Research Article

First insights into insecticidal activity against *Aedes aegypti* and partial biochemical characterization of a novel low molecular mass chymotrypsin-trypsin inhibitor purified from *Lonchocarpus sericeus* seeds

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Abstract

BACKGROUND: Arboviroses such as dengue, Zika and chikungunya represent a serious public health issue as a consequence of the absence of approved vaccines or specific antiviral drugs against the arboviruses that cause them. One way to prevent these diseases is by combating the vector mosquito, *Aedes aegypti* (Diptera), which has serine proteases in the midgut. Protease inhibitors are molecules that can block enzyme activity, impairing digestion and nutrition, which can lead to death. Thus, we purified and characterized a novel chymotrypsin-trypsin inhibitor (LsCTI) from *Lonchocarpus sericeus* seeds and investigated its effect upon *Ae. aegypti* egg hatching, larval development and digestive proteases.

RESULTS: LsCTI showed a single protein band in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and the molecular mass determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was 8870.45 Da. Kinetics analyses revealed a noncompetitive type of inhibition and low inhibition constant (K_i) for chymotrypsin (8.24×10^{-8} M). The thermal resistance was remarkable, even at 100 °C for 180 min. The inhibitor concentration required for 50-percent enzyme inhibition (IC_{50}) of LsCTI was 4.7×10^{-7} M for *Ae. aegypti* midgut larval enzymes. LsCTI did not affect egg hatchability at 0.3 mg mL^{-1}, but caused a high larval mortality rate (77%) and delayed development (37%).

CONCLUSIONS: LsCTI is a novel protease inhibitor with remarkable biochemical characteristics and is a potential tool to control *Ae. aegypti* development.
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Keywords: chymotrypsin inhibitor; *Lonchocarpus*; legume seeds; dengue; zika; midgut enzymes

1 INTRODUCTION

The arboviruses (arthropod-borne viruses) are a large group of viruses carried and spread by several arthropods, most commonly blood-sucking insects, and represent one of the greatest challenges to current and future human well-being. Among the arboviroses are malaria, yellow fever and Japanese encephalitis, as well as a group of so-called 'neglected tropical diseases' including chikungunya, dengue, leishmaniasis and Chagas' disease.[1,2] In addition, the increase in international trade and human migration has facilitated the accidental introduction of vectors and/or pathogens from one continent to another.[3]

Mosquitoes transmit about 30 arboviruses of great importance to global public health, among which *Aedes aegypti* (Diptera: Culicidae), an anthropophilic mosquito, is widespread throughout the world and is the main vector of chikungunya, dengue and Zika viruses. Zika virus deserves special attention in view of its ability to cross the transplacental barrier and to lodge in the fetal nervous system, causing not only microcephaly, but also vision problems, deafness, arthrogryposis and dysphagia.[4] Zika virus can also settle and survive in immunoprivileged tissues, such as the germ tissues present in the testicles, and thus Zika fever is a potential sexually transmitted infection.[5] These *Ae. aegypti*-related diseases are particularly challenging because of the absence of specific antiviral drugs or vaccines approved for their prevention.[6] Epidemiological studies suggest that about 3.9 billion people in more than 120 countries live in risk areas for these diseases, which, along with other arboviroses, represent about 20% of infectious diseases, causing the deaths of more than 1 million people every

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year.[7,8] Combating the vector is thus a crucial strategy to reduce the incidence of these diseases. [9]

One of the strategies to eliminate this mosquito is the use of synthetic insecticides belonging to the classes organochlorines, organophosphates and pyrethroids, which generally act on the nervous systems of insects.[10] Although effective, the inadequate management of these insecticides led to the emergence of resistant insect populations, and as a consequence of increased resistance of pests to chemical insecticides, research on natural and biodegradable insecticides that promote larval mortality and avoid the emergence of resistant strains has increased.[11,12]

Bioinsecticides are ecofriendly pesticides (low toxicity to the environment) which do not cause problems in terms of air and water quality, often targeting only specific insects without having negative effects on non-target insects. They can be used in the management of pests harmful to plants and also to combat vectors of diseases.[13,14]

Among the molecules of the primary metabolism of plants, pathogen-related proteins are often associated with protection of organs and reproductive tissues against herbivory. These proteins constitute an arsenal capable of acting against several types of pathogens and are potential insecticides; among them are lectins, ribosome-inactivating proteins (RIPs) and inhibitors of proteolytic enzymes or of glucosidases.[15–17]

Protease inhibitors are proteins or peptides capable of interacting with proteolytic enzymes and inhibiting their catalytic activities.[18] These molecules can play various biological roles in plants, acting as regulators of endogenous proteases, reserve proteins and plant defense agents against microorganisms, insects and other herbivorous animals.[19] In the last few decades, protease inhibitors have attracted much attention as a consequence of their involvement in plant defense and possible applications in plant bioengineering to increase resistance to insects and other pathogens. For insect population control, the digestive enzymes of the insects have become a potential target of protease inhibitors.[20]

The insecticidal potential of protease inhibitors against different insect classes has been reported; for example, protease inhibitors from *Adenanthera pavonina* (ApTI)[11], *Cassia leiandra* (CITI)[21] and *Leucaena leucocephala* (LTI)[22] have been reported to have effects on larvae of *Ae. aegypti* (Diptera), those from *Inga vera* (IVTI)[23] and *Poincianella pyramidalis* (PpyTI)[24] have been reported to have effects on *Anagasta kuehniella* neonates, and a protease inhibitor from *Dimorphandra mollis* (DMTI-II)[25] has been reported to have effects on *Callosobruchus maculatus* (Coleoptera).

Lonchocarpus sericeus (Poir.) Kunth ex DC is a legume belonging to the Fabaceae (subfamily Faboideae) that occurs in the semi-arid region of Brazil and is present in America, Africa, Asia and Oceania. The presence of phytochemicals such as alkaloids, saponins, carotenoids, flavonoids, tannins, triterpenes and steroids has been reported in its stem bark, seeds and roots.[26] Other studies have shown cytotoxic activity of its flavonoids derricin and lonchocarpine on several cell types. [27,28]

In addition to being a good source of secondary metabolites, *L. sericeus* is also a good source of proteins and lipids, as its seeds contain about 40 and 30% of these macronutrients, respectively. Among the proteins, protease inhibitors were identified.[29] The present study reports the purification and partial biochemical characterization of a novel low molecular mass protease inhibitor from *L. sericeus* seeds (LsCTI) as well as its ability to promote negative and deleterious effects on the growth and development of *Ae. aegypti* larvae.

2 MATERIALS AND METHODS

2.1 Chemicals
Bovine serum albumin (BSA) and enzymes were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The synthetic substrates $N\alpha$-benzoyl-DL-arginine-*p*-nitroanilide (BApNA), *N*-benzoyl-L-tyrosine-*p*-nitroanilide (BTpNA), *n*-succinyl-ala-ala-val-*p*-nitroanilide (SAAVpNA) and $N\alpha$-benzoyl-DL-arg-β-naphthylamide (BANA) and the protein substrate azocasein were purchased from Sigma-Aldrich Co. Molecular mass markers for electrophoresis, immobilized pH gel strips and ampholites were obtained from GE Healthcare Life Sciences (St. Louis, MO, USA).

