bridges, CSTI was incubated with DTT 1 mM at 37 °C for 3 h and far-UV spectrum was measured, showing a slight decrease in the ellipticity at 230 nm which can be attributed to the chirality of the cysteine bonds (Fig. 7b). When the reduction was followed by carbamidomethylation and dialysis, the secondary structure was lost, as shown in the far-UV CD spectrum. These results suggest that, even after disulfide bridge rupture, the interaction between the two polypeptide chains remains strong enough to maintain a near native structure, and that the conformational changes become apparent only after carbamidomethylation, probably due to steric hindrance. These changes are consistent with the loss of inhibitory activity observed in these conditions.

3. Conclusions

CSTI is a two-chain protein belonging to the Kunitz-type family, devoid of lectin-like properties. Circular dichroism analyses, fluorescence spectroscopy and thermal denaturation studies clearly demonstrate that the protein is rich in beta-sheet structure and highly stable over a wide range of pH and temperature. Surprisingly, reduction of disulfide bridges did not produce considerable changes in secondary structure, as judged by circular dichroism, and only when reduction was followed by carbamidomethylation the molecule fell apart.

4. Experimental

4.1. Materials

Calliandra selloi MacBride seeds were manually collected from trees growing in Misiones, Argentina and were kindly provided by Dr. Teresa Argüelles y Andrés, from the Universidad Forestal of Misiones (Misiones, Argentina). Bovine serum albumin (BSA), bovine pancreatic trypsin, bovine pancreatic chymotrypsin, human plasma kallikrein, porcine pancreatic elastase, *N*-α-benzoyl-L-arginine ethylester (BAEE), *N*-benzoyl-L-tyrosine ethylester (BTEE), HD-Pro-Phe-Arg-pNa, N-Suc-Ala-Ala-Pro-Val-pNa, trypsin-agarose, DEAE-cellulose, RPMI medium, penicillin, streptomycin, glutamine, iodoacetamide, guanidine hydrochloride, 4-vinylpyridine, gel filtration markers and electrophoresis reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). SDS–PAGE molecular weight markers were provided by Pro-Jump (Buenos Aires, Argentina), dithiothreitol (DTT) and β-mercaptoethanol were provided by Promega (Madison, WI, USA) and Merck (Darmstadt, Germany), respectively. Pro-blot membranes for sequencing were purchased from Applied Biosystems (Foster City, CA, USA). Phastgel™ IEF 3–9) and pI markers were provided by GE Healthcare (Sweden).

4.2. Purification of Calliandra selloi trypsin inhibitor (CSTI)

C. selloi Macbride seeds, free of integument, were finely ground and extracted with 15 volumes (w/v) of 150 mM NaCl, 5 mM $CaCl_2$ for 18 h at 4 °C, followed by filtration and adjustment of the crude extract to pH 8.2 with Tris–HCl buffer plus addition of $CaCl_2$ up to 20 mM. A clear supernatant was obtained after centrifugation at 10,000 × g for 45 min at 4 °C and filtration with Whatman paper. This supernatant was applied to a trypsin-agarose column previously equilibrated with 20 mM Tris–HCl buffer, pH 8.2, 20 mM $CaCl_2$, 150 mM NaCl. The column was washed with the same buffer and eluted with 100 mM glycine–HCl buffer, pH 2.6, 150 mM NaCl. The elution profile was monitored at 280 nm, and fractions were collected, dialyzed against 20 mM Tris–HCl buffer, pH 8.2, 5 mM $CaCl_2$ and loaded on a DEAE cellulose column equilibrated in the same buffer. The column was washed with the equilibration buffer plus 60 mM NaCl and elution was performed with addition of 125 mM NaCl (isocratic elution). Fractions were collected and concentrated by ultrafiltration on Millipore tubes.

The purified fraction was submitted to reversed phase HPLC on a C_8 column. Elution was achieved with a linear gradient of 0–80% acetonitrile in 0.1% (v/v) TFA.

Protein concentrations were determined as described by Bradford (1976), using bovine serum albumin as standard.

Isoelectric focusing was performed loading 2 μg of protein on precast polyacrylamide gels in the pH range of 3.0–9.0, on a Phastsystem apparatus. Proteins were detected by staining with 0.1% Coomasie brilliant blue G-250.

4.3. Molecular weight determination

Molecular mass was determined on a LCQ Duo Spectrometer (Finnigan Corporation), equipped with electrospray ionization and ion trap analyzer.

