RESEARCH ARTICLE

Purification and structural stability of a trypsin inhibitor from Amazon *Inga cylindrica* [Vell.] Mart. seeds

Leonardo A. Calderon[1], Humberto A. Almeida Filho[2], Rozeni C. L. Teles[2], Francisco J. Medrano[3], Carlos Bloch Jr[4], Marcelo M. Santoro[5] and Sonia M. Freitas[2*]

[1] Centro de Estudos de Biomoléculas Aplicadas a Medicina, Núcleo de Saúde, Universidade Federal de Rondônia, UNIR. Porto Velho, RO. Brazil;

[2] Laboratório de Biofísica, Depto de Biologia Celular, Universidade de Brasília, UnB. Brasília, DF. Brazil;

[3] Laboratório de Genômica e Expressão, Depto de Genética e Evolução, Universidade de Campinas, UNICAMP, Campinas, SP. Brazil;

[4] Laboratório de Espectrometria de Massa, Embrapa Recursos Genéticos e Biotecnologia. Brasília, DF. Brazil;

[5] Laboratório de Físico-Química de Proteínas, Depto de Bioquímica e Imunologia, Universidade Federal de Minas Gerais, UFMG. Belo Horizonte, MG. Brazil.

* Corresponding author: Campus Universitário Darcy Ribeiro, Asa Norte. Brasília, DF. Brazil. CEP: 70910-900; Tel.: +55 61 33072192; Fax: +55 61 33498411; e-mail: nina@unb.br
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ABSTRACT

Inga cylindrica Trypsin Inhibitor (ICTI) was purified as a single polypeptide chain by one step anion-exchange chromatography from a crude extract of *Inga cylindrica* (Vell.) Mart. seeds. ICTI is a 19.5 kDa protein presenting a remarkable inhibitory activity against bovine trypsin (EC 3.4.21.4) (K_i = 4.3 nM). Circular dichroism analysis revealed that this inhibitor is a β type protein (40.4% of β-strand; 24.6% of β-turn and 6.7% of α-helix) in accordance with properties displayed in Kunitz type inhibitors. ICTI is a thermal stable protein within a wide range of pH (1.6 to 10.0) exhibiting highest stability at pH 7.0 as indicated by T_m of 70.0 °C and ΔG^{25} of 48.5 ± 0.7 kJ.mol^{-1}. The values of ΔG^{25} at pH 1.6 (22.5 ± 1.2 kJ.mol^{-1}) and pH 10.0 (31.5 ± 1.0 kJ.mol^{-1}) indicate a reduced structural stability of the protein under these conditions. This is likely to result from pK_a differences of the acid and basic side chains reflecting the changes in the non-covalent interactions in the folded state.

Key words: *Inga cylindrica* [Vell.] Mart; Leguminosae; Mimosoideae; protease inhibitor; protein stability; trypsin inhibitor.

INTRODUCTION

Plant protease inhibitors (PIs) are small proteins, generally present in high concentrations in storage tissues (up to 10% of protein content), and also detectable in leaves in response to the attack from insects and pathogenic microorganisms (Ryan 1990). PIs' contribution to plant defense mechanisms relies on inhibition of proteases present in insect gut or produced by microorganisms, causing a reduction in the availability of amino acids necessary for their growth and development (De Leo et al. 2002). PIs are considered to be part of an array of constitutive and inducible chemical resistence that protect plants against herbivore predators (Ryan 1990; Gatehouse et al. 1999; Jouanin et al. 2000), and/or act as storage protein for the plant embryo (Xavier-Filho 1992), as regulators of endogenous proteolytic activity (Ryan 1990), as well as participating in mechanisms of programmed plant cell death (Solomon et al. 1999).

