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A trypsin inhibitor purified from *Cassia leiandra* seeds has insecticidal activity against *Aedes aegypti*

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ABSTRACT

A trypsin inhibitor from *Cassia leiandra* seeds, named *Cl*TI, was purified, characterized, and its insecticidal activity against *Aedes aegypti* evaluated. *Cl*TI was purified by DEAE-Cellulose and trypsin-Sepharose 4B chromatography, with a 15.5-fold purification and 2.4% yield. *Cl*TI is composed of a 19,484 Da polypeptide chain as revealed by mass spectrometry, it is not a glycoprotein, its amino acid sequence is similar to other Kunitz-type inhibitors, and it comprises 35% β-sheets, 14% β-turns, and 50% disordered secondary structures. *Cl*TI is an uncompetitive inhibitor of bovine trypsin (IC_{50} of 33.81×10^{-8} M, *Ki* of 6.25×10^{-8} M) stable over a broad range of pHs (2.2–10.0) and temperatures (30–70 °C), but dithiothreitol led to a partial loss of the inhibitory activity. *Cl*TI, at 4.65×10^{-6} M, reduced in 50% the activity of the *Ae. aegypti* midgut proteases. *Cl*TI also promoted acute toxicity on the 3rd instar larvae of *Ae. aegypti*, with an LC_{50} of 2.28×10^{-2} M. Moreover, it caused a 24-h delay of the larvae development and 44% mortality after ten days of exposure. Altogether, these results suggest that *Cl*TI has potential as a natural compound to control *Ae. aegypti*, a vector of several infection diseases.

1. Introduction

The World Health Organization considers dengue virus infection one of the most important public health problems. Studies estimate that the disease affects approximately 390 million people annually and more than 90 million of those infected with the virus developed severe symptoms. To further aggravate the problem, approximately 3.9 billion people in 128 countries live in risk areas of dengue infection [1,2]. *Aedes aegypti* (Diptera: Culicidae), a blood-sucking insect that lives and cohabits with the human population in tropical and subtropical areas, is the principal vector responsible for dengue virus transmission [3]. *Ae. aegypti* is also the main transmitter of Chikungunya and Zika viruses. Chikungunya has been identified in more than 60 countries, its symptoms are sometimes milder than those of dengue, and rarely cause death. Zika virus has been reported in 65 countries, and it was estimated that by the end of 2016, approximately 4 million people in the Americas were infected, with 1.5 million cases in Brazil. Presently, the major concern is the association of microcephaly and Guillain-Barré syndrome with Zika virus infection [2]. As there are no antiviral drugs or vaccines available to prevent virus infection and specific medicine to treat infected patients, the main strategy for the disease control is to combat the vector through improvement of basic sanitation and environmental practices to eliminate all sites where mosquitoes can lay eggs [4]. To eliminate dengue mosquitoes, chemical control with insecticides (organophosphates, carbamates, and pyrethroids) has been widely used. However, prolonged and indiscriminate application of these compounds has favored the appearance of resistant insects [5]. This concern has driven scientists to search for natural compounds with insecticidal activity, and plants are potential sources of these constituents. Indeed, numerous extracts and various compounds isolated from different plant parts have shown activity against *Ae. aegypti* [6–8].

Several plant proteins, including protease inhibitors (PIs), are among these compounds with insecticidal activity. PIs are proteins or peptides that inhibit the catalytic action of proteases by forming

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stoichiometric complexes with their target enzymes, blocking or altering the active site [9]. Such inhibition mechanism has been observed with digestive proteases of some insects leading to decreased or complete interruption of the dietary protein digestion, which is essential to make the amino acids available for larval growth and development [10]. Plant protease inhibitors (PPIs) are present in storage tissues as seeds and tubers and also in leaves, flowers, and fruits [11,12], where they function as storage proteins and/or endogenous regulators of proteolytic activity [13]. Additionally, PPIs are involved in programmed cell death [14] and other events related to the protection of plants against pests and pathogens [15]. These PPIs can be categorized as serine, cysteine, aspartic, and metalloproteinase inhibitors, according to their target proteases [16]. PPIs have also been grouped into families according to their sequence relationships and structural properties [17,18]. The Kunitz-type PPI family is one of the most studied and characterized. This inhibitor family generally consists of a single polypeptide chain with a molecular mass in the range of 18–22 kDa, containing two intrachain disulfide bridges and a single reactive site [19].

Several Kunitz inhibitors purified from legume seeds have insecticidal activity. Their mode of action involves reduction of the hydrolytic processes of the dietary proteins in the insect gut, which diminishes the amino acid availability, particularly those essentials for the larval development [20–22]. Indeed, PPIs do inhibit *in vitro* the hydrolytic activity of *Ae. aegypti* midgut proteases [23]. This present study reports the purification and biochemical characterization of *Cl*TI, a trypsin inhibitor from the seeds of *Cassia leiandra* Benth. (Fabaceae, Caesalpinioideae), a plant species native to the Amazon rainforest. In addition, the *Cl*TI action on *Ae. aegypti* midgut proteases and its effect on the development and survival of this mosquito are assessed. The results suggest that *Cl*TI is a strong candidate as a biologically active molecule to control *Ae. aegypti* and could be potentially exploited for future biotechnological applications.

2. Materials and methods

2.1. General

C. leiandra mature seeds were collected at the campus of the Federal University of Mato Grosso do Sul (UFMS), Ivinhema City, Mato Grosso do Sul State, Brazil (Authorization and Information System on Biodiversity – SISBIO # 47978-1). The plant species was identified by Professor Almeida G. M. (UFMS). *Ae. aegypti* (Rockefeller strain) larvae and eggs were obtained from NUVET/SESA (Center for Vector Control – Healthy Secretary of Ceara, Brazil). Molecular mass markers, chromatographic matrices and immobilized pH gradient gel strips were obtained from GE Healthcare Life Science (New York, USA). All other chemicals were purchased from Sigma–Aldrich Co. (St. Louis, USA).

2.2. Protein determination

The total protein concentration was estimated following the method described by Bradford [24]. BSA (bovine serum albumin) was used as a protein standard.

2.3. Trypsin inhibitory activity assay

This was performed according to Erlanger et al. [25] by measuring the residual hydrolytic activity of bovine trypsin on BApNA (Nα-benzoyl-DL-arginine-*p*-nitroanilide). Different sample concentrations, dissolved in 0.05 M Tris–HCl, pH 8.0, were incubated with 20 μL trypsin (0.3 mg mL^{-1} in 0.001 M HCl) at 37 °C for 10 min. Then, 500 μL of 1.25×10^{-3} M BApNA (prepared in 100% dimethyl sulfoxide and 0.05 M Tris–HCl buffer, pH 8.0) was added to the mixture. After 15 min incubation, the reaction was stopped by adding 250 μL of 30% (v/v) glacial acetic acid. BApNA hydrolysis was monitored at 410 nm. One trypsin inhibitor activity unit (TIU) was defined as a decrease of 0.01 units of absorbance at 410 nm in relation to the control sample (without inhibitor).