2.2 Protein quantification and chymotrypsin activity
Protein concentration was estimated using the Pierce® BCA (Thermo Fisher Scientific, Waltham, MA, USA) protein assay kit with soybean trypsin inhibitor ($25 – 2000$ µg mL^{-1}) as standard. The assay to measure chymotrypsin inhibitory activity was performed using BTpNA as the substrate for the reaction.[30] Two hundred microliters of bovine chymotrypsin (EC 3.4.21.1) (0.01 mg mL^{-1} in 0.001 M HCl solution containing 0.002 M CaCl$_2$) was incubated for 2 min with 300 µL of 0.05 M Tris–HCl, pH 7.5, containing 0.02 M CaCl$_2$ and 100 µL of the different samples. Next, 200 µL of 5 x 10^{-3} M BTpNA (50% dimethyl sulfoxide in 0.05 M Tris–HCl buffer, pH 7.5) was added and the mixture was incubated at 37 °C. After 30 min, the reaction was stopped by adding 100 µL of 30% (v/v) acetic acid in distilled water. BTpNA hydrolysis was monitored at 410 nm. One unit of chymotrypsin inhibitor activity was defined as the amount of inhibitor capable of producing a reduction in absorbance of 0.01 relative to the standard control. Blank reactions and enzymatic standard activity reactions were performed in parallel. Results are expressed as mean of three independent assays.

2.3 Purification of *Lonchocarpus sericeus* protease inhibitor (LsCTI)
Lonchocarpus sericeus seeds were collected in Fortaleza, State of Ceará, in northeastern Brazil (3°44'34.5"S, 38°34'34.4"W). The seeds were processed using an electric mill, then sieved to generate a homogeneous flour, which was then defatted by hexane extraction 1:3 flour/hexane (w/v). Seed proteins were extracted using 0.05 M Tris–HCl buffer, pH 7.5 (1:10 w/v), under constant stirring for 2 h at room temperature (24 ± 2 °C) followed by centrifugation for 15 min at 15 000 ***g*** to obtain the supernatant [crude protein extract (CPE)]. To the CPE, a 20% (w/v) solution of trichloroacetic acid (TCA) was added so that the final concentration was 5%; this mixture was then left at 4 °C for 30 min to precipitate high molecular mass proteins. The resulting suspension was centrifuged at 15 000 ***g*** for 10 min, and the supernatant was collected (LsF5) and dialyzed against distilled water for 48 h before lyophilization.

In order to purify the protease inhibitor present in LsF5, one-step chromatography was performed. We solubilized 6 mg of lyophilized proteins in 3 mL of 0.05 M Tris–HCl buffer, pH 7.5, and loaded them into a trypsin-agarose affinity chromatography column (1 mL of beaded agarose matrix) equilibrated with the same buffer. After loading, the column was washed with the buffer at a flow rate of 0.5 mL min^{-1} until absorbance of <0.005 at 280 nm was achieved. This process was monitored using a Spectronic™ Genesys™ 10 VIS Spectrophotometer 335900 (Thermo Fisher Scientific Inc.). Then, adsorbed proteins were eluted by applying a 0.05 M HCl solution and the protein elution was monitored by measuring absorbance at 280 nm. The chromatographic fractions

showing the highest absorbances were pooled together (LsCTI), dialyzed against distilled water for 36 h and lyophilized. Along the inhibitor purification procedure, all samples were subjected to protein quantification and chymotrypsin assay.

2.4 Characterization of LsCTI
2.4.1 Mass determination
The purified protein (10 mg mL^{-1}) was analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). One milliliter of the protein solution was mixed with 1 mL of the matrix solution (5 mg 1-acetil-2,4-dihydroxybenzene, 2'-4'-dihydroxyacetophenone dissolved in 250 mL of acetonitrile, 0.5 mL of trifluoroacetic acid and 250 mL of water). This mixture (1 mL) was spotted onto a MALDI target plate and left at room temperature to dry. The protein masses were obtained using the positive linear mode of a Bruker Microflex LT MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA) equipped with a 337-nm nitrogen laser operated by Flexcontrol 3.3 software (Bruker Daltonics). The spectra were acquired in the mass range of m/z 2000 to 25 000 and external mass calibration was performed using Protein Standard II (Bruker Daltonics).

2.4.2 Determination of purity, isoforms and isoelectric point
To verify the purity of the purified protein, we applied 10 μg of LsCTI to an electrophoretic gel containing 15% polyacrylamide and sodium dodecyl sulfate (SDS) [SDS–polyacrylamide gel electrophoresis (PAGE)]. This process was performed according to the methodology proposed by Laemmli.[31] After electrophoresis, the gel containing LsCTI and molecular mass markers [GE Healthcare Life Sciences: phosporylase b (97 kDa); bovine albumin (66 kDa); ovalbumin (45 kDa); bovine carbonic anidrase (30 kDa); soybean trypsin inhibitor (20.1 kDa); bovine α-lactalbumin (14.4 kDa)] was stained with a solution of 0.08% (w/v) Coomassie brilliant blue, 1.6% (w/v) ortho-phosphoric acid and 8% (w/v) ammonium sulfate in 20% (v/v) methanol.

Isoelectric focusing (IEF) was carried out using the Ettan™ IPG-Phor II™ system (GE Healthcare Life Sciences). Immobilized pH gradient (IPG) 3–10 gel strips (7 cm) were first rehydrated overnight at 25 °C by adding 60 μg of LsCTI diluted in 125 μL of rehydration buffer composed of 7 M urea, 2 M thiourea, 10 μg μL^{-1} of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 10 μg μL^{-1} of dithiothreitol (DTT), 0.01 μg μL^{-1} of bromophenol blue, and 0.6% (v/v) of ampholites (IPG buffer) and covered with mineral oil in a reswelling tray. The IEF was performed in a stepwise mode: 300 V for 30 min, 500 V for 30 min, 1000 V for 1 h and 5000 V until reaching a total of 10 000 V h^{-1} per gel strip. A focused gel strip was placed in a tube with 5 mL of equilibration buffer [50 mM Tris–HCl, pH 8.8, containing 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 1% (w/v) bromophenol blue containing 1% (w/v) DTT] and gently shaken for 15 min. The strips were then placed in an equilibration solution containing 2.5% (w/v) iodoacetamide. For the second dimension, SDS-PAGE was conducted in a vertical system (MiniVE; GE Healthcare Life Sciences) under 20 mA constant current per gel and 200 V of maximum tension. Electrophoresis in two gels with identical dimensions (100 × 100 × 1 mm) was performed using 15% polyacrylamide gels without a stacking gel. The staining process was conducted as previously described. Gel images were scanned using an Imager Scanner (GE Healthcare Life Sciences) and Labscan software (GE Healthcare Life Sciences). The number

of isoforms was determined using acquired images and the isoelectric point was analyzed using Imagemaster 2d Platinum software (GE Healthcare Life Sciences).

2.4.3 Enzyme specificity
In order to assess the enzyme specificity of LsCTI, we first determined the minimum inhibitor concentration required to achieve maximum chymotrypsin inhibition.