Relative M_r was obtained by SDS–PAGE (12.5%) (Shagger and von Jagow, 1987). SDS–PAGE M_r standards

were bovine serum albumin (66 kDa), intracellular fragment of PTP-IA-2 (44 kDa), *B. licheniformis* β lactamase (32 kDa), eggwhite lysozyme (14 kDa) and bovine aprotinin (6 kDa). The proteins were detected by silver or Coomassie brilliant blue staining.

Native protein M_r was assessed by gel filtration on a Superdex 75 on an ÄKTA purifier system. The column was loaded with 100 μl of a 3 mg/ml protein solution in 150 mM NaCl, 5 mM $CaCl_2$. Chromatograms were run at a flow rate of 0.5 ml/min and the eluant was monitored at 280 nm. The column was calibrated with bovine serum albumin (66 kDa), ovoalbumin (45 kDa), bovine erythrocyte carbonic anhydrase (29 kDa), soybean trypsin inhibitor (22 kDa) and bovine heart cytochrome *c* (12 kDa) as standards. Blue dextran and sodium azide were used to determine the void volume and the total solvent-accessible column volume, respectively.

4.4. Cysteine modification with 4-vinylpyridine

Liophilized CSTI was denaturated with 6 M guanidine hydrochloride in 4 mM EDTA, 20 mM Tris–HCl buffer, pH 8.5 in the presence of 5 mg/ml DTT. After one hour incubation at room temperature, 1 μl 4-vinylpyridine was added and the mixture was incubated for another hour in the darkness. Then, HPLC separation was performed on a C_8 column, with a 2–96% CH_3CN gradient in 2% acetic acid and the eluate CH_3CN analyzed by mass spectrometry.

4.5. Protease inhibitory activity assays

Inhibitory activity was assayed by incubating different amounts of CSTI (final concentration ranging from 0 to 34 μM) with trypsin (final concentration 38 nM), chymotrypsin (final concentration 36 nM), kallikrein (final concentration 11 nM) and elastase (final concentration 0.11 nM). Activities were determined by using 1 ml of *N*-α-benzoyl-L-arginine ethylester (BAEE, final concentration 79.5 μM), *N*-benzoyl-L-tyrosine ethylester (BTEE, final concentration 87 μM), HD-Pro-Phe-Arg-pNa (final concentration 9 μM) and Suc-Ala-Ala-Pro-Val-pNa (final concentration 9 μM) as substrates, respectively. Increasing concentrations of CSTI (50 μl) were pre-incubated with the enzymes (50 μl) for 10 min at 37 °C in the assay buffer (20 mM Tris–HCl buffer, pH 8.2, containing 20 mM $CaCl_2$). After substrate addition, the reaction was monitored by the absorbance change at 253 nm (trypsin and chymotrypsin) or 405 nm (kallikrein and elastase) for 5 min at 37 °C. The dissociation constant (K_i) of the enzyme-inhibitor complex was determined according to Morrison (1982).

4.6. N-terminal amino acid sequence determination

Protein bands obtained by SDS–PAGE were electroblotted onto Pro-blot membranes and amino acid sequence analysis of the N-terminal region was performed by Edman degradation in an Applied Biosystem Model 477A Automatic Sequencer (Applied Biosystems), according to the manufacturers instructions.

4.7. Internal amino acid sequence determination

After reduction and alkylation with DTT and iodoacetamide, the alfa and beta chains were isolated by reversed phase chromatography on a C4 column (2.1 × 100 mm), eluted with a gradient of acetonitrile in 0.1% TFA and operated in SMART System from GE Healthcare Bio-Sciences, Uppsala, Sweden. For sequence analysis of the chains, the material was digested with trypsin (porcine, modified sequence grade Promega, Madison, WI, USA) or LysC protease (Achromobacter lyticus, Wako Chemicals, Osaka, Japan). The peptide maps were established by MS, and after lysine blockage by LysTAG (Peters et al., 2001) and sulfonation using the Ettan CAF reagent (GE Healtcare) (Hellman and Bhikhabhai, 2002) another MS was performed. Peptides that had increased their mass by 136 Da were candidates for easy sequencing by MALDI TOF/TOF. These were selected and amino acid analyses were performed by post source decay, using the LIFT procedure (Bruker proprietary).

The sequences were submitted to automatic alignment through the NBCI-Blast search system.

4.8. Biological activities

Rabbit erythrocytes were obtained by venous puncture, treated with trypsin, and fixed with glutaraldehyde as described by Nowak and Barondes (1975). Hemagglutinating activity was registered as described by Iglesias et al. (1996).