PIs from Leguminosae seeds have been classified under either Kunitz type or Bowman-Birk type on the basis of molecular mass and disulfide bond patterns (Ikenaka and Norioka 1986; Oliva and Sampaio 2008). Members of the Kunitz family have

a molecular mass of approximately 18-26 kDa, with one or two polypeptide chains cross-linked by two or three disulfide bonds. These PIs usually have one reactive site, but recently, a secondary reactive site has been observed (Franco et al. 2002; Gomes et al. 2005; Teles et al. 2005). In contrast, members of the Bowman-Birk PI family are smaller (6 to 15 kDa) and often present a conserved pattern of five to seven disulfide bonds that confer a compact and very stable tertiary structure with two opposite reactive loops (Lawrence and Koundal 2002). These PIs interact reversibly with proteinases forming stoichiometric complexes and competitively influencing the catalytic activity (Radisky et al. 2004). PIs have been studied as model systems for elucidating proteinase inhibition mechanisms, as well as the study of protein-protein associations (Oliva and Sampaio 2008).

Bowman-Birk and Kunitz PIs have been long used in the development of transgenic plants by the expression of heterologous inhibitors in order to improve resistance against pests and pathogens that are responsible for severe impacts on agriculture production (Lopes et al. 2004; Zavala et al. 2004). Moreover, many of these PIs have often been used as an effective insecticide against several pest insect species, including dipterans as *Ceratitis capitata* (Gomes et al. 2005; Oliveira et al. 2007), lepidopterans such *Alabama argillacea* (Oliveira et al. 2007), *Anagasta kuehniella* (Macedo et al. 2003), *Heliothis zea* (Broadway and Duffey 1986), *Lucila cuprina* (Reed et al. 1999), *Manduca sexta* (Johnson et al. 1989), *Plodia interpunctella* (Oliveira et al. 2007), *Spodoptera litura* (Yeh et al. 1997), and coleopterans such *Anthonomus grandis* (Franco et al. 2003, 2004), *Callosobruchus maculatus* (Gomes et al. 2005; Oliveira et al. 2007), *Zabrotes subfasciatus* (Oliveira et al. 2007), and others (Araujo et al. 2005).

Beyond the well known role of seeds PIs in plant defense against herbivory, it was postulated that these proteins can regulate plant cell proteolysis by inhibition of endogenous proteases and thus control protein turnover and metabolism (Ryan 1989). In fact, it had already been demonstrated that some PIs play a crucial role in seed development (Sin et al. 2006).

In this paper, we report the purification and partial structural characterization of a PI with molecular mass of 19.5 kDa from Amazon *Inga cylindrica* seeds. *I. cylindrica* is a Leguminosae belonging to the Mimosoideae family. It is a species distributed from southeast Costa Rica to the Brazilian Amazon, as well as some central areas and coastal regions (Pennington 1997). Its fruit represent an important alimentary source for the Amazon primate population which in turn disperse the seeds (Andresen 2002). In this context, the purification and characterization of this new PI can also represent a first step in exploring some important aspects of seed development of this Amazon species.

MATERIAL AND METHODS

Purification of *Inga cylindrica* inhibitor: One hundred grams of seeds were crushed and stirred for 1 minute in acetone at –20ºC in order to remove its lipid content. A part of this material (ten grams of pulverized seeds) was homogenized in 100 mL of 150 mM NaCl and stirred for 16 hours, at 4ºC. The extract was centrifuged at 8.000 *g* for 30 min. The resulting supernatant was dialyzed against water and lyophilized. The purification of ICTI was performed by anion-exchange chromatography in DEAE-Celulose column (8.0 x 2.5 cm i.d.) equilibrated with 20 mM Tris-HCl buffer (pH 8.0). A sample of 100 mg of *I. cylindrica* crude extract eluted in 5 mL of 20 mM Tris-HCl buffer (pH 8.0) was applied into the column. Elution was carried out at a flow rate of 2.5 $mL.min^{-1}$ with increasing concentrations of NaCl (0 to 1.0 M). Fractions were pooled and assayed to inhibitory activity against bovine trypsin according to Erlanger et al. 1961.