2.4.3.1 Serine proteases.
The human leucocyte elastase (EC 3.4.21.37) inhibitory activity was evaluated using SAAVpNA as the substrate.[32] Ten microliters of enzyme (0.5 mg mL^{-1} in 0.05 M sodium phosphate buffer, pH 7.4) was incubated for 15 min at 37 °C with 390 μL of 0.1 M sodium phosphate buffer, pH 7.4, and 100 μL of LsCTI (49.6 x 10^{-8} M). The reaction was started by adding 100 μL of 0.125 M SAAVpNA in 0.1 M sodium phosphate buffer, pH 7.4. The enzymatic reaction was allowed to proceed for 30 minutes and stopped by adding 300 μL of 2% (w/v) citric acid in distilled water. The absorbance was measured at 405 nm. The inhibitory activity toward pancreatic porcine elastase (EC 3.4.21.36) was evaluated using azocasein as the substrate. Fifty microliters of enzyme (0.2 mg mL^{-1} in buffer) was incubated for 15 min at 37 °C with 350 μL of 0.05 M Tris–HCl buffer containing 0.02 M CaCl$_2$, pH 7.5, and 100 μL of LsCTI (49.6 x 10^{-8} M). The reaction was initiated by adding 200 μL of 1% (w/v) azocasein in 0.05 M Tris–HCl buffer. The enzymatic reaction was carried out for 30 minutes and stopped by adding 300 μL of 20% (w/v) TCA solution in distilled water. The resulting mixture was centrifuged for 10 min at 10 000 *g* at 25 °C and 2 M of NaOH in distilled water was added to the supernatant at 1:2 (v/v). The absorbance at 440 nm was measured. Trypsin inhibitor activity was determined using BApNA as the substrate for the reaction.[33] Two hundred microliters of enzyme (EC 3.4.21.4) (0.01 mg mL^{-1} in 0.001 M HCl) was incubated for 10 min with 250 μL of 0.05 M Tris–HCl, pH 7.5, containing 0.02 M CaCl$_2$ and 100 μL of LsCTI (49.6 x 10^{-8} M). The reaction was started by adding 250 μL of 1.25 mM BApNA in 0.05 M Tris–HCl, pH 7.5, and carried out for 15 min until it was stopped by the addition of 0.1 mL of 30% acetic acid in distilled water (v/v). The absorbance at 410 nm was measured. All tests were accompanied by standard enzymatic and blank tests and performed in triplicate, and results are expressed as the mean of three independent assays.

2.4.3.2 Cysteine proteases.
The inhibitory activity of bromelain (EC 3.4.22.32) was evaluated using azocasein as the substrate prepared in 0.05 M sodium acetate buffer, pH 5.5. One hundred microliters of enzyme (0.01 mg mL^{-1} in 0.05 M sodium acetate buffer, pH 5.5) was incubated for 15 min at 37 °C with 360 μL of 0.3 M sodium acetate buffer, pH 5.5, 40 μL of activator solution [0.003 M DTT and 0.002 M ethylenediaminetetraacetic acid (EDTA) in 0.3 M sodium acetate buffer, pH 5.5] and 49.6 x 10^{-8} M of LsCTI. The reaction was initiated by adding 200 μL of 1% (w/v) azocasein and stopped after 30 min by the addition of 300 μL of 20% (w/v) trichloroacetic acid in distilled water. The mixture was centrifuged for 10 min at 10 000 *g* and 25 °C, and 2 M of NaOH in distilled water was added to the supernatant at 1:2 (v/v). The absorbance at 440 nm was measured. The assay of inhibitory activity against papain (EC 3.4.22.2) was carried out using BANA as a substrate.[34] Ten microliters of enzyme (0.1 mg mL^{-1} in 0.25 M sodium phosphate buffer, pH 6.0) was incubated for 10 min at 37 °C with 0.25 M sodium phosphate buffer, pH 6.0, papain activator solution (0.003 M DTT and 0.002 M EDTA in 0.25 M sodium

phosphate buffer, pH 6.0) and 49.6 x 10^{-8} M LsCTI. The reaction was started by adding 200 μL of 1 mM BANA. The enzymatic reaction was carried out for 20 min and terminated by adding 500 μL of 2% HCl in ethanol. Next, 500 μL of the color reagent p-dimethylaminocinnamaldehyde (DMACA) (0.06% in ethanol) was added and after 10 min, the absorbances were read at 540 nm. All tests were accompanied by standard enzymatic and blank tests and performed in triplicate, and results are expressed as the mean of three independent assays.

2.4.3.3 Glucosidases. The porcine pancreatic *α*-amylase (EC 3.2.1.1) inhibition assay was performed using 3,5-dinitrosalicylic acid (DNS), as previously described by Dias *et al.*[21] Fifty microliters of enzyme (1.0 mg mL^{-1} in 0.02 M sodium phosphate buffer, pH 6.9, containing 0.006 M NaCl) was incubated with 100 μL of the above buffer and 100 μL (49.6 x 10^{-8} M) of LsCTI. After 10 min at 37 °C, the reaction was initiated by adding 250 μL of 1% (m/v) starch solution in the buffer assay. The reaction proceeded for 15 min and was stopped by the addition of 500 μL of 1% (w/v) DNS in 1 M NaOH aqueous solution containing 1 M sodium potassium tartrate. The mixture was boiled in a water bath for 10 min, diluted with 2.5 mL of distilled water and cooled to room temperature (23 ± 2 °C) and the absorbance was measured at 540 nm. All tests were accompanied by standard enzymatic and blank tests and performed in triplicate, and results are expressed as the mean of three independent assays.

2.4.4 Inhibition kinetics of LsCTI for chymotrypsin and trypsin

The LsCTI concentration required to reduce in 50% of chymotrypsin activity was determined, as described in Section 2.2, using inhibitor concentrations ranging from 1.24 x 10^{-8} to 49.6 x 10^{-8} M. The kinetic measurements of chymotrypsin inhibition by LsCTI were conducted according to Dias *et al.* with modifications.[21] LsCTI was solubilized in 0.05 M Tris–HCl buffer, pH 7.5, at different concentrations (3.95 x 10^{-8}, 9.22 x 10^{-8}, and 13.16 x 10^{-8} M) and incubated with 200 μL of chymotrypsin (0.01 mg mL^{-1} in 0.001 M HCl) at 37 °C. The reaction was initiated adding 300 μL of BTpNA at different concentrations (0.23 x 10^{-3} to 12.18 x 10^{-3} M) and stopped 30 min later by adding 100 μL of 30% (v/v) acetic acid. The absorbance was measured at 410 nm. A Lineweaver–Burk plot was obtained by plotting the reciprocal of the rate of the enzyme reaction (1/*v*) versus the reciprocal substrate concentration (1/[S]) in the absence and presence of LsCTI. The inhibition constant (K_i) was determined according to Dixon.[35] K_i was obtained as the intersection of the five lines at the *x*-axis, corresponding to the substrate concentrations (0.23 x 10^{-3}, 0.40 x 10^{-3}, 0.60 x 10^{-3}, 0.91 x 10^{-3} and 1.22 x 10^{-3} M).

The LsCTI concetration required to reduce in 50% of trypsin activity was determined, as described in Section 2.4.3.1, using concentrations of LsCTI ranging from 5.85 x 10^{-8} to 116.85 x 10^{-8} M. The kinetic of LsCTI was determined by its solubilization in different concentrations (9.17 x 10^{-8}, 18.33 x 10^{-8}, and 36.66 x 10^{-8} M) in 0.05 M Tris–HCl buffer, pH 7.5, and it was incubated with 200 μL of trypsin (0.01 mg mL^{-1} in 0.001 M HCl) at 37 °C. The reaction was initiated by adding 300 μL of BApNA at different concentrations (0.7 x 10^{-3} to 22.55 x 10^{-3} M) and after 15 min 100 μL of 30% (v/v) acetic acid was added. The absorbance was measured at 410 nm. As previous described for chymotrypsin kinect inhibition, a Lineweaver–Burk plot was obtained and K_i was obtained as the intersection of the four lines at the *x*-axis, corresponding to the substrate concentrations (0.70 x 10^{-3}, 1.06 x 10^{-3}, 1.40 x 10^{-3} and 2.25 x 10^{-3} M).