2.4. Purification of ClTI

C. leiandra mature seeds were ground in a coffee mill to obtain a fine flour, which was defatted with *n*-hexane (1:10, m/v). The defatted flour was air-dried at room temperature (23 ± 2 °C) and stored at −20 °C until use. For protein extraction, the fine flour was brought into contact with 0.05 M sodium phosphate buffer, pH 7.5 (1:10, m/v) and was subjected to moderate stirring (3 h, 4 °C). The suspension was filtered through a fine-screen cloth, and the filtrate was centrifuged at 10,000 × *g* for 30 min at 4 °C. The precipitate was discarded, the supernatant was dialyzed against 0.05 M sodium phosphate buffer, pH 7.5, recovered, and designated as SPE (soluble protein extract). Ten milliliters of SPE (80 mg protein) was loaded on a DEAE-Cellulose column (1.5 cm × 16 cm) equilibrated with 0.05 M sodium phosphate buffer, pH 7.5, and the chromatography carried out at 45 mL h^{-1} flow rate. The non-retained proteins were eluted with the equilibration buffer and the fraction with trypsin inhibitory activity was eluted with 0.1 M NaCl prepared in the equilibration buffer. Fractions of 2 mL were collected and the absorbance was read at 280 nm (Spectrophotometer Biochrom Libra S21, Cambridge, England). The fractions with anti-trypsin activity were pooled, dialyzed against distilled water at 4 °C, and freeze-dried. The lyophilized sample (10 mg) was dissolved in 2.0 mL of 0.05 M sodium phosphate buffer, pH 7.5, containing 0.2 M NaCl. After centrifugation (10,000 × *g*, 4 °C, 15 min), the resulting supernatant was applied to a trypsin-Sepharose 4B column (2.5 cm × 5 cm) that was pre-equilibrated with 0.05 M sodium phosphate buffer, pH 7.5, containing 0.2 M NaCl. Chromatography was developed at 45 mL h^{-1} flow rate, 2 mL protein fractions were collected, and the absorbance readings taken at 280 nm, as above. The non-retained proteins were eluted with the equilibration buffer. The fractions possessing trypsin inhibitor activity were eluted with 0.1 M HCl, pooled, dialyzed against distilled water at 4 °C, and lyophilized for further analysis. The purified trypsin inhibitor was named *Cl*TI (*C. leiandra* trypsin inhibitor).

2.5. Characterization of ClTI

2.5.1. Purity and molecular mass determination

*Cl*TI was subjected to PAGE (polyacrylamide gel electrophoresis) in the absence (native-PAGE) and presence of sodium dodecyl sulfate (SDS-PAGE) in a vertical system [26]. The samples (10 μg) were heated at 100 °C for 10 min in the presence or absence of 5% (v/v) β-mercaptoethanol and loaded on a 12.5% (m/v) polyacrylamide gel (8.5 cm × 8.0 cm) that was prepared in 0.025 M Tris–HCl buffer, pH 8.9, containing 1% (m/v) SDS (only in SDS-PAGE), at 20 mA constant current. Protein bands were stained with 0.1% (m/v) Coomassie Brilliant Blue R-250. Phosphorylase B (97 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), and lactalbumin (14.2 kDa) were used as molecular mass markers.

To determine the native molecular mass, 1 mg *Cl*TI was dissolved in 0.05 M sodium phosphate buffer, pH 7.5, containing 0.4 M NaCl. After centrifugation (12,000 × *g*, 15 min, room temperature), the supernatant was loaded on a HiLoad® 16/600 Superdex® 75 column (1.6 cm × 60 cm), pre-equilibrated with the same buffer, and the protein eluted at a 0.5 mL min^{-1} flow rate. The elution profile was monitored at 280 nm. Alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), trypsinogen (24 kDa), and cytochrome c (12.4 kDa) were used as molecular mass standards.

The molecular mass of *Cl*TI (1 mg mL^{-1} in water/acetonitrile [1:1, v/v]) was also determined using a Synapt G1 HDMS Acquity UPLC instrument (Waters Co.). The spectrometer was operated in the 'V'

mode at a minimum resolution of 12,000 *m/z*. The analysis was performed using nanoelectrospray ionization in positive ion mode (ESI+) and a NanoLockSpray source. Data were collected, processed, and analyzed using MassLynx 4.1 and 2.4 ProteinLynx software (Waters Co.).

2.5.2. Reverse zymography

Visualization of the in-gel inhibition profile of *Cl*TI was performed according to Prasad et al. [27]. *Cl*TI (50 μg) was applied to 12.5% (m/v) SDS-PAGE containing 0.1% (m/v) gelatin. After the electrophoretic run, the gel was incubated in 2.5% (v/v) Triton X-100 for 20 min at 37 °C, washed with distilled water, and incubated with a trypsin solution (0.06 mg mL^{-1} in 0.05 M Tris–HCl buffer, pH 7.5, containing 0.02 M $CaCl_2$) for 2 h at 37 °C. Staining was performed with a solution containing 0.1% (m/v) Coomassie Brilliant Blue R-250 in methanol, acetic acid, and water (4:1:5, v/v/v) for 4 h. Excess dye was removed from the gel using a solution of methanol, acetic acid, and water (4:1:5, v/v/v). The commercial SBTI (Kunitz soybean trypsin inhibitor, Sigma) was also analyzed under the same conditions.

2.5.3. N-terminal amino acid sequencing

N-terminal amino acid sequencing of *Cl*TI was performed on a PPSQ 23A automated protein sequencer (Shimadzu) in which the phenylthiohydantoin-amino acid derivatives were detected at 269 nm after separation on a reversed-phase C18 column (4.6 mm × 2.5 mm) under isocratic conditions according to the manufacturer's instructions. The amino acid sequence was subjected to automatic alignment using the NCBI-BLAST system [28].

2.5.4. Mass spectrometry analysis

Capillary liquid chromatography/nanoelectrospray ionization tandem mass spectrometry (LC–ESI–MS/MS) was performed using a Synapt G1 HDMS Q-ToF mass spectrometer (Waters Co.) coupled to a Waters ultra-high-performance liquid chromatography (UPLC) unit. *Cl*TI (50 μg) was digested with 1 μg trypsin (Promega®) at 37 °C for 16 h. The tryptic peptides were separated using a nanoACQUITY UPLC System (Waters) equipped with a C18 HSS T3 reversed-phase column. After elution, the peptides were ionized in a nanoESI source through a one-spray cone-shaped 22-μm silica capillary. The spectrometer was operated under the same conditions described in Section 2.5.1. The data were processed using Protein Lynx Global Server software (Waters Co.) and subjected to a database search using the Mascot search engine [29]. The searches were performed with the assumptions that there was a maximum of one missed trypsin cleavage and that the experimental masses of the peptides were monoisotopic. Cysteine carbamidomethylation and methionine oxidation were set as fixed and variable modifications, respectively. MS/MS ion searches were performed against the NCBI non-redundant database (last accessed on September 04, 2016) using a significance threshold of $P < 0.05$. The peptide mass tolerance and fragment mass tolerance were both initially set to ± 0.1 Da for MS/MS ion searching. Candidate peptide IDs were only accepted if the *m/z* values were within 0.1 Da (typically less than 0.05 Da) of the theoretical mass of the candidate ID, as determined by manually reviewing the MASCOT search results.

2.5.5. Circular dichroism (CD) measurement

CD spectra measurements were recorded on a JASCO J-715 spectropolarimeter (Jasco Instruments) under nitrogen atmosphere at 25 °C. *Cl*TI (50 μg) was solubilized in 500 μL 0.01 M sodium phosphate buffer, pH 7.5, and subjected to analysis. Measurements were recorded as an average of eight successive scans performed at a 20 nm min^{-1} scan rate, with 4 s response time, in the 190–250 nm region using a circular quartz cuvette with a 1-mm optical path. Three methods were used to quantify the secondary structure elements using CDPro software (CDSSTR CONTINLL, and SELCON3). CD spectroscopy was also used to assess the *Cl*TI thermal stability. *Cl*TI (200 μg mL^{-1} in 0.01 M sodium phosphate buffer, pH 7.5) was incubated at temperatures ranging from 25 to 90 °C for 20 min in a TC-100 circulating water bath (Jasco Instruments), and the spectra were recorded using the above parameters. To evaluate the structural stability as a function of pH, *Cl*TI (200 μg mL^{-1}) was incubated for 30 min in 0.02 M acetate/phosphate/borate buffer at different pH values (2.5, 4.0, 6.0, 8.0, and 10.0) at room temperature (23 ± 2 °C) and the CD spectra recorded.

2.5.6. Carbohydrate determination

The glycoprotein nature of *Cl*TI was evaluated after SDS-PAGE and periodic acid-Schiff staining, as previously described [30]. Briefly, *Cl*TI (20 μg) electrophoresis was performed in SDS-PAGE, as above, and was then fixed in 7.5% (v/v) acetic acid solution for 2 h, immersed in 0.2% (v/v) periodic acid solution at 4 °C for 45 min, followed by immersion in the Schiff reagent at 4 °C for 45 min. To reveal the glycoprotein nature of the protein, the gel was immersed in a 0.5% (m/v) potassium metabisulfite solution prepared in 0.05 M HCl.