Bovine protease inhibition assays: The inhibitory activities of the crude extracts and the purified ICTI against bovine trypsin (EC 3.4.21.4) and bovine α-chymotrypsin (EC 2.4.21.1) were performed as described by Erlanger et al. (1961) using the chromogenic substrates N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) and N-glutaryl-L-phenylalanine p-nitroanilide (GPNA), respectively. Briefly, 100 μL of ICTI at a concentration of 300 $\mu g.mL^{-1}$ in water were incubated with 100 mL of 0.8 $mg.mL^{-1}$ α-chymotrypsin or 100 mL of 0.64 $mg.mL^{-1}$ trypsin, at 25ºC for 15 minutes. After that, 500 mL of 0.43 $mg.mL^{-1}$ BAPNA in 50 mM Tris-HCl, 20 mM $CaCl_2$ buffer (pH 8.2) for trypsin assay or 0.40 $mg.mL^{-1}$ GPNA in 50 mM Tris-HCl, 20 mM $CaCl_2$ buffer (pH 7.6) for chymotrypsin assay were added. Following a reaction time of 10 minutes, 100 μL of 30% acetic acid was added in order to stop the reactions. The relative enzymatic activities were evaluated by the liberation of *p*-nitroanilide which was measured at 410 nm with a Hitachi U–1100 spectrophotometer. The residual activities of the enzymes, in the presence of the inhibitor were estimated considering the free enzyme activity to be 100%. The obtained values are an average of three independent measurements. Inhibition curves were obtained by plotting decreasing relative activities of the protease *versus* ICTI concentration. Inhibition constant of the enzyme-inhibitor

complex, K_i, was calculated from the fitted inhibition curve following the procedure described by Morrison, 1982, using the GRAFIT program version 3 (Erithacus software Ltd.).

Molecular mass determinations: Molecular mass of the proteins present in the crude extracts and purified ICTI were determined using a Voyager-DE STR from PerSeptive Biosystems. Samples were prepared for Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) analysis by mixing equal volumes (9 μL) of a protein solution (5 pmol) and 25 mg.mL^{-1} ferulic acid (*trans*-4-Hydroxy-3-methoxycinnamic acid) in acetone (matrix solution). The mixture was vigorously stirred for 30 seconds in a Vortex Genie 2. One μL of each solution was applied to a MALDI sample plate and allowed to dry at room temperature. The instrument was operated in linear mode with delayed extractions on. Ions were generated by irradiation with a nitrogen laser with fixed wavelength on 337 nm. Ions were accelerated with a voltage of 25 kV and delay of 500 ns. Signals were captured at 500 MHz and the obtained data was processed, using the GRAMS V.4.03 program and Galatic software. Spectra were obtained through internal calibration by using the Sequazyme molecular mass standards set out by PerSeptive Biosystems.

Number of polypeptide chains estimation by MALDI-TOF and electrophoresis: In order to determine the number of polypeptide chain content, samples were incubated with 50 mL of 50 mM NH_4HCO_3 and 1.5 mL of 50 mM dithiothreitol (DTT) for 30 minutes. After incubation, molecular mass was determined by MALDI-TOF Mass Spectrometry and differences in the molecular mass of the native and reduced protein were analyzed. The approximate molecular mass of ICTI was calculated using 13% SDS–PAGE (Laemmli 1970) in reducing conditions, employing molecular mass standards purchased from Sigma: bovine serum albumin (66,200 Da), ovalbumin (45,000 Da), glyceraldehyde 3-phosphate dehydrogenase (36,000 Da), carbonic anhydrase (29,000 Da), trypsinogen (24,000 Da), soybean trypsin inhibitor (20,100 Da) and α-alactoalbumin (14,200 Da). For protein detection, gels were stained with 0.01% Coomassie blue in MeOH/H_2O (4/6) and HOAc/H_2O (1/9).