2.4.5 Stability of inhibitory activity in the presence of physical and chemical denaturing agents

The stability of the inhibition of the chymotrypsin and trypsin was assessed using aliquots of LsCTI, at respective concentrations of 49.6 x 10^{-8} and 116.8 x 10^{-8} M, dissolved in 0.05 M Tris–HCl buffer, pH 7.5. For the assessment of thermal stability, these aliquots were incubated in a water bath at different temperatures (37, 40, 50, 60, 70, 80, 90 and 100 °C) for 30 min. Further aliquots were also incubated at 100 °C for different times (30, 60, 90, 120 and 180 min). After the thermal treatment, samples were cooled to room temperature (23 ± 3 °C) and the residual inhibitory activity was assessed as previously described for these enzymes. The stability after exposure to a range of pH values was evaluated according to Klomklao *et al.*[36]; for this purpose, aliquots of LsCTI were dissolved in different buffer solutions (0.1 M): glycine-HCl (pH 2), sodium acetate (pH 4), sodium phosphate (pH 6), Tris–HCl (pH 8), glycine-NaOH (pH 10) and sodium phosphate (pH 12). After incubation in each buffer for 16 h at 4 °C, the pH was adjusted to pH 7.5 and residual inhibitory activity for chymotrypsin and trypsin was assessed. To verify resistance in the presence of the reducing chemical agent DTT, we used the methodology proposed by Bezerra *et al.*[23] Aliquots were incubated at 37 °C with DTT at different final concentrations (0.001, 0.01 and 0.1 M) for different times (15, 30, 60 and 120 min). The reaction was terminated by the addition of a concentration of iodoacetamide equal to twice each DTT concentration. After treatments, samples were subjected to determination of residual inhibitory activity for chymotrypsin and trypsin. All experiments were run in triplicate and the results are the mean of three independent assays.

2.5 Activities against *Ae. aegypti*

2.5.1 Mosquitoes

Aedes aegypti (Rockefeller strain) eggs and larvae were obtained from Núcleo de Controle de Endemias Transmissíveis por Vetores/Secretaria de Saúde do Estado do Ceará (NUVET/SESA) and maintained at 27 ± 2 °C, with a photoperiod of 14:10 h light:dark. Larvae were fed *ad libitum* with turtle food from Alconpet (Camboriú, SC, Brazil) until the stage of third-or fourth-instar larvae for further assays.

2.5.2 In vitro inhibition of Aedes aegypti midgut proteases

To measure the inhibitory activity of LsCTI against *Ae. aegypti* midgut proteases, azocasein was used as a substrate for the enzyme reaction. To obtain *Ae. aegypti* proteases, 200 midguts were dissected from fourth-instar larvae, and transferred to microtubes containing 250 μL of 0.05 M Tris–HCl buffer, pH 7.5, in an ice bath; the midguts were then gently macerated and centrifuged at 10 000 ***g*** for 10 min at 4 °C, and the supernatant was collected and used in further enzymatic assays. Sixty microliters of enzymatic solution was incubated with different concentrations of LsCTI (0.15 x 10^{-6}, 0.30 x 10^{-6}, 0.60 x 10^{-6} and 0.90 x 10^{-6} M) and 340 μL of 0.05 M Tris–HCl, pH 7.5, containing 0.02 M CaCl$_2$ at 37 °C for 10 min. To start the reaction, 200 μL of 1% azocasein (w/v) in 0.05 M Tris–HCl buffer, pH 7.5, was added. After 30 min, the reaction was stopped by adding 300 μL of 20% TCA solution in distilled water. The mixture was centrifuged for 10 min at 10 000 ***g*** for 25 °C and 2 M of NaOH in distilled water was added to the supernatant at 1:2 (v/v). Absorbances were measured at 440 nm. As an enzymatic standard, the homogenate without LsCTI was incubated under the same conditions. All tests were run in triplicate and results are expressed as the mean of three independent assays.
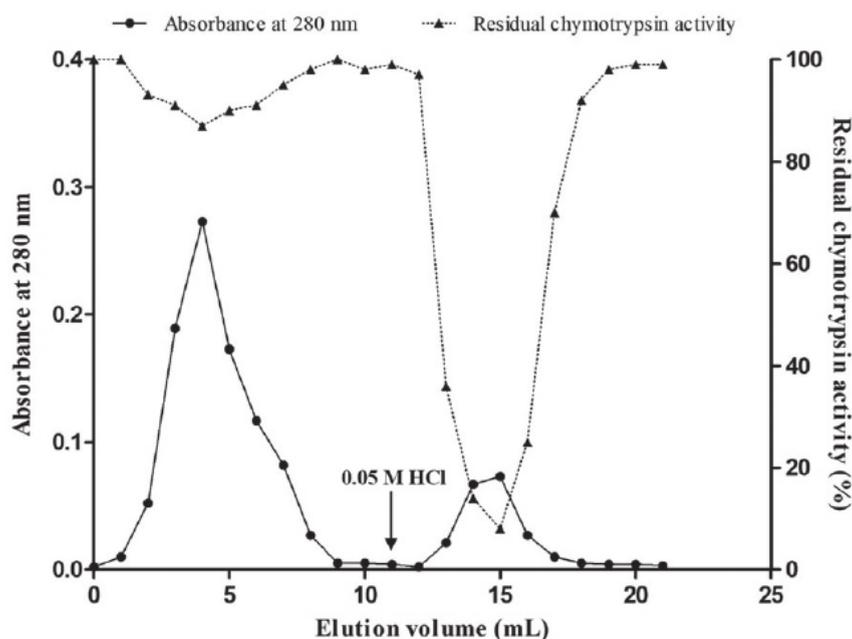
Figure 1. Chromatographic step on trypsin agarose column equilibrated with 0.05 M Tris-HCl pH 7.5 buffer of protein fraction obtained from the crude protein extract of *Lonchocarpus sericeus* seeds (LsF5). Adsorbed proteins were eluted by adding 0.05 M HCl (arrow tip). Fractions of 1.0 mL were collected at a flow rate of 0.5 mL min^{-1} and subjected to chymotrypsin inhibition assay.

2.5.3 Egg hatching

The effect of LsCTI on hatching of *Ae. aegypti* eggs was determined as described by Souza *et al.*[37] with slight modifications. Ten eggs of *Ae. aegypti* were selected on the basis of their integrity using a stereomicroscope (Luxeo 4Z; Labo America, Inc., Fremont, CA, USA) and placed into tubes containing 5 mL of LsCTI solubilized in distilled water [final concentration 0.3 mg mL^{-1} (3.38 x 10^{-5} M)]. Positive (0.3 mg mL^{-1} soybean Bowman–Birk inhibitor; 3.75 x 10^{-5} M) and negative controls (distilled water and 0.3 mg mL^{-1} bovine serum albumin) were also tested under the same conditions. Each sample was run in three replicates and results are presented as means.