2.5.7. IC_{50} and kinetic analysis

The *Cl*TI concentration capable of reducing 50% of the trypsin activity (IC_{50}) was determined, as described in Section 2.3, using the inhibitor in the concentration range of 45.6×10^{-8} M to 4.5×10^{-8} M. The kinetic measurements of trypsin inhibition by *Cl*TI were conducted according to Costa et al. [31]. *Cl*TI was prepared in 0.05 M sodium phosphate buffer, pH 7.5, at different concentrations (9.12×10^{-8} M, 18.24×10^{-8} M, and 27.36×10^{-8} M) and was incubated with 20 μL trypsin (0.3 mg mL^{-1} in 0.001 M HCl) at 37 °C. The reaction was initiated by adding 500 μL of BApNA at different concentrations (0.8×10^{-3} M to 1.6×10^{-3} M) and was stopped after 15 min by the addition of 120 μL 30% (v/v) acetic acid. The liberated *p*-nitroaniline was measured at 410 nm. A Lineweaver–Burk plot was obtained by the reciprocal of the rate of the enzyme reaction ($1/v$) *versus* the reciprocal of the substrate concentration ($1/[S]$) in the absence and presence of *Cl*TI. The inhibition constant (*Ki*) was determined according to Dixon [32]. *Ki* was obtained by the intersection of the three lines at the x-axis, corresponding to the substrate concentrations (0.40×10^{-3} M, 0.80×10^{-3} M, and 1.25×10^{-3} M).

2.5.8. Enzyme specificity

The ability of *Cl*TI to inhibit chymotrypsin was evaluated according to Erlanger et al. [25] using azocasein as the substrate. Twenty microliters of bovine chymotrypsin (0.1 mg mL^{-1} in 0.05 M Tris–HCl buffer, pH 7.5, containing 0.02 M $CaCl_2$) was incubated with 100 μL of *Cl*TI (10 μg mL^{-1}) for 15 min at 37 °C. A 200 μL aliquot of 1% (m/v) azocasein in 0.05 M Tris–HCl buffer, pH 7.5, was added, and after 30 min, the reaction was stopped by adding 300 μL of 20% (v/v) trichloroacetic acid. The mixture was centrifuged (10,000 × *g*, 10 min), aliquots were withdrawn from the supernatants, 2 M NaOH was added at a ratio of 1:1 (v/v) to intensify the color of the cleavage product, and the absorbance was measured at 440 nm. The papain inhibitory assay was performed as described by Abe et al. [33] using BANA (*N*α-benzoyl-DL-arginine β-naphthylamide hydrochloride) as the substrate. To 60 μL of papain solution (0.02 mg mL^{-1} in 0.25 M sodium phosphate buffer, pH 6.0), 40 μL of the activating solution (0.25 M sodium phosphate buffer, pH 6.0), 2×10^{-3} M EDTA (ethylenediaminetetraacetic acid), 3×10^{-3} M DTT (dithiothreitol), 200 μL 0.25 M sodium phosphate buffer, pH 6.0, and 200 μL of *Cl*TI (10 μg mL^{-1}) were added. The mixture was incubated at 37 °C for 10 min, and 200 μL of 1×10^{-3} M BANA (solubilized in dimethyl sulfoxide and 0.25 M sodium phosphate buffer, pH 6.0) were added. After 20 min, the reaction was stopped by the addition of 500 μL 2% (v/v) HCl in 95% (v/v) ethanol. Next, 500 μL of 0.06% (m/v) DMACA (4-[dimethylamino]-cinnamaldehyde), dissolved in 95% (v/v) ethanol were added and incubated for 30 min, and the absorbance was measured at 540 nm. The porcine pancreatic α-amylase inhibition assay was performed using DNS (3,5-dinitrosalicylic acid), as previously described [34]. The

enzyme (1.0 mg) was solubilized in 0.02 M sodium phosphate buffer, pH 6.9, containing 0.006 M NaCl (0.1 mg mL^{-1}). The reaction medium was prepared by combining 20 µL of α-amylase, 60 µL of *Cl*TI (0.2 mg mL^{-1}), and 170 µL of the above buffer. This mixture was preincubated at 37 °C for 10 min and the reaction initiated by the addition of 250 µL of 1% (m/v) starch solution. After 15 min the reaction was stopped by the addition of 500 µL of DNS (1% in 1 M NaOH and 25% sodium potassium tartrate). The mixture was boiled in a water bath for 10 min, diluted with 2.5 mL of distilled water, cooled to room temperature (23 ± 2 °C), and the absorbance readings taken at 540 nm. In all assays, the residual inhibitory activity in the presence of *Cl*TI was calculated in relation to the fully enzyme activity (100%) achieved in the absence of *Cl*TI.

2.5.9. Stability of the inhibitory activity against bovine trypsin

The thermal stability of *Cl*TI was evaluated according to Jamal et al. [35]. *Cl*TI (6 µg mL^{-1}) was dissolved in 0.05 M sodium phosphate buffer, pH 7.5, and 500 µL aliquots were incubated in a water bath at different temperatures (30, 40, 50, 60, 70, 80, 90, and 100 °C) for 20 min. The samples were cooled to room temperature (23 ± 2 °C) before testing the residual inhibitory activity. The *Cl*TI stability at different pH was evaluated according to Klomklao et al. [36]. *Cl*TI (6 µg mL^{-1}) was dissolved in different buffers: 0.05 M glycine–HCl, pH 2.2; 0.05 M sodium acetate, pH 5.2; 0.1 M sodium phosphate, pH 7.8; 0.1 M Tris–HCl, pH 8.0, or 0.1 M borate, pH 10.0. After 30 min incubation in each buffer at room temperature (23 ± 2 °C), the residual trypsin inhibitory activity was evaluated, as previously described. The effect of reducing agent on the activity of the trypsin inhibitor was evaluated according to Bezerra et al. [6]. *Cl*TI samples (6 mg mL^{-1}) were incubated with different DTT concentrations (0.001–0.1 M) for different amounts of time (15, 30, 60, and 120 min). The reaction was terminated by adding iodoacetamide at twice the DTT concentration, and the residual inhibitory activity was determined.

2.6. Activity of ClTI against Ae. aegypti

2.6.1. Mosquitoes

Ae. aegypti larvae and eggs were maintained at 27 ± 2 °C, 70 ± 10% relative humidity, 12-h light and 12-h dark photoperiod, and were fed a diet for turtles (ReptoLife Alcon Club®). Third instar larvae were collected with a Pasteur pipette and transferred to a beaker containing distilled water until the bioassays were conducted.

2.6.2. Inhibition assay of Ae. aegypti gut proteases

Homogenates of the larval midgut were prepared according to Almeida Filho et al. [37], with minor modifications. For the assay, *Ae. aegypti* larvae were cold-immobilized, and their midguts were surgically removed, macerated in 0.05 M Tris–HCl buffer, pH 8.0, containing 0.02 M $CaCl_2$, and centrifuged at 10,000 × *g* for 20 min at 4 °C. The supernatants were used immediately as the source of proteases. Aliquots (60 µL) of the supernatant were incubated with 100 µL of *Cl*TI (100 µg mL^{-1}) and 640 µL of 0.05 M Tris–HCl buffer, pH 8.0, containing 0.02 M $CaCl_2$ for 10 min at 37 °C, and then 500 µL of 1.25×10^{-3} M BApNA was added. After 15 min, the reaction was stopped by adding 120 µL of 30% (v/v) acetic acid, and the absorbance was measured at 410 nm. The activity of midgut samples without *Cl*TI was considered as 100% activity. To determine the *Cl*TI concentration required to reduce 50% (IC_{50}) the activity of the intestinal proteases of *Ae. aegypti*, the assay was performed under the same conditions described above, using increasing concentrations of *Cl*TI (25–175 µg mL^{-1} or 1.28×10^{-3} M to 8.98×10^{-3} M).