Fluorescence emission analysis: For the emission fluorescence measurement at temperatures ranging from 25°C to 95°C, samples of protein in 20 mM MOPS buffer (pH 7.0), 20 mM sodium citrate buffer (pH 1.6), 20 mM sodium citrate buffer (pH 3.8) and 20 mM glycine-NaOH buffer (pH 10.0) were used. Emission spectra were recorded at 300-400 nm in a 1x1 cm length thermostated quartz cell with a Peltier-type temperature controller, after 295 nm excitation, with 5 nm bandwidth for both excitation and emission, using a JASCO FP-777 fluorescence spectrophotometer. The transition curves were obtained using centered 359 nm emission bands and the protein fraction present in the folded (f_N) and unfolded conformation (f_U). The f_U, equilibrium constants (Keq), and Gibbs free energy (ΔG) were calculated using the following equations:

$$f_U = (y_F - y) / (y_F - y_U) \quad \text{(Eq. 1)}$$

$$K\text{eq} = [U] / [N] = f_U / (1 - f_U) = (y_F - y) / (y - y_U) \quad \text{(Eq. 2)}$$

$$\Delta G = - RT \ln K\text{eq} = - RT \ln [(y_F - y) / (y - y_U)] \quad \text{(Eq. 3)}$$

where y_F and y_U represent the amount of y in the folded and unfolded states, respectively. These data were fitted according to Eq. 4 considering the van´t Hoff approximation (Eq. 5):

$$Yobs = \frac{(Yd + Md.T)\exp[(\Delta S/R)-(\Delta H/RT)] + (Yn + Mn.T)}{1 + \exp[(\Delta S/R)-(\Delta H/RT)} \quad \text{(Eq. 4)}$$

$$\text{Ln } Keq = (\Delta S / R) - (\Delta H / R).(1/T) \quad \text{(Eq. 5)}$$

where *Keq* is the experimentally observed equilibrium constant, T is the temperature in Kelvin (K), ΔH is the slope (the van'T Hoff change in enthalpy) and ΔS is the intersection from the linear regression (the change in entropy). In Eq. 4, these parameters have the same meaning. Additionally, *Yobs* is the experimentally observed spectroscopic data, *Yn* and *Mn* represent the intercept and slope of the pretransition straight line, respectively, whereas *Yd* and *Md* represent the intercept and slope of the posttransition straight line, respectively.

The correspondent stability at 25 °C (ΔG^{25}) was estimated from the Gibbs-Helmholtz equation (Eq. 6), which is the result of the approximation of the change in heat capacity value that accompanies protein unfolding (ΔC_p) to zero, seeing the values of $\Delta H_{(t)}$ and $\Delta S_{(t)}$ constant in the temperature range where unfolding occurs.

$$\Delta G_{(t)} = \Delta H_m (1 - T/T_m) \quad \text{(Eq. 6)}$$

The transition temperature T_m corresponding to the midpoint of the thermal unfolding curve was calculated from the ΔG *versus* T plot, for $\Delta G = 0$ ($\Delta G = 0 = \Delta H_m - T_m \Delta S_m$). The slope of this plot at T_m corresponds to ΔS_m and the enthalpy at T_m is $\Delta H_m = T_m \Delta S_m$ (Santoro, Bolen, 1992).

Circular dichroism spectrum analysis: Far-UV (190-260 nm) circular dichroism measurements were carried out on a JASCO J-815 (Jasco, Tokyo, Japan) spectropolarimeter equipped with a Peltier type temperature controller and thermostatized cuvette cell linked to a thermostatic bath. Spectra were recorded in 0.1 cm path length quartz cells at a protein concentration of 300 μg.mL^{-1} in 50 mM MOPS buffer (pH 7.0). Five consecutive scans were accumulated and the average spectrum was stored. The observed ellipticities were converted into the molar ellipticities [θ] based on a mean molecular mass per residue of 112 Da. Data was corrected for the baseline contribution of MOPS buffer.

Secondary structure contents were estimated from the CD curves adjustments using the program CDNN (Böhm, 1997), considering the database that resulted on total sum of secondary structures closest to 100%.