2.5.4 Development assay

To evaluate the effect of LsCTI and its precursor protein fraction (LsF5) on *Ae. aegypti* development, we used the methodology proposed by Almeida Filho *et al.*[22] Ten eggs were gently placed into glass tubes containing 5 mL of 0.3 mg mL^{-1} LsCTI (3.38 x 10^{-5} M) or LsF5 solution (0.3 mg mL^{-1}), both in distilled water. A 0.3 mg mL^{-1} solution of the soybean Bowman–Birk inhibitor (BBI) (3.75 x 10^{-5} M) was used as a positive protein control and bovine serum albumin (0.3 mg mL^{-1}) as a negative, using the same solvent. Only distilled water was also used as a standard for development time. The number of hatched larvae after 48 h was recorded for treatments. The developmental stages, number of individuals and survival rates for each treatment were monitored for 11 days. To prevent microbial growth, larvae were transferred to a new test solution every 72 h and received a standard amount of turtle feed (0.2 mg per larva). The experimental results are presented as the average of ten independent replicates.

2.6 Statistical analysis

Results are expressed as mean ± standard deviation (SD) for each set of results. Analyses of variance (ANOVAs) were used followed by Bonferroni's test ($P < 0.05$) and/or Tukey's test ($P < 0.05$) to determine whether there was a significant difference among treatments.

3 RESULTS AND DISCUSSION

Plants are sessile organisms which are constantly exposed to environmental stress factors. In addition to these, they are also subject to constant attack from predators and pathogens. To overcome unfavorable conditions, plants have acquired evolutionary adaptations culminating in the development of bioactive compounds, some of which are toxic.[38] Among these compounds, protease inhibitors have been attracting considerable attention as a consequence of their involvement in plant defense and possible applications in plant engineering to increase resistance to pest insects and other pathogens. Regarding insects, inhibitors targeting chymotrypsin-like and trypsin-like proteases are of particular interest in view of their roles in insect nutrition, growth and development.[20,39,40] There are many reports on the presence of protease inhibitors in Fabacea family plants[21,41] and our group has previously described the presence of trypsin inhibitors in *L. sericeus* seeds[29], a legume belonging to Papilionoideae, which is a Fabaceae subfamily. In view of the biotechnological potential of protease inhibitors, we aimed to purify this novel inhibitor present in *L. sericeus* seeds and assess its insecticidal effect on *Ae. aegypti*, a multiple disease vector.

Before inhibitor purification, the crude protein extract from *L. sericeus* seeds was assessed for its chymotrypsin inhibition ability and the specific activity achieved for that protein sample was 1808.51 UI mg^{-1}. Next, the crude extract was subjected to protein precipitation by the addition of TCA to obtain the TCA–protein fraction (LsF5) which exhibited an activity of 5631.77 UI.mg^{-1}. This protein fraction was loaded into a trypsin-agarose column and two peaks were obtained; the first was eluted with equilibration buffer and the second eluted by applying 0.05 M HCl (Figure 1). Eluted

Table 1. Purification steps for *Lonchocarpus sericeus* chymotrypsin/trypsin inhibitor (LsCTI) from 1000 mg of defatted seed flour

Sample	Volume (mL)	Total protein (mg)	Total activity (CIU)[a]	Specific activity (CIU.mg^{-1})[b]	Yield (%)[c]	Purification index[d]
Crude protein Extract	8.8	181.88	328,931.92	1,808.51	100	1
LsF5	12.4	22.92	129,102.67	5,631.77	12.60	3.11
LsCTI	10.4	1.60	23,925.97	14,935.06	0.88	8.26

[a] Inhibitory activity on bovine chymotrypsin. One unit of chymotrypsin inhibitor activity was defined as the amount of inhibitor that caused a 0.01 decrease in absorbance (410 nm), at 37 °C after 30 min.
[b] Specific activity was calculated using the ratio of total inhibitory activity (CIU) to total protein content (in milligrams).
[c] Yield was based on protein recovered after each purification stage relative to the crude protein extract content.
[d] Purification was measured as the ratio between the specific activity in the purification stage and the specific activity of the crude protein extract.
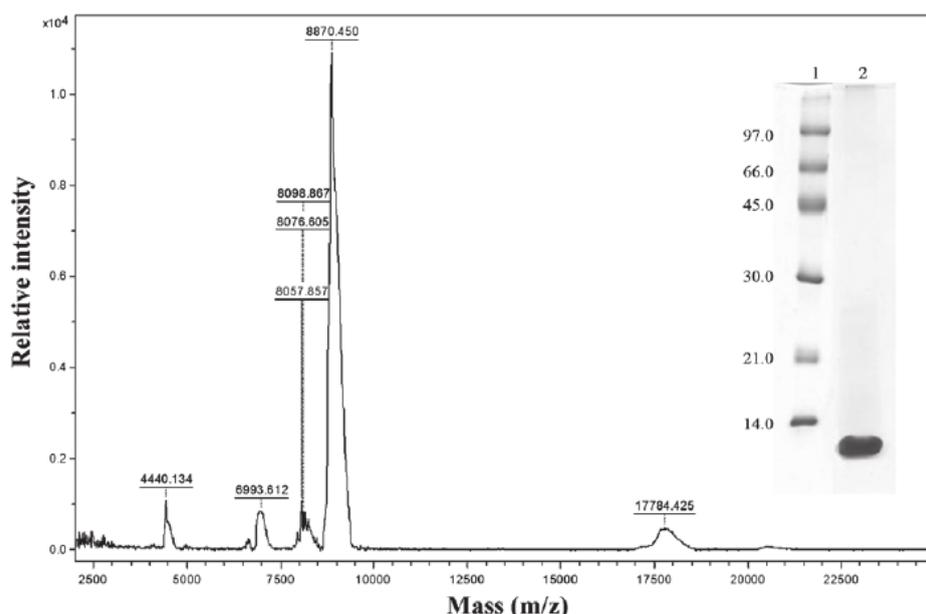
Figure 2. Mass spectra (MALDI-TOF-MS) of the *Lonchocarpus sericeus* chymotrypsin and trypsin inhibitor (LsCTI) under native conditions revealed a major peak at 8870.45 Da. Inset: SDS-PAGE (15% w/v) profile of LsCTI: (1) molecular mass markers (kDa); (2) LsCTI.

fractions from the second peak were pooled (LsCTI) and exhibited a higher specific activity of 14 935.06 UI mg^{-1}. LsCTI was purified to 8.26-fold based on its to chymotrypsin inhibitory activity. The purification is summarized in Table 1.

According to Carvalho *et al.*,[29] *L. sericeus* seeds have a high content of protein, around 35% on a dry weight basis; similarly, we observed a high content of soluble proteins in the crude extract, 18% on a dry weight basis. This finding reinforces our hypothesis that *L. sericeus* is a good source of proteins with biological activity. To date, no chymotrypsin inhibitor has been reported for this plant. Among the bioactive proteins already reported are lectin, trypsin inhibitor and urease.[29,42] The purification of LsCTI by a single chromatography step achieved a 0.88% protein yield. LsCTI recovery was lower than those reported for *Butea monosperma* (7.7%)[43] and *Artocarpus heterophyllus* (7.1%)[44] protease inhibitors, but higher than that of *Pipitadenia moniliformis* (0.15%).[45]

In order to characterize this novel inhibitor, we subjected the molecule to many assays. Electrophoresis of LsCTI showed a protein band with an apparent molecular mass < 14 000 Da (Figure 2B) and when it was analyzed by mass spectrometry (MALDI-TOF-MS) under native conditions we obtained a spectrum (m/z) with a major peak at 8870.45 Da (Figure 2A).