Inhibition of the proteolytic activity of the midgut extract of *Ae. aegypti* larvae was also assessed after SDS-PAGE with a gel (12.5%, m/v) containing gelatin (1%, m/v) as substrate, according to Ashouri et al. [38]. Aliquots (3.5 µL) of the midgut extract were incubated with 0.05 M Tris–HCl buffer, pH 7.5, plus 3.5 µL of *Cl*TI (3 mg mL^{-1}) for 15 min at 37 °C. The midgut extract incubated in the absence of *Cl*TI was used as control. After electrophoresis of the samples, the gel was rinsed with distilled water and washed by shaking gently with 2.5% (v/v) Triton X-100 for 45 min at 37 °C. Then, the gel was incubated at 37 °C with 0.05 M Tris–HCl buffer, pH 7.5, for 30 min and subsequently stained as described previously in Section 2.5.2. The clear bands visualized against the blue background indicated the zones of proteolysis of the gelatin carried out by the proteases present in the midgut extract of the *Ae. aegypti* larvae.

2.6.3. Egg hatching and larval development assay

The effect of *Cl*TI on the hatching of *Ae. aegypti* eggs was assessed as described by Almeida Filho et al. [37]. Ten *Ae. aegypti* eggs were placed in plastic tubes containing 5 mL of 1.54×10^{-5} M *Cl*TI in distilled water (0.3 mg mL^{-1}), with 5 replicates. BSA and distilled water were also tested under the same conditions. After 48 h incubation, the hatched larva number in each treatment was recorded. The number of individuals at the different stages of development and the survival rate were monitored for 10 days. To prevent microbial growth, larvae were transferred to a new solution containing turtle feed (0.2 mg per larva) every 48 h. The experimental results were taken as the average of three independent bioassays.

2.6.4. LC_{50} bioassay

The *Cl*TI concentration required to cause 50% mortality of *Ae. aegypti* larvae (LC_{50}) was determined as previously described [39]. *Ae. aegypti* eggs were allowed to hatch in distilled water at a temperature range of 25–27 °C and the 3rd instar larvae were collected and used in the bioassays. Aliquots of the stock solutions of *Cl*TI in distilled water (1000 µg mL^{-1}) were diluted to provide test solutions with different protein concentrations (1000–31 µg mL^{-1} or 5.13×10^{-2} M to 1.60×10^{-3} M). The assay final volume was set to 20 mL and BSA and water, instead of *Cl*TI, were used as controls. The experiment was repeated thrice with five replicates per concentration, and 10 larvae per replicate ($n = 150$). Mortality rate (%) was determined after 48 h incubation at 25–27 °C. Larvae that were unable to reach the surface solution or did not respond to mechanical stimulus were considered dead. Data were analyzed and LC_{50} value was determined by linear regression.

2.7. Statistical analysis

All analyses were conducted at least in triplicate and were reported as the means with the standard deviation. For each set of results, analysis of variance (ANOVA) was applied, followed by the Dunnett or Tukey test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Purification of ClTI

SPE presented trypsin inhibitory activity (specific activity 15.4 TIU mg^{-1} protein). Purification of *Cl*TI from SPE required two chromatography steps. SPE was separated into three protein fractions after DEAE-Cellulose, one of which, eluted with 0.1 M NaCl (Fig. 1A), displayed inhibitory activity against trypsin. At this stage, the trypsin inhibitor was purified to 6.0-fold with a protein yield of 8.2% and specific activity of 93.1 TIU mg^{-1} protein (Table 1). This fraction was further purified by trypsin-Sepharose 4B affinity chromatography, and the unique retained fraction (Fig. 1B), which exhibited antitryptic activity, presented a single protein band in the native-PAGE (Fig. 2A), as well as when examined by SDS-PAGE under non-reducing conditions or in the presence of 5% β-mercaptoethanol (Fig. 2B). A single antitryptic activity band was also visualized by zymography, which showed a dark blue band on a light background (Fig. 2C). The purity of

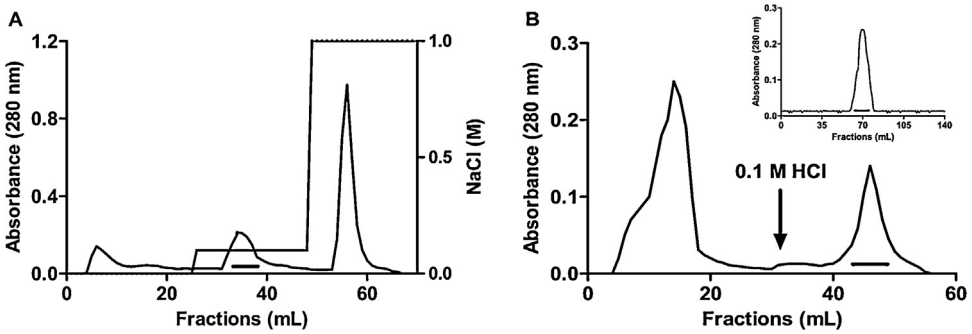

Fig. 1. *Cl*TI purification. (A) SPE (80 mg protein) was loaded on a DEAE-Cellulose column that was previously equilibrated with 0.05 M sodium phosphate, pH 7.5. The fraction with trypsin inhibitory activity was eluted with equilibration buffer containing 0.1 M NaCl. (B) The pooled fractions with antitrypsin activity (10 mg) were applied to a trypsin-Sepharose 4B column that was equilibrated with 0.05 M sodium phosphate buffer, pH 7.5, containing 0.2 M NaCl. *Cl*TI was eluted with 0.1 M HCl. In both chromatographic steps, 2 mL fractions were collected at a 45 mL h^{-1} flow rate. Inset: Gel filtration of *Cl*TI (1 mg) on a HiLoad® 16/600 Superdex 75 column, pre-equilibrated with 0.05 M sodium phosphate buffer, pH 7.5, containing 0.4 M NaCl at a 0.5 mL min^{-1} flow rate, showing a single peak at 21,900 kDa. Horizontal bars indicate fractions exhibiting trypsin inhibitory activity.

*Cl*TI was also evaluated by exclusion chromatography in a HiLoad 16/600 Superdex 75 pg column, from which a prominent single peak emerged (Fig. 1B, inset). The purification strategy adopted for *Cl*TI resulted in 15.5-fold purification and 2.4% protein yield (Table 1).

3.2. Characterization of ClTI

3.2.1. Molecular mass and assessment of the glycoprotein nature

SDS-PAGE (12.5%) showed that *Cl*TI has an apparent molecular mass of 20,000 Da, regardless of the presence of β-mercaptoethanol (Fig. 2B). Native gel filtration chromatography of *Cl*TI using a HiLoad 16/600 Superdex 75 pg column indicated a molecular mass of 21,900 Da (Fig. 1B, inset). ESI mass spectrometry under native conditions revealed a major peak at 19,484 Da (Fig. 3), which is very close to the molecular mass of *Cl*TI found by SDS-PAGE and gel filtration. *Cl*TI was not stained by periodate-Schiff's reagent on SDS-PAGE, suggesting that it is not a glycoprotein.

3.2.2. Protein identification

The N-terminal 40 residues of the intact chain of *Cl*TI were identified as SVELDSDGEPIRNGGGLYYILPVVQGKGGGLELAKTGSQS (UniProt accession number C0HK48). Alignment of this N-terminal sequence with known sequences deposited in the NCBI non-redundant database showed that *Cl*TI has identity with trypsin inhibitors purified from the *Caesalpinioideae*, *Papilionoideae*, and *Mimosoideae* subfamilies (Table 2). The highest degree of N-terminal sequence identity was found with Kunitz-type inhibitors from *Prosopis juliflora* (76%), *Enterolobium contortisiliquum* (70%), *Copaifera langsdorffii* (68%), and *Acacia confusa* (68%). In addition, the peptide sequences obtained by mass spectrometry analysis confirmed the identity of *Cl*TI with other trypsin inhibitors (Table 3).

3.2.3. Secondary structure content

The CD spectrum of *Cl*TI in 0.01 M sodium phosphate buffer, pH 7.5, at room temperature, exhibited a very weak positive ellipticity maximum at 227 nm and a strong negative minimum at 197.2 nm (Fig. 4). Deconvolution of this CD spectrum using the CDPro program (CDSSTR method) showed that *Cl*TI is composed of 35% β-sheets, 14% β-turns, and 50% disordered structures.