RESULTS AND DISCUSSION

Purification of *I. cylindrica* trypsin inhibitor (ICTI): The PI from the *Inga cylindrica* seed crude extract was identified by trypsin and chymotrypsin inhibitory activity assays. In order to purify the trypsin inhibitor, the dialyzed and lyophilized crude extract was submitted to an anion-exchange chromatography on DEAE-Cellulose. The elution with NaCl gradient yielded ten peaks related to protein fractions. The proteins presenting trypsin inhibitory activity was eluted in the fifth (non-retained), eighth (80 mM NaCl) and ninth (120 mM NaCl) fractions (Figure 1). The active fractions were pooled and analyzed by Coomassie Blue-stained SDS-PAGE and MALDI-TOF mass spectrometry. Analysis reveals that the fractions V and IX present proteins ranging from 6 to 20 kDa. The purification of the trypsin inhibitor was achieved in the fraction VIII, which presented a protein with a molecular mass of 19,465.19 Da $[M+H]^+$ (Figure 2). This fraction was pooled and analyzed by SDS-PAGE in the presence of the reducing agent. After Coomassie blue staining, a single band with an apparent molecular mass of 20 kDa was observed (Figure 2 inset), showing the presence of a single polypeptide chain. This result was confirmed by mass spectrometry analysis of the protein reduced with DTT. This protein was named *Inga cylindrica* Trypsin Inhibitor (ICTI).

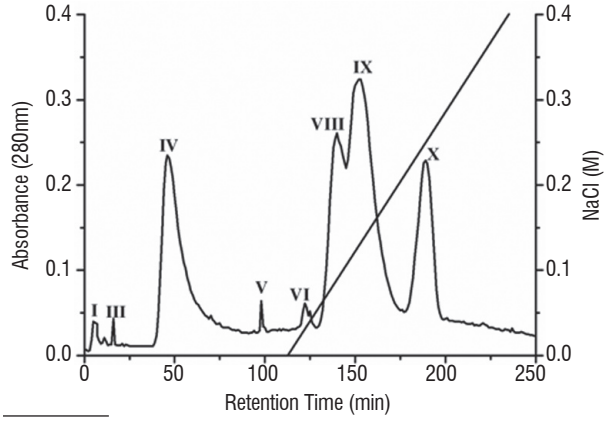

Figure 1. DEAE-Celulose ion exchange chromatography showing four mainly peaks (IV, VII, IX and X). The inhibitors were eluted with 20 mM Tris-HCl buffer (pH 8.0) in a crescent and linear salt gradient (0-1 M) NaCl. The peaks IV and VIII correspond to eluted proteins with inhibitory activity against bovine trypsin.

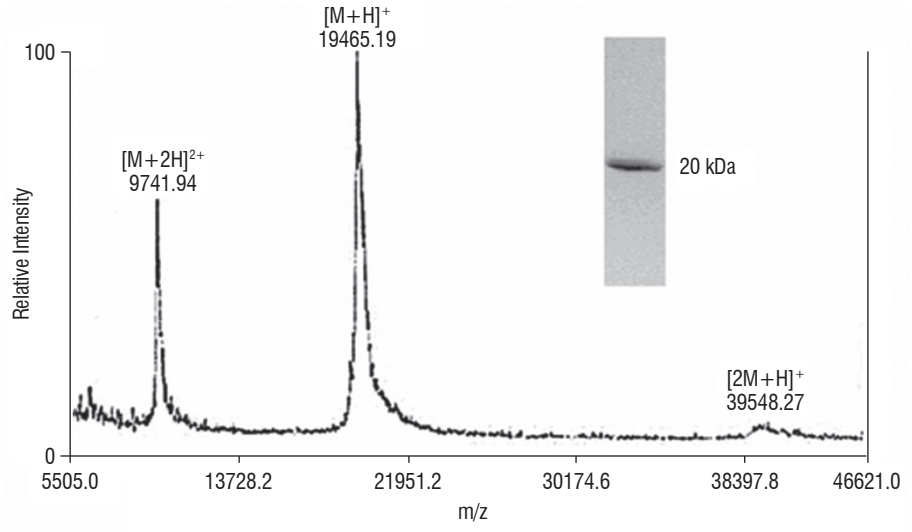

Figure 2. MALDI-TOF MS analysis performed on peak VIII sample (pure ICTI) resulted in a molecular mass value ($[M+H]^+$) value of 19,465.19, a $[M+2H]^{2+}$ of 9,741.94, and a $[2M+H]^+$ of 39,548.27. Fig. 2 inset, 13% SDS–PAGE analysis of ICTI. Protein band was stained with Coomassie blue R-250.