The molecular mass observed for LsCTI was similar to those of other chymotrypsin inhibitors reported, such as the black eyed-pea chymotrypsin inhibitor (BCTI), from *Vigna unguiculata* (9.1 kDa) and the wild emmer chymotrypsin inhibitor (WeCl) from *Triticum dicoccoides* (13 kDa).[46,47] It is well known that protease inhibitors generally have more than one isoform.[48,49] To verify the presence of isoforms, LsCTI was subjected to a 2D electrophoresis process and in the obtained profile four isoforms were verified with isoelectric points ranging from 4.19 to 4.92 (Figure 3). Morrison *et al.* have described the presence of isoforms of trypsin inhibitors in pea (*Pisum sativum* L.) seeds,[50] which had distinct isoelectric points ranging from 4.6 to 7.6. Ee *et al.*[49] described three isoforms and respective isoelectric points varying between 4.27 and 5.13 for trypsin inhibitors present in *Acacia victoriae* seeds. The presence of multiple isoforms is a common phenomenon which can be explained by post-translational modifications or gene duplication phenomena.[25] As a practical example, insects have biochemical mechanisms enabling them to escape from inhibitor insecticidal activity, while plants, during prey–predator co-evolution, developed strategies to overcome insect adaptations. Such examples suggest the presence of more than one isoform, which, although quite similar to each other, have different characteristics such as different isoelectric points, inhibitory capacities and resistance to digestion.[50,51]

Enzyme inhibitors may show different enzyme-specific inhibitory activities. A few inhibitors are specific for chymotrypsin
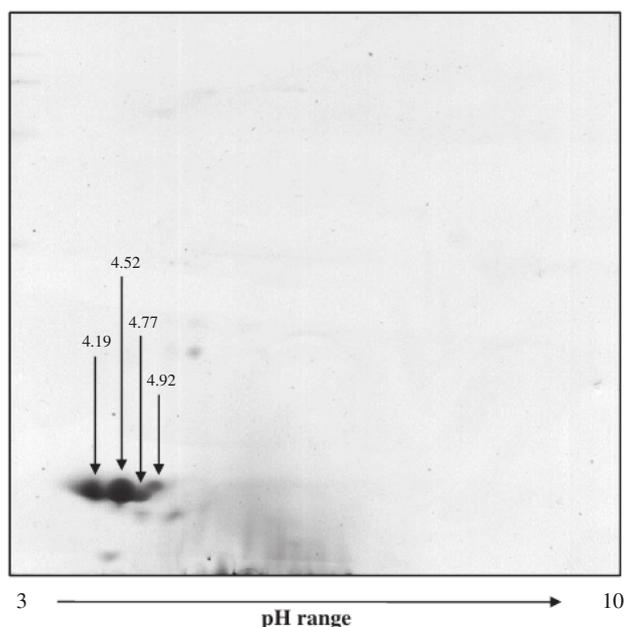
Figure 3. Electrophoretic profile on SDS-PAGE (15% w/v) of LsCTI after isoelectric focusing on a 7-cm gel strip with an immobilized pH range (3–10). Arrows indicate the isoelectric points for the observed isoforms.

Table 2. Enzyme-specific inhibitory activity of LsCTI toward different enzyme classes

Enzyme class		Inhibition[a](%)
Serine	Chymotrypsin	92 ± 0.5
	Trypsin	49 ± 1.2
	Neutrophilic elastase	25 ± 1.6
	Pancreatic elastase	13 ± 0.7
Cysteine	Bromelain	0
	Papain	0
Glucosidase	Pacreatic α-amylase	0

[a] Values are mean $\pm$ standard deviation of three independent assays relative to an enzyme standard.

but do not inhibit trypsin,[19] whereas others are specific for trypsin but do not exert activity on chymotrypsin.[21] Some are potent trypsin inhibitors and also inhibit chymotrypsin, such as the inhibitor purified from *Derris trifoliata* seeds,[48] or have the ability to inhibit enzymes of different classes, such as the *A. pavonina* kunitz trypsin inhibitor (ApKTI), which inhibits trypsin and papain.[41] Tests for the enzyme inhibition specificity of LsCTI were performed for three enzyme classes (serine and cysteine proteases and glucosidase). Among the enzymes tested, LsCTI was able to inhibit only serine proteases (Table 2), showing higher values for chymotrypsin and trypsin. Interestingly, the chymotrypsin inhibitor from *T. dicoccoides* (WeCI) did not exert inhibitory activity toward trypsin but was able to promote inhibition of α-amylase, and thus was characterized as a bifunctional inhibitor.[47]

Considering that LsCTI is an inhibitor of multiple serine proteases which acts preferably towards chymotrypsin and trypsin, we performed the following characterization assays by using only those two enzymes. To characterize the type of inhibition exerted by LsCTI on these enzymes, kinetic assays were performed. Analyzing the generated Lineweaver–Burk diagrams, we observed that LsCTI was a noncompetitive inhibitor for both chymotrypsin (Figure 4A) and trypsin (Figure 4B) since K_m and V_{max} values were altered. This type of inhibition was reported for the *Tamarindus indica* trypsin inhibitor (TTI)[52] and ApTI from *A. pavonina*.[53] Although LsCTI showed the same inhibition type for both tested enzymes, different enzymatic affinities were previously noted. This was confirmed by constructing Dixon plot diagrams to determine the inhibition constant (K_i), which was 8.24×10^{-8} and 7.61×10^{-7} M for chymotrypsin (Figure 4C) and trypsin (Figure 4D), respectively. The lower K_i value for LsCTI obtained for chymotrypsin is consistent with its higher affinity for that enzyme. The LsCTI K_i value for trypsin was higher than those for BmPI from *B. monosperma* (1.2×10^{-9} M)[43] and IVTI from *Inga vera* (1.19×10^{-9} M)[23], but the K_i value for chymotrypsin was similar in magnitude to those for the inhibitor from *Ricinus communis* (1.9×10^{-8} M)[54] and AHLTI from *A. heterophyllus* (3.47×10^{-8} M).[44]

An important step in identifying proteins that might be used as biotechnological tools is to determine their optimal activity conditions, especially optimal ranges of temperature and pH, as well as their stability against chemicals, such as DTT, which can reduce the number of disulfide bonds. Protease inhibitors without disulfide bonds are more susceptible to variations of pH and temperature.[55] Disulfide bonds play a well-known role in the stabilization of protease inhibitor structure and biological functions.[33] Before incubating LsCTI with different concentrations of DTT, we determined the minimum amount of LsCTI required to achieve maximum inhibition. We obtained LsCTI values of 49.6×10^{-8} and 116.8×10^{-8} M for chymotrypsin and trypsin, respectively. After DTT incubation, the inhibitory activity of LsCTI towards chymotrypsin and trypsin was reduce, this reduction increasing as the DTT concentration increased. The lowest DTT concentration (1×10^{-3} M) did not affect the activity even after 120 min of incubation, whereas at 10×10^{-3} M only 30 min of incubation was necessary to reduce the inhibitory activity almost totally for chymotrypsin (Figure 5A) and totally for trypsin (Figure 5B). Although the LsCTI reduction caused by higher DTT concentrations led to loss of activity for both enzymes assessed, the molecule was more resistant to reduction than the *Clitoria fairchildiana* protease inhibitor (CFPI), which was found to lose 77% of its inhibitory activity for trypsin and 44% for chymotrypsin after 120 min of incubation with DTT (1×10^{-3} M),[56] and BgPI from *Vigna mungo*, which completely lost its activity after 15 min at the same DTT concentration.[57] The incubation of LsCTI with DTT (10×10^{-3} M) for 30 min caused complete loss of its inhibitory activity on trypsin and a > 80% loss for chymotrypsin. This finding suggests the existence of more disulfide bonds near the anti-trypsin reactive site than near the anti-chymotrypsin reactive site. Similar findings have been reported for CFPI from *C. fairchildiana*[56] and horsegram BBI from *Dolichos biflorus*.[58]