The conformational stability of *Cl*TI to pH and temperature variation was investigated by CD spectra analysis. Heating at different temperatures resulted in a progressive loss of the conformational pattern of *Cl*TI (Fig. 4B). *Cl*TI incubation at temperatures greater than 70 °C for 20 min caused complete loss of the secondary structure. These conformational changes were reverted even when *Cl*TI was cooled to 25 °C. However, incubation of *Cl*TI at pH extremes (2.5 and 10.0) did not cause any significant changes in its secondary structure (Fig. 4C).

3.2.4. Enzyme specificity, IC_{50}, inhibition mechanism and Ki determination

*Cl*TI specifically inhibited bovine trypsin, and did not show inhibitory activity to chymotrypsin, papain, and porcine pancreatic α-amylase. The IC_{50} of *Cl*TI on trypsin was 33.81×10^{-8} M (Fig. 5A). The Lineweaver–Burk (Fig. 5B) and Dixon (Fig. 5C) diagrams revealed that *Cl*TI is an uncompetitive inhibitor, with a *Ki* of 6.25×10^{-8} M.

3.2.5. Temperature, pH, and DTT stability

The ability of *Cl*TI to inhibit trypsin was stable up to 70 °C, but higher temperatures caused steady decreases in the inhibitory activity (Fig. 6A). At 100 °C for 20 min, approximately 48% of the activity was lost. However, the trypsin inhibitory activity remained constant in the pH range of 2.2–10.0 (Fig. 6B). Treatment of *Cl*TI with increasing DTT concentrations (0.001 M, 0.01 M, and 0.1 M) decreased progressively the inhibitory activity (Fig. 6C) and incubation with 0.1 M DTT for 2 h, promoted 40% loss of the inhibitory activity of *Cl*TI.

3.3. Insecticidal activity of ClTI against Ae. aegypti

Increased *Cl*TI concentrations resulted in a gradual inhibition of *Ae. aegypti* midgut digestive proteases, reaching 50% reduction at 4.65×10^{-6} M (Fig. 7). This was confirmed by in gel inhibition of

Table 1
Purification steps of *Cl*TI.

Steps	Total protein[a] (mg)	Total activity[b] (TIU)	Specific activity (TIU mg^{-1} protein)	Yield[c] (%)	Purification index[d]
SPE (soluble protein extract)	1279.0	19,701.0	15.4	100	1.0
DEAE-Cellulose	105.1	9780.0	93.1	8.2	6.0
Trypsin-Sepharose 4B	31.0	7404.0	239.0	2.4	15.5

Results are presented as the means of six similar runs.
[a] The total amount of protein recovered from 10 g of defatted flour from *C. leiandra* seeds.
[b] One TIU (trypsin inhibitory activity unit) was defined as the decrease in 0.01 unit of absorbance at 410 nm.
[c] The recovery of protein at each purification step (SPE, 100%).
[d] Purification index is calculated as the ratio between the specific activity obtained at each purification step and that of the SPE taken as 1.0.

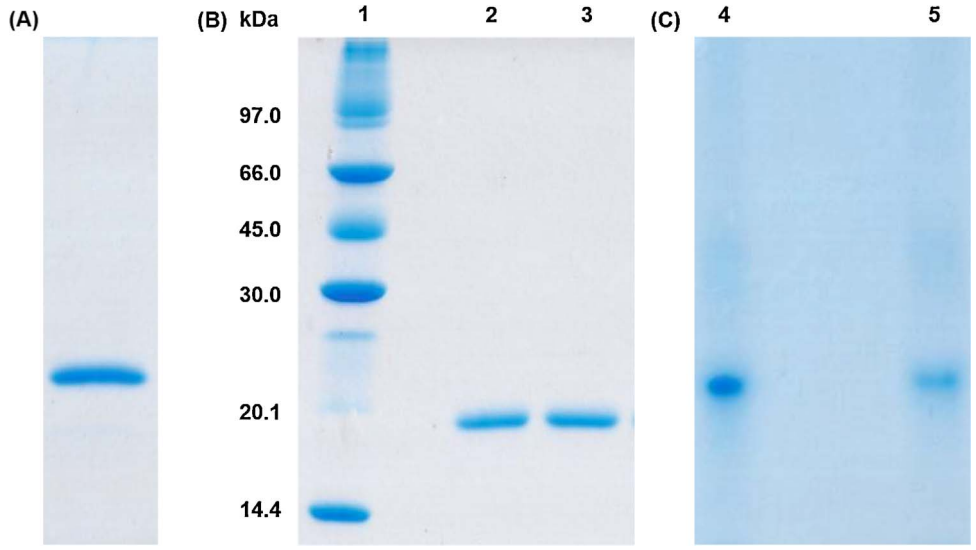

Fig. 2. Polyacrylamide gel electrophoresis (12.5%, m/v) and zymography of *Cl*TI. (A) Native-PAGE. (B) SDS-PAGE. Lane 1: Molecular mass markers; lanes 2 and 3: *Cl*TI (10 μg) in the absence or presence of 5% (v/v) β-mercaptoethanol. (C) lanes 4 and 5: Activity of SBTI (50 μg) and *Cl*TI (50 μg), respectively, on gelatin SDS-PAGE.

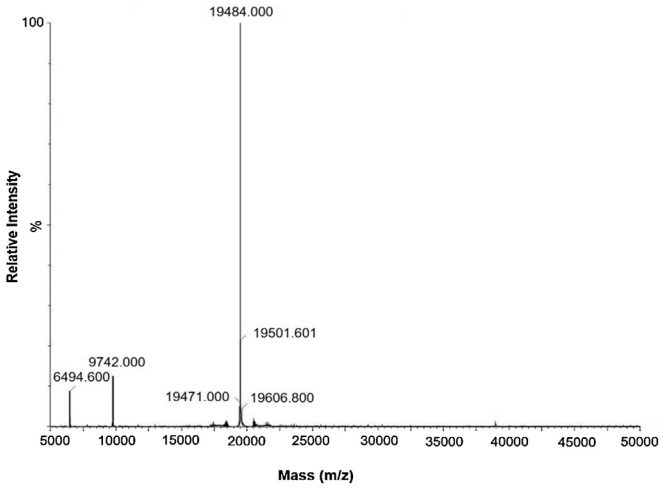

Fig. 3. Mass spectrometry of *Cl*TI. ESI–MS of *Cl*TI (1 mg mL^{-1} in water/acetonitrile [1:1, v/v]) under native conditions revealed a major peak at 19,484 Da.

the gelatin proteolysis by the intestinal proteases of *Ae. aegypti* in the presence of *Cl*TI (Fig. 7, inset). Based on these results, the bioinsecticidal potential of *Cl*TI on *Ae. aegypti* egg hatching and larval development was evaluated 10 days after 48 h egg exposure to this protease inhibitor (chronic test). *Cl*TI, at a final concentration of 1.54×10^{-5} M (0.3 mg mL^{-1}), did not inhibit egg hatching. However, *Ae. aegypti* larvae were negatively affected as they did not develop properly or survive longer than controls (Table 4). *Cl*TI also delayed adult emergence by 24 h and caused 44% mortality.

Acute toxicity of *Cl*TI on 3rd instar *Ae. aegypti* larvae was dose-dependent, with an LC_{50} value of 445 μg mL^{-1}, which is equivalent to 2.28×10^{-2} M (Fig. 8).

4. Discussion

Amazonian traditional communities use the leaves, fruits and entire *C. leiandra* plants for human and animal feed, medicinal and ornamental purposes, and as fishing lure [40]. However, its potential as a source of new molecules of biotechnological interest has not been exploited, especially with regard to bioactive proteins, such as PIs. In this study, a new trypsin inhibitor, named *Cl*TI, was purified from *C. leiandra* seeds, its biochemical characterization was conducted, and its potential insecticidal activity toward *Ae. aegypti* was evaluated.