Inhibitory properties and dissociation constant determination: The inhibitory activity of ICTI against bovine trypsin (EC 3.4.21.4) and bovine chymotrypsin (EC 2.4.21.1) was determined by measuring the hydrolytic activity toward BAPNA and GPNA, respectively. The ICTI inhibited trypsin at a molar ratio of 1:1 but did not show any significant inhibition against α-chymotrypsin. The dissociation constant (K_i) value of ICTI was calculated using the inhibition curve according to the Morrison method. The dissociation constant (K_i) value was found to be 4.3 nM which clearly indicates that ICTI is a potent and competitive inhibitor of bovine trypsin (Figure 3).

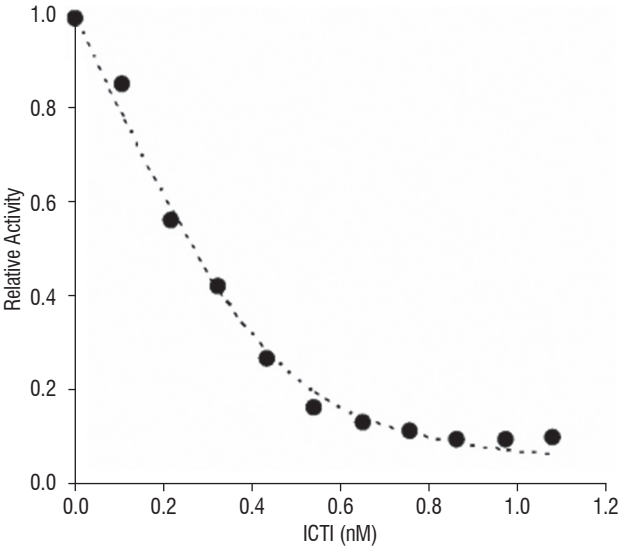

Figure 3. Inhibitory activity of ICTI against trypsin. Residual activities of trypsin were measured at 25 ºC with BAPNA at pH 8.2 (K_i = 4.3 nM).

Stability studies: Fluorescence studies at increasing temperatures demonstrated the thermal stability of ICTI structure. The fluorescence spectra were recorded at 300-400 nm with temperatures ranging from 25 to 95 ºC at different pHs (1.6, 3.8, 7.0 and 10.0). The fluorescence intensities at 359 nm were used to obtain the unfolding curves assuming a two state process (Figure 4). The thermodynamic parameters were obtained from the linear fitted curves in accordance with van't Hoff approximation (Figure 4 inset). The data suggest that the thermal stability of this PI is clearly dependent on pH. The ΔG^{25} value decreases in both extreme pHs, showing the major value at pH 7.0 with ΔG^{25} of 48.5 kJ.mol^{-1} (Table 1). This result is in accordance with the most cases of thermostable globular proteins with ΔG^{25} ranging from 21.0 to 63.0 kJ.mol^{-1} (Pace 1990).

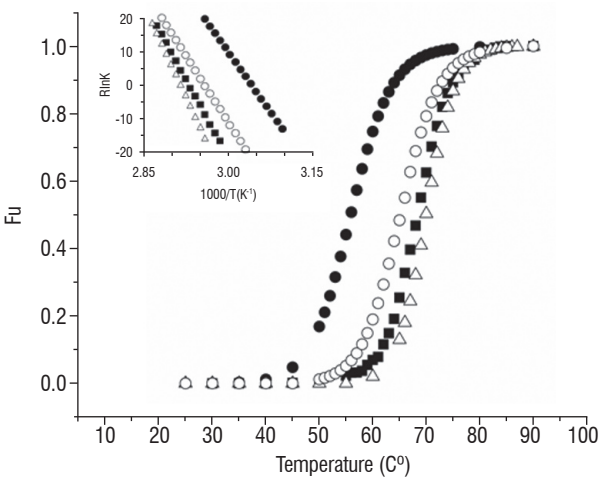

Figure 4. Heat-induced unfolding curves of ICTI according to Eq. 4 (see Materials and Methods) obtained at pH 1.6 (solid circles); 3.8 (solid squares); 7.0 (open triangles) e 10.0 (open circles). These data are calculated considering the decrease of fluorescence intensity at 359 nm. Fig. 4 inset, van't Hoff Plot (Eq. 5). The estimated thermodynamic parameters derived from these analysis are presented in Table 1.