When exposed to treatment with a range of pHs, LsCTI was stable, with none of the pHs tested being able to reduce its inhibitory activity on chymotrypsin (Figure 5C) and trypsin (Figure 5D). Similarly, the protease inhibitor from *T. dicoccoides* (WeCI) was shown to be stable in a wide pH range (2–12)[47] but, in contrast, the BmPI from *B. monosperma* was shown to maintain activity only in a pH range of 5–10.[43] The stability of LsCTI after heat treatment was remarkable; the molecule was able to maintain its inhibitory activity at all temperatures tested (37–100 ℃) (Figure 5E), even when heated at 100 °C for 180 min (Figure 5F), for both chymotrypsin and trypsin. Protease inhibitors with disulfide bonds generally are stable to thermal and pH variations. In contrast to the higher thermal stability of LsCTI, the BBI from *Vicia faba* was stable only until 60 ℃[59] and PpyTI from *P. pyramidalis* until 70℃.[24] Similar to
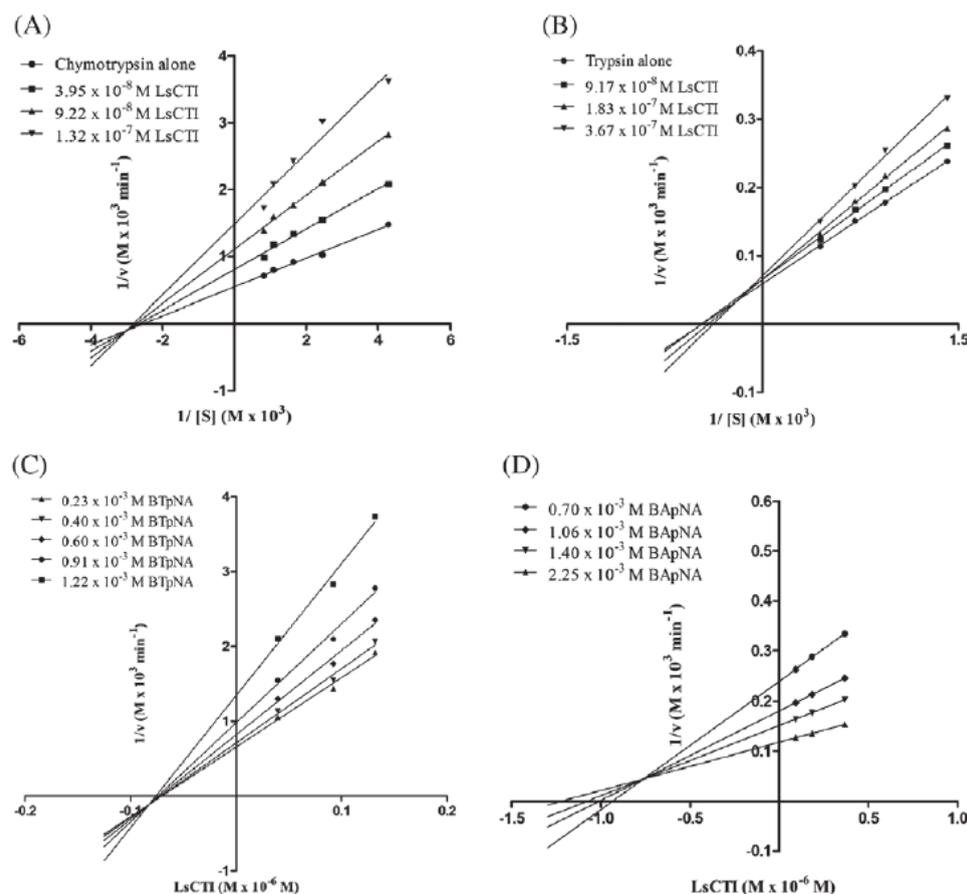
Figure 4. Inhibition kinetics. (A, B) Lineweaver–Burk plot analysis of the inhibitory activity of LsCTI toward serine enzymes (A) chymotrypsin and (B) trypsin, representing the noncompetitive inhibition type. (C, D) A Dixon plot for determination of the LsCTI dissociation constant (K_i) at different substrate concentrations for (C) chymotrypsin and (D) trypsin; the calculated K_i was 8.24×10^{-8} and 7.61×10^{-7} M, respectively.

the results for LsCTI, the BBI from soybean retains its inhibitory activity even after heating at 100 °C.[60] Protease inhibitors from Fabaceae plants are generally stable up to 80 °C; however, there are cases of stability loss above this temperature.[61] The stability of these protease inhibitors may be associated with the rigidity of their tridimensional structure and the number of disulfide linkages. Together, these factors allow the inhibitors to undergo slight conformational changes and yet be able to renature after exposure to adverse conditions.[21]

Among serine, cysteine, aspartic and metallo-protease inhibitors, the first type are the most common and best studied.[62] Within serine protease inhibitors, there are two families that should be highlighted, Kunitz and Bowman–Birk. These families are abundant in various legumes.[56] The characteristics of LsCTI, such as its low molecular mass (8.87 kDa), its inhibitory activity towards several serine enzymes and its remarkable stability in the presence of denaturing agents, suggest that the inhibitor is a novel BBI. Furthermore, *L. sericeus* belongs to Papilionoideae, the most evolutionarily derived Fabaceae subfamily which typically expresses BBIs in contrast to other subfamilies expressing Kunitz-type inhibitors.[45] According to Domoney *et al.*,[63] BBIs are products of multigene families, justifying the existence of multiple isoforms, as we have shown for LsCTI. Similarly, other BBIs have been purified from Papilionoideae species and have been showed to have isoforms, such as *P. sativum*[63] and *Lens culinaris*.[64]

Plant protease inhibitors can have diverse physiological functions, such as regulation of self-proteases, sulfur amino acids

reserve and defense against herbivorous animals such as insect pests. Those proteins can bind to insect proteases, blocking their activity, which can impair the absorption of nutrients necessary for growth, development and survival.[39] There are many reports, both *in vitro* and *in vivo*, on the activity of protease inhibitors on larval gut enzymes. Silva *et al.*[54] reported the *in vitro* inhibition of *Ae. aegypti* larvae midgut proteases by a trypsin inhibitor from *R. communis* and Sasaki *et al.*[11] demonstrated the *in vivo* inhibition of *Ae. aegypti* enzymes after incubation of larvae in solutions of ApTI, an inhibitor from *A. pavonina*. In order to characterize the insecticidal activity of LsCTI, we firstly evaluated its ability to inhibit *in vitro* the midgut proteases extracted from *Ae. aegypti* larvae (Figure 6). This inhibitor at the highest tested concentration (0.90×10^{-6} M) was able to bind insect proteases and block their activity, causing about 60% inhibition, and the calculated IC_{50} was 4.7×10^7 M. The inhibition value was similar to that reported for the *C. leiandra* trypsin inhibitor (CITI)[21], but lower than that reported for the *R. communis* trypsin inhibitor.[54] The IC_{50} was 10-fold lower than that of CITI, suggesting that LsCTI had more affinity for these insect midgut proteases and may have insecticidal activity against immature developmental stages.