*Cl*TI was purified by anion exchange chromatography followed by affinity chromatography. These two steps constituted a simple and effective purification strategy, which achieved a 2.4% protein yield (Table 1). After this purification process, the *Cl*TI recovery (2.4%) was 15–16 times higher than recorded for *Trigonella foenum-graecum* (0.16%) [41] and *Piptadenia moniliformis* (0.15%) [42] trypsin inhibi-

Table 2
Identity of the N-terminal sequence of *Cl*TI with other protease inhibitors from *Fabaceae* seeds (NCBI databases).

Subfamily	Species	Sequence	Accession number (NCBI)	Identity (%)
	Cassia leiandra	01SVELDSDGEPIRNGGGLYYILPVVQGKGGGLELAKTGSQS40	C0HK48	
Caesalpinioideae	*Copaifera langsdorffii*	03 VDTDGKPIENDGAEYYILPSVRGKGGGLVLAKSG 36	1R8O_A	68
	Phanera variegata	04 LDTDGEVVRNNGGPYYIIPAFRGNGGGLTLTRVGSET40	P83595.1	57
	Bauhinia rufa	01SVVLDTKGQPVRNAADAYYLEPVARG-DGGLALAKVGNEA39	P84882.1	50
	Bauhinia bauhinioides	01SVILDTKGEPVSNAADAYYLVPVSHGE-GGLALAKVGNEA39	P83051.2	50
Papilionoideae	*Glycine max*	27 IVFDTEGNPIRNGG-TYYVLPVIRGKGGGIEFAKTETET64	NP_001236275.1	56
	Cajanus cajan	30 LDTDGKLLRNGGS-YYVVPVKRGSGGGIELAATGNET65	KYP60026.1	57
	Erythrina caffra	01 VLLDGNGEVVQNGGT-YYLLPQVWAQGGGVQLAKTGEET38	P09943.1	54
Mimosoidae	*Prosopis juliflora*	04 LDVDGEILRNGGS-YYILPAFRGKGGGLELAKT 35	P32733.1	76
	Enterolobium contortisiliquum	04 LDVDGEILRNGGS-YYILPAFRGKGGGLELAKT 35	P32733.1	70
	Acacia confusa	04 LDADGDILRNGGA-YYILPALRGKGGGLTLAKTGDES39	AAB26177.1	68
	Adenanthera pavonina	04 LDVDGNFLRNGGS-YYIVPAFRGKGGGLELARTGSET39	P09941.1	65

Conserved residues are shaded in gray.

Table 3
Amino acid sequences of tryptic peptides from *Cl*TI identified by LC–ESI–MS/MS.

Peptide sequence[a]	Mass (Da)		Species/trypsin inhibitor with identity	GenBank accession number	Identity (%)
	Experimental	Calculated			
$_{26}$GKGGGLELAK$_{35}$	928.5388	928.5342	*Enterolobium contortisiliquum*	ABQ42566	100
$_{162}$DGDPLAVRFVK$_{172}$	1215.6104	1215.6612	*Enterolobium contortisiliquum*	4J2K_A	100
$_{148}$VSDDEFNNYK$_{157}$	1229.5258	1229.5200	*Glycine max*	BAD04941.1	100
$_{13}$NGGLYYILPVFR$_{25}$	1410.5182	1410.7660	*Psophocarpus tetragonolobus*	P32877.1	88
$_{194}$SSNPLVVQFVK$_{205}$	1216.4697	1216.6815	*Cajanus cajan*	KYP60029.1	78
$_{1}$LPVFDSDGEPLR$_{12}$	1343.4414	1343.6721	*Solanum tuberosum*	AIT42210.1	77

[a] The numbers before and after each sequence indicate the predicted residue positions relative to Ser[1], which is the N-terminal residue of the mature *Cl*TI.

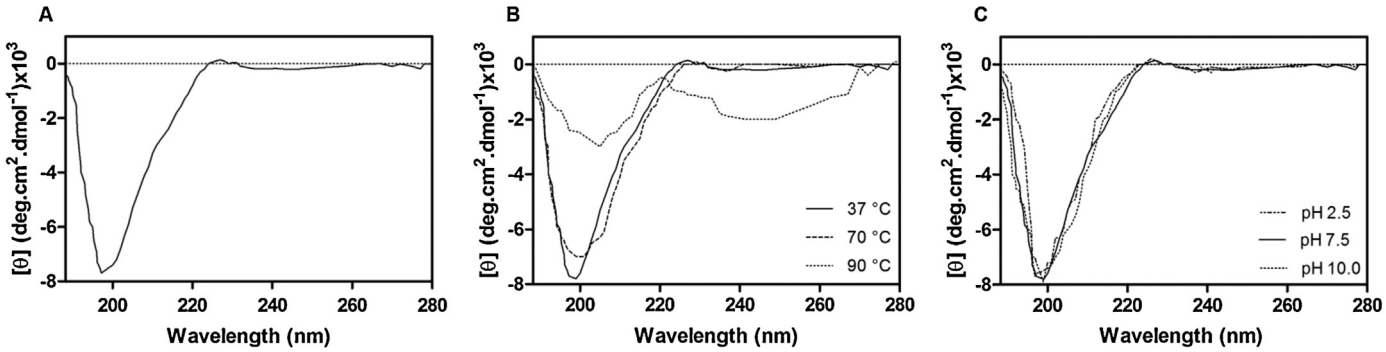

Fig. 4. Circular dichroism spectra of *Cl*TI. Far-UV CD spectra of *Cl*TI (5.1 × 10^{-3} M) in 0.01 M sodium phosphate buffer, pH 7.5, using a circular quartz cuvette with a 1-mm optical path.

tors, but similar to that of *Vigna radiata* (2.2%) [36]. PIs represent approximately 1–10% of the constitutive proteins in plants and are consistently more abundant in seeds than in tubers, leaves, and flowers [17]. The *de novo* PI synthesis can be induced by jasmonates, which are hormone signals involved in plant defense against herbivores, in addition to mechanical wounding [43,44].

The molecular mass of *Cl*TI, around 20 kDa, did not change even under reducing conditions. The trypsin inhibitors from *Dimorphandra mollis* [45] and *Senna tora* [46] have a molecular mass of 20,000 Da and are also composed of a single polypeptide chain. Thus, based on these biochemical characteristics, the properties of *Cl*TI are similar to those of other classic PIs purified from plants belonging to the Caesalpinioideae subfamily. Further, *Cl*TI has no covalently linked carbohydrates, as indicated by the periodic acid-Schiff staining assay. Indeed, most trypsin inhibitors are non-glycoproteins, such as those from *Sapindus trifoliatus* seeds [47] and *Ricinus communis* cake [23]. However, some Kunitz-type inhibitors are glycoproteins, such as those from *Peltophorum dubium*, *Acacia victoriae*, and *Solanum tuberosum* [48–50].

Regarding *Cl*TI sequencing, 103 amino acids residues were identified corresponding to 58% of the entire primary sequence of this polypeptide. Moreover, alignment of the amino acid sequences of *Cl*TI with other proteins revealed strong identity with Kunitz-type PIs present in species of all the subfamilies in the Fabaceae family, particularly those belonging to the Mimosoideae subfamily (Table 2). Different PI types have evolved during the evolution of the Fabaceae family, and the Kunitz-type PIs are mainly present in the species of the most primitive Caesalpinioideae and Mimosoideae subfamilies, whereas species of the Papilionoideae subfamily, the most advanced, typically contain Bowman–Birk inhibitors [42]. Thus, PIs from the most primitive subfamilies might be closely related. In addition, *Cl*TI has an asparagine residue at position 13 (Asn^{13}), which is conserved among members of the Kunitz-type inhibitor family, and it is crucial to the hydrogen bond stabilization of the primary binding loop and contributes to the inhibitory activity [51]. These findings suggest that *Cl*TI is a new member of the Kunitz-type inhibitors.