Table 1. Thermodynamic parameters for the thermal unfolding of ICTI obtained from fluorescence measurement at different pH values.

pH	T_m (ºC)	ΔG^{25} (kJ.mol^{-1})	ΔH_m (kJ.mol^{-1})	ΔS_m (kJ.mol^{-1}.K^{-1})
1.6	55.9	22.5 ± 1.2	239.6 ± 9.8	0.728 ± 0.029
3.8	68.3	38.7 ± 0.3	305.0 ± 2.2	0.893 ± 0.006
7.0	70.0	48.5 ± 0.7	369.7 ± 12.0	1.074 ± 0.035
10.0	65.1	31.5 ± 1.0	265.3 ± 9.8	0.784 ± 0.029

CD spectroscopy: Far-UV CD spectroscopy study (250-190 nm wavelength range) was carried out in order to estimate the secondary structure content. The ajusted CD spectrum of the native ICTI with a negative band at approximately 201 nm (Figure 5) showed that this PI is a β protein type composed by 40.4% of β-strand, 24.6% β-turn, 33% of unordered and 6.7% of α-helix secondary structures. This results are in accordance with most trypsin inhibitors particularly the Kunitz type inhibitors that are predominantly structurally organized in β-strand with little helical content (Azarkan et al. 2006; Chaudhary et al. 2008).

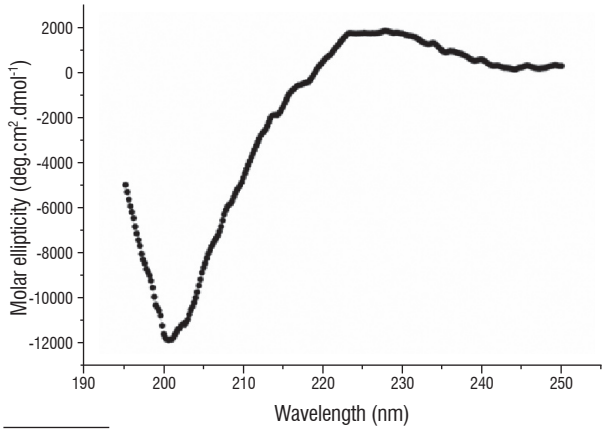

Figure 5. Far-UV CD spectrum of the ICTI. The deconvoluted spectrum results in a predominance of β-strand (40.4%) and unordered secondary structure (33%) presenting a low content of α-helix structure (6.7%).

CONCLUSIONS

Species-rich genera such as the *Inga* (Leguminosae – Mimosoideae), which is composed of 300 species is one important characteristic of the Amazon biodiversity (Richardson et al. 2001; Bermingham and Dick 2001). Amazonian leguminosae seeds are an under explored source of new PIs, with a few number of species researched (Calderon 2004). To date, four PIs have been described from *Inga* seeds (i.e. IUCI-1, IUCI-2, IUCTI from *I. umbratica* and ILTI from *I. laurina*) (Calderon et al. 2001, 2005; Macedo et al. 2007). Results show a new PI named *Inga cylindrica Trypsin Inhibitor* (ICTI) that is a single thermal stable polypeptide chain with a molecular mass of 19.5 kDa, which differs from the majority of other Kunitz inhibitors purified from the Mimosoideae species that have two polypeptide chains. ICTI displayed a remarkable thermal stability at pH 7.0 as indicated by T_m of 70.0 °C and ΔG^{25} of 48.5 kJ.mol^{-1}. CD analyses of ICTI revealed that it is an β-strand type protein in accordance with well-characterized Kunitz type PIs. The K_i value of 4.3 nM demonstrated a high affinity between bovine trypsin (EC 3.4.21.4). However, in order to place ICTI under the Kunitz family of PIs possessing a single disulfide bridge and its role in seed development, complete amino acid sequencing of the inhibitor and further experiments need to be carried out.

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