Chymotrypsin- and trypsin-like proteases are present in insect orders such as lepidopterans and dipterans. These enzymes have been reported to be involved in protein digestion for nutrition and development throughout the *Ae. aegypti* life cycle.[65,66] After LsCTI had been demonstrated *in vitro* to inhibit the midgut enzymes of
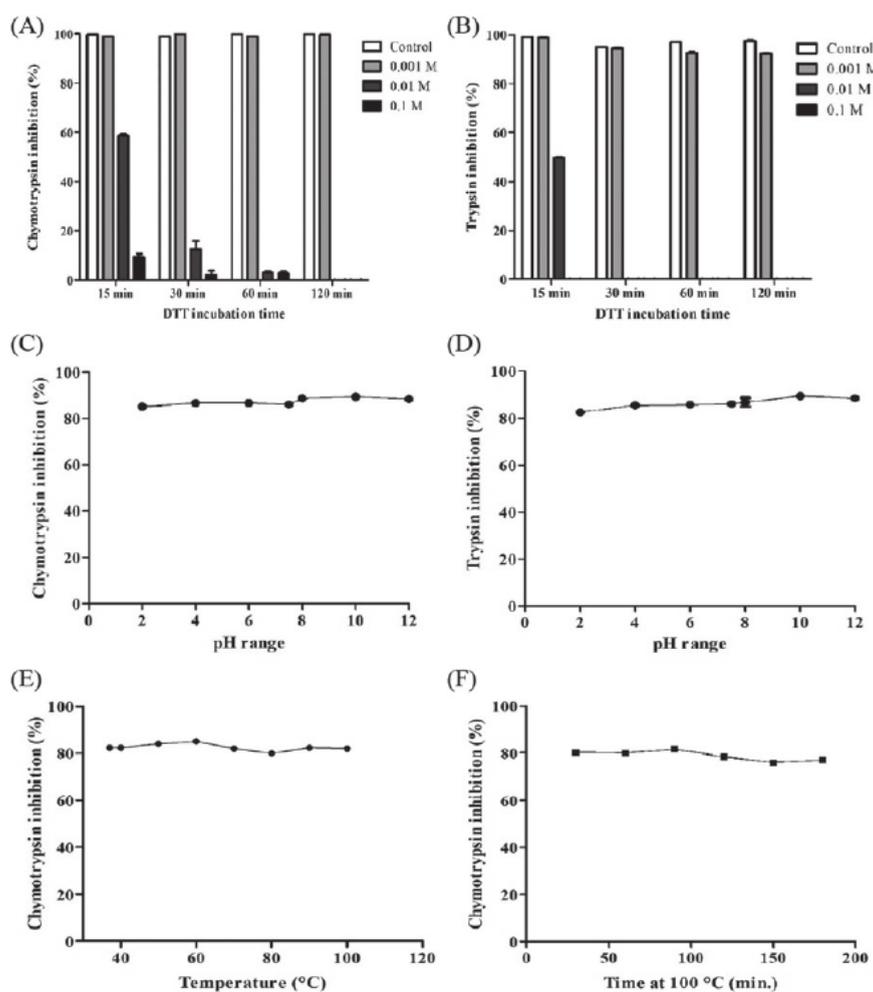
Figure 5. LsCTI inhibitory stability (A, B) after incubation with different DTT concentrations for different times at 37 °C and (C, D) after incubation at different pHs for 16 h at 4 °C. (E) Thermal stability after 30 min of incubation at different temperatures and (F) at 100 °C for different times for chymotrypsin and trypsin. Bars correspond to SD from triplicate measurements and (*) indicates a significant difference by Bonferroni test (p < 0.05).

the larvae of this mosquito, *in vivo* assays were performed to determine how this inhibitor affects mosquito development. For the *in vivo* assays, both LsCTI and its precursor protein fraction (LsF5) were used. The *Ae. aegypti* egg hatchability was about 80% and was not affected by incubation in LsCTI or LsF5 solutions (0.3 mg mL^{-1}) for 48 h, being similar to that for protein controls. Similarly, other protease inhibitors did not affect egg hatchability, such as CITI[21] and the *Moringa oleifera* flowers trypsin inhibitor (MoFTI).[12] In contrast, a protease inhibitor from *L. leucocephala* inhibited egg hatching by 50% in the same conditions.[22] Other classes of proteins have also been reported to inhibit egg hatchability, such as lectins[67] and cysteine proteases.[68]

Although LsCTI did not affect egg hatching, this inhibitor was able to delay larval development and to cause high larval mortality. The delays produced by bioactive proteins LsCTI, LsF5 and BBI were 25, 12.5 and 37.5%, respectively. Interestingly, the innocuous protein assessed (BSA) reduced the time required for emergence of adult mosquitoes, generating a negative value for development time (Table 3). After 96 h of treatment, larvae were observed visually, and protease inhibitor (BBI and LsCTI)-treated larvae (Figure 7C and 7D) were found to be smaller and to have underdeveloped external structures, such as the cuticle and siphon, compared with the control (Figure 7A) and LsF5-treated
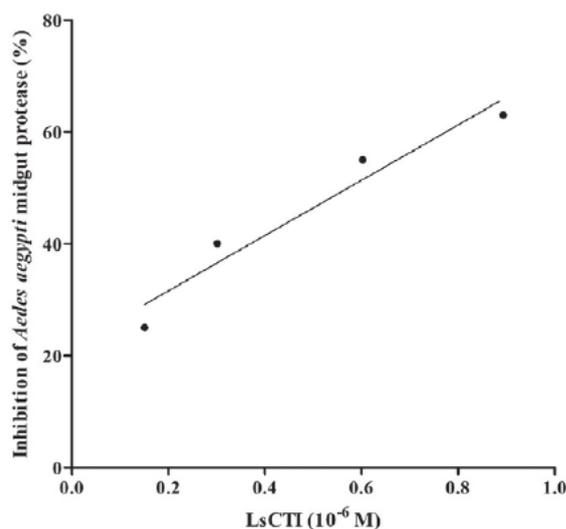

Figure 6. Inhibition of *Aedes aegypti* midgut digestive proteases by different concentrations of LsCTI. The inhibition percentage was calculated with respect to midgut proteases in the absence of inhibitor. The IC$_{50}$ value was determined as 4.7 x 10^{-7} M LsCTI.

Table 3. Effect of LsCTI on the development of *Aedes aegypti* larvae derived from eggs treated with aqueous solution (0.3 mg mL^{-1}). The mortality, number of survivors at different stages and delay in development were determined after 11 days of exposure. Bovine serum albumin, a soybean Bowan–Birk inhibitor and LsF5 were used under the same conditions. Distilled water was used as a control. The mortalitty values are presented as the mean and standard deviation of ten replicates