*Cl*TI presents disordered structures and β-sheets but no α-helix

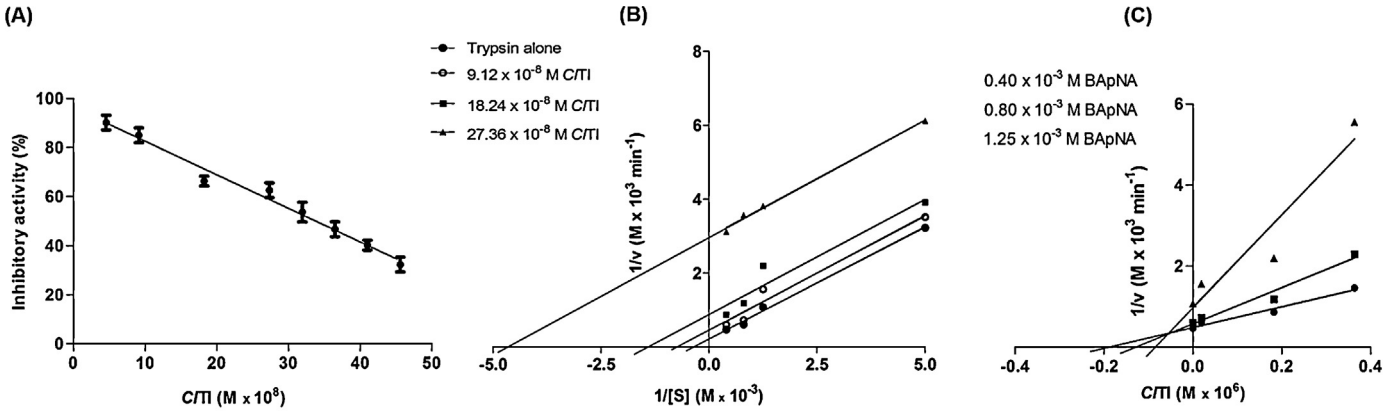

Fig. 5. Inhibition kinetics of *Cl*TI. (A) Effect of *Cl*TI against bovine trypsin. The IC_{50} of *Cl*TI on trypsin was 33.81 × 10^{-8} M. (B) Lineweaver–Burk plot analysis of the inhibition of trypsin by *Cl*TI. The parallel lines represent different *Cl*TI concentrations and are typical of uncompetitive inhibitors. (C) Dixon plot for the determination of the dissociation constant (*Ki*) of *Cl*TI at three BApNA concentrations. The *Ki* value for *Cl*TI was 6.25 × 10^{-8} M. The standard deviation was less than 10%.

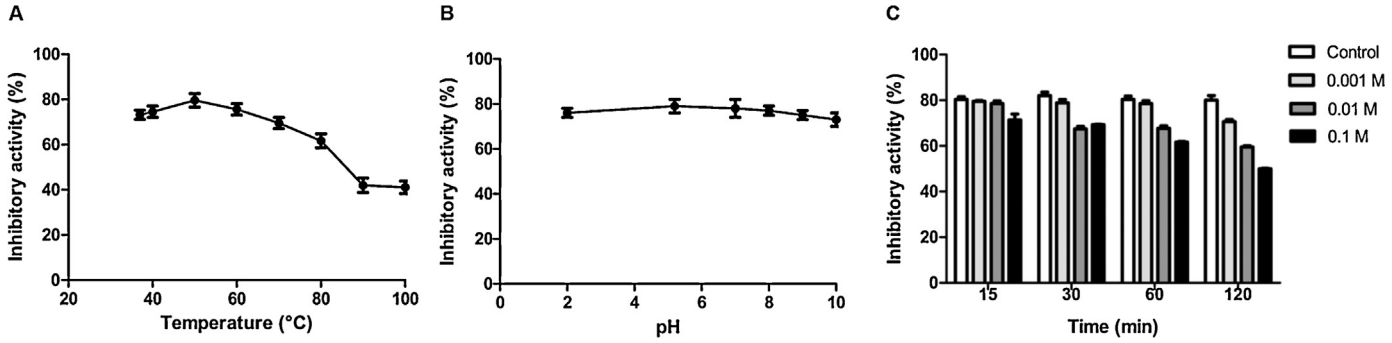

Fig. 6. Stability of *Cl*TI. (A) Temperature stability of *Cl*TI after 20 min incubation at different temperatures. (B) pH stability of *Cl*TI after incubation at different pH for 30 min at 37 °C. (C) Residual trypsin inhibitory activity of *Cl*TI in the presence of different DTT concentrations and incubation times at 37 °C. Bars indicate the standard deviation from triplicate measurements.

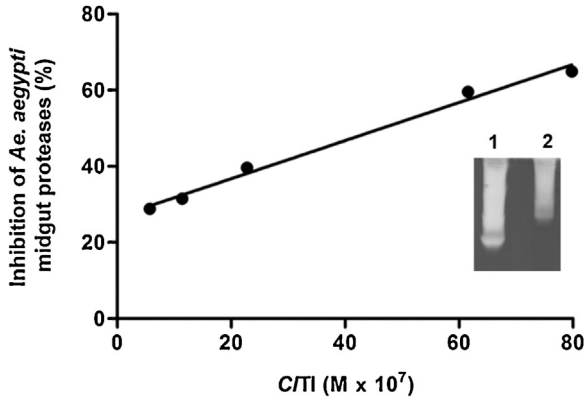

Fig. 7. Inhibitory activity of *Cl*TI against *Ae. aegypti* midgut digestive proteases. Intestinal homogenates of *Ae. aegypti* were incubated with increasing concentrations of *Cl*TI. After 15 min incubation with BApNA, the reaction was stopped, and the inhibition percentage was calculated and compared with the activity of the homogenate in the absence of the inhibitor. The IC_{50} value for *Cl*TI was 4.65×10^{-6} M. Inset: In-gel inhibitory activity of *Cl*TI against *Ae. aegypti* digestive proteases. Lane 1 – Midgut extract; lane 2 – Midgut extract + *Cl*TI.

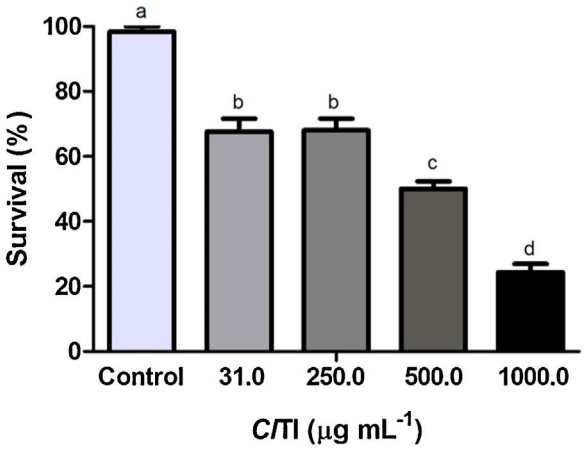

Fig. 8. Effect of *Cl*TI on *Ae. aegypti* survival. Larvae (3rd instar) were exposed to different concentrations (1000–31.0 µg mL^{-1} or 5.13×10^{-2} M to 1.60×10^{-3} M) of *Cl*TI for 48 h. *Cl*TI presented an LC_{50} of 2.28×10^{-2} M. The values correspond to the mean of 5 replicates ($n = 50$). Different letters indicate significant differences ($P < 0.05$).

Table 4
Survival and development of *Ae. aegypti* larvae arising from eggs treated with *Cl*TI.

Treatment	*Ae. aegypti*							
	Total number of individuals 10 days after egg treatment	Number of individuals who reached each developmental stage[a]						Mortality (%)
		Larvae instar				Pupae	Mosquito	
		L1	L2	L3	L4			
BSA	39	0	0	0	2	20	17	22.0 ± 4.5[a]
*Cl*TI	28	0	0	3	8	9	8	44.0 ± 5.7[b]
Control	40	0	0	0	3	19	18	20.0 ± 6.9[a]

[a] Survival and development of *Ae. aegypti* were determined after 10 days of egg exposure to *Cl*TI (1.54×10^{-5} M or 0.3 mg mL^{-1}, final concentration). The results are the means of three independent experiments. Different letters indicate significant differences ($P < 0.05$) based on the Tukey test.

structure (Fig. 4A). Kunitz-type inhibitors typically possess few α-helix structures and 12 antiparallel β-strands connected by long loops [18]. Disordered structures may provide flexibility to certain inhibitors to inhibit enzymes of different classes [41]. Some PIs inhibit trypsin and chymotrypsin [41,52] and even different protease classes, such as trypsin and papain [42,53], but there are PIs highly specific for trypsin [54]. *Cl*TI is probably included in the last case. Moreover, *Cl*TI promoted an uncompetitive inhibition of bovine trypsin (Fig. 5B), but was inactive against chymotrypsin, papain, and α-amylase.