The viability of Nb2 rat lymphoma cells was evaluated using CellTiter 96 AQ_{ueos} non-radioactive cell proliferation assay (Promega Corp. Madison, WI, USA) as described by Troncoso et al. (2003). Different concentrations of CSTI (from 500 μg/ml to 5 μg/ml) were tested. Antifungal activity was determined according to Trindade et al. (2006) against the following fungal strains: *Aspergillus chevalier*, *A. fumigatus*, *A. niger*, *Colletotrichum gloeosporioides*, *C. lindemuthianum*, *C. musae*, *C.* sp. *F37*, *C.* sp. *P10*, *C. truncatum*, *Fusarium moniliforme*, *F. oxysporum*, *F. pollidoroseum*, *Mucor* sp, *Phomopsis* sp, *Pythium oligandrum*, *Thielaviopis paradoxa* and *Trichoderma viridae*.

4.9. Thermal and pH stability

The heat stability of the trypsin inhibitory activity of CSTI was determined by incubation of the CSTI solution (20 μg/ml in 150 mM NaCl, 5 mM $CaCl_2$) at various temperatures (37–100 °C) for 30 min in a water bath. After treatment, the aliquots were cooled on ice.

To measure the pH stability, a solution of trypsin inhibitor (500 μg/ml) was diluted with an equal volume of different buffers (100 mM): sodium citrate (pH 2.0–4.0), sodium

acetate (pH 4.5–5.5), sodium citrate (pH 6.0), Tris–HCl (pH 7.0–8.5), sodium bicarbonate (pH 9.0–10.0) and sodium borate (pH 11.0). After 1 h incubation in each buffer at 37 °C, the pH was adjusted to pH 8.2 by addition of 20 mM Tris–HCl buffer, pH 8.2, 20 mM $CaCl_2$ to achieve a final CSTI concentration of 20 μg/ml.

After these treatments, the residual inhibitory activity on trypsin was measured as described above.

4.10. DTT treatment

A solution of 1 mg/ml of CSTI in 20 mM Tris–HCl buffer, pH 8.2, 20 mM $CaCl_2$, was incubated with an equal volume of 1 or 10 mM DTT in the same buffer for time intervals ranging from 20 to 180 min at 37 °C. The reaction was terminated by adding iodoacetamide at twice the amount of each DTT concentration and the mixture was diluted with 20 mM Tris–HCl buffer, pH 8.2, 20 mM $CaCl_2$, in order to achieve a final CSTI concentration of 20 μg/ml. The residual inhibitory activity on trypsin was measured as described above.

4.11. Circular dichroism spectroscopy

Circular dichroism (CD) spectral measurements were performed on a JASCO J810 spectropolarimeter at a scan speed of 20 nm/min. The entire instrument, including the sample chamber, was constantly flushed with N_2 gas during the operation. CSTI concentration was 1 mg/ml for both far-UV CD (0.1 cm pathlength) and near UV-CD (1 cm pathlength) in neutral pH buffer (20 mM Tris–HCl buffer, pH 7.5) and extreme pH buffers (20 mM sodium citrate buffer, pH 3.0/20 mM sodium borate buffer, pH 11.0). Buffer scans were recorded under the same conditions and subtracted from the protein spectra before further analysis. The secondary structure analysis was performed by CD spectrum deconvolution using the Cluster program and CONTINLL method (Sreerama et al., 2000, 2001). For temperature stability studies, a protein sample (0.5 mg/ml in 20 mM Tris–HCl buffer, pH 7.5) was heated gradually in 5 °C steps, from 10 to 80 °C using a circulating bath TC-100. At each temperature, the protein was incubated for 5 min and the spectrum was recorded from 195 to 250 nm. To evaluate the effect of disulfide bridge reduction on conformation, far-UV CD spectra were determined in 1 mM DTT, 20 mM Tris–HCl buffer, pH 7.5, after 180 min incubation at 37 °C, either directly or after addition of 2 mM iodoacetamide followed by dialysis against the same buffer without DTT.

4.12. Fluorescence spectroscopy

Fluorescence measurements were made on a KONTRON SFM-25 fluorescence spectrophotometer, using a 1 cm pathlength rectangular quartz cuvette. Protein concentration was 200 μg/ml in the respective buffer (20 mM sodium citrate buffer, pH 3, 5 mM $CaCl_2$/20 mM Tris–HCl buffer, pH 7.5, 5 mM $CaCl_2$/20 mM sodium borate buffer, pH 11) The excitation wavelength was 295 nm and the emission spectra was recorded from 305 to 500 nm.

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