Uncompetitive inhibition is unusual among Kunitz-type inhibitors, although it was previously reported for the trypsin inhibitor from *M. oleifera* leaves [55]. *Cl*TI had a *Ki* of 6.25×10^{-8} M (Fig. 5C), similar in order of magnitude to those determined for the *amara* (1.24×10^{-8} M) [56] and *R. communis* (1.90×10^{-8} M) [23] inhibitors. *Ki* is an important parameter to indicate the degree of interaction between an enzyme and its corresponding inhibitor [55]. The low *Ki* value of *Cl*TI indicates its high affinity to trypsin. Indeed, the IC_{50} value of *Cl*TI (33.81×10^{-8} M) (Fig. 5A) for trypsin was lower than that (60.00×10^{-8} M) calculated for the *M. oleifera* inhibitor [55].

Generally, Kunitz-type trypsin inhibitors maintain their activity after exposure to high temperatures and large pH variations. *Cl*TI was heat stable (Fig. 6A) like the trypsin inhibitors from *Poincianella pyramidalis* [57] and *Adenanthera pavonina* [58] seeds, which maintained their inhibitory activity after incubation within wide temperature range (37–70 and 25–70 °C, respectively) for 30 min. Moreover, a trypsin inhibitor obtained from *Pithecellobium dumosum* seeds maintained approximately 90% of its activity after exposure to temperatures varying from 37 to 100 °C for 30 min [59]. The high thermal stability of trypsin inhibitors from species of the Fabaceae family is associated with the presence of intramolecular disulfide bridges. Kunitz-type inhibitors have four cysteine residues involved in two disulfide bridges [17]. Oliva et al. [22] reported that the most important disulfide bridge for the stability of Kunitz-type inhibitors is that established between the cysteine residues 39 and 86, which is also responsible for stabilizing the loop involved with the inhibitor reactive site. In addition, *Cl*TI was also stable over a wide pH range (Fig. 6B), as the trypsin inhibitors from *P. dumosum* [59] and *Entada acaciifolia* [60] seeds, which maintained

their functional stability at extreme pH conditions. In general, PPIs remain active in a wide pH range (2.0–10.0). Maintenance of the functional stability of trypsin inhibitors after exposure to a large range of temperature and pH might be associated with the rigidity of their tridimensional structure, which allows only slight conformational changes under adverse conditions and/or their capacity to renature as soon as they return to appropriate physiological conditions. Nevertheless, such stability is an important feature of bioactive molecules with the potential to be employed in biotechnology-based processes [55].

The conformational stability of *Cl*TI was confirmed by circular dichroism (Fig. 4B and C), which showed only slight changes in its tridimensional structure when the inhibitor was incubated at extreme pH (2.5 and 10.0). A similar result was reported for the *Cajanus cajan* trypsin inhibitor, which also showed small changes in its CD spectra when incubated at extreme pH [61]. In contrast, significant changes in the spectra were observed when *Cl*TI was subjected to thermal treatment (70–90 °C). However, the inhibitory activity of *Cl*TI was recovered after the heat treatments, which suggested that it has the ability to renature, within a given limit, under the physiological conditions of the assay.

*Cl*TI incubation with 0.1 M DTT for 120 min (Fig. 6C) induced a partial loss of its inhibitory activity. This suggests the importance of the disulfide bridges for the maintenance of the *Cl*TI structure and functionality, a characteristic presented by other PPIs. For instance, incubation of the trypsin inhibitor of *Inga vera* with 0.1 M DTT for 120 min reduced its inhibitory activity by approximately 30% [6]. A trypsin inhibitor from *P. dubium* lost 90% of its activity after 45 min of 0.1 M DTT incubation [48]. Most Kunitz-type inhibitors have two disulfide bridges that are accessible to reducing agents, such as DTT. However, these bridges, in some cases, are not directly involved in the functionality of trypsin inhibitors, as the reactive site structures are maintained predominantly by weak interactions, such as hydrogen bonds, Van der Waals forces, and hydrophobic interactions. In contrast, the loss of activity of some inhibitors by reducing agents indicates that the disulfide bridges might, in this case, be involved in the maintenance of their reactive site architecture and are thus fundamental to the preservation of the structural conformation and functionality [62,63].

Most PIs bind to the gut proteases of insects of different orders [64–66]. Trypsin inhibitors from *I. vera* [6], *P. moniliformis* [42], and *Clitoria fairchildiana* [65] inhibited the proteases of coleopterans, lepidopterans, and/or dipterans, and caused various deleterious effects, such as retarded growth and development, increased mortality, and deformations. *Cl*TI inhibited the gut proteases of *Ae. aegypti* (Fig. 7), as did the *R. communis* inhibitor [23]. Trypsin and chymotrypsin are the main enzymes involved in the dietary protein digestion by *Ae. aegypti* throughout the entire insect developmental stages. Both enzymes are very active during the larval stage, but trypsin is the most abundant [67,68]. Because *Cl*TI is a trypsin inhibitor, interaction with *Ae. aegypti* intestinal proteases might act like an antimetabolic agent, which could interfere with the insect physiology and development. Chronic treatment of *Ae. aegypti* with *Cl*TI delayed the larval development and increased the mortality rate (Table 4), which might be related to the diminished uptake of dietary essential amino acids, resulting in starvation and death [69]. In addition to the protease inhibitor activity, the deleterious effect of PIs against insects is thought to come from other functional domains that can elicit signals leading to insect death [70]. Other inhibitors showed effects similar to those of *Cl*TI. *Ap*TI, a trypsin inhibitor from *A. pavonina* seeds, besides reducing the proteolytic activity of *Ae. aegypti* midgut enzymes, diminished the larval weight and survival. Furthermore, larvae fed on *Ap*TI-containing diets showed microvillus degeneration at the posterior region of the midgut epithelial cells, hypertrophy of the gastric caeca cells, and increased ectoperitrophic space [71]. The inhibitor of *M. oleifera* flowers delayed the development rate of *Ae. aegypti* first-instar larvae, although did not affect pupa survival [72]. Similar negative effects of PIs on the life cycle of herbivores belonging to different insect orders were previously reported. The *C. fistula* inhibitor (CFTI-1) prolonged the *H. armigera* life cycle, increased the larval mortality, and decreased the larval weight [73]. *Ap*TI caused deleterious effects on the larvae and pupae of *Diatraea saccharalis* [74]. The *Bauhinia rufa* inhibitor negatively affected *Callosobruchus maculatus* development [70]. Importantly, one strategy to decrease insect populations is the interruption or delay of the insect life cycle [75]. Within this context, and according to the findings described above and elsewhere, PIs represent promising tools for insect control [42], with the advantage of offering low potential risk for insect populations to evolve resistance to these natural insecticides [73].

5. Conclusions

In the present study, a novel trypsin inhibitor from *C. leiandra* seeds (*Cl*TI) was purified, characterized, and evaluated for its insecticidal activity. *Cl*TI showed similar structural features to Kunitz-type inhibitors and activity against *Ae. aegypti* by inhibiting its midgut proteases, delaying the larval development, and increasing the mortality rate. Our results constitute an important contribution to a better understanding of the insecticidal activity of PIs and indicate that *Cl*TI has biotechnological potential as an alternative strategy to control the *Ae. aegypti* vector, which transmit multiple virus disease, by employing this inhibitor alone or in combination with other insecticidal compounds toward synergistically enhancing its toxicity.

Authors contributions

Study conception and design: LPD, JTAO, IMV. Acquisition of data: LPD, LCBRB, DOBS, HPSC, NMSA, PMST, MDPL, FBMBM, JLSL, IMV. Analysis and interpretation of data: LPD, JTAO, LCBRB, AFUC, ACOMM, BAMR, LMB, IMV. Drafting of manuscript: LPD, JTAO, AFUC, IMV. Critical revision: JTAO, IMV.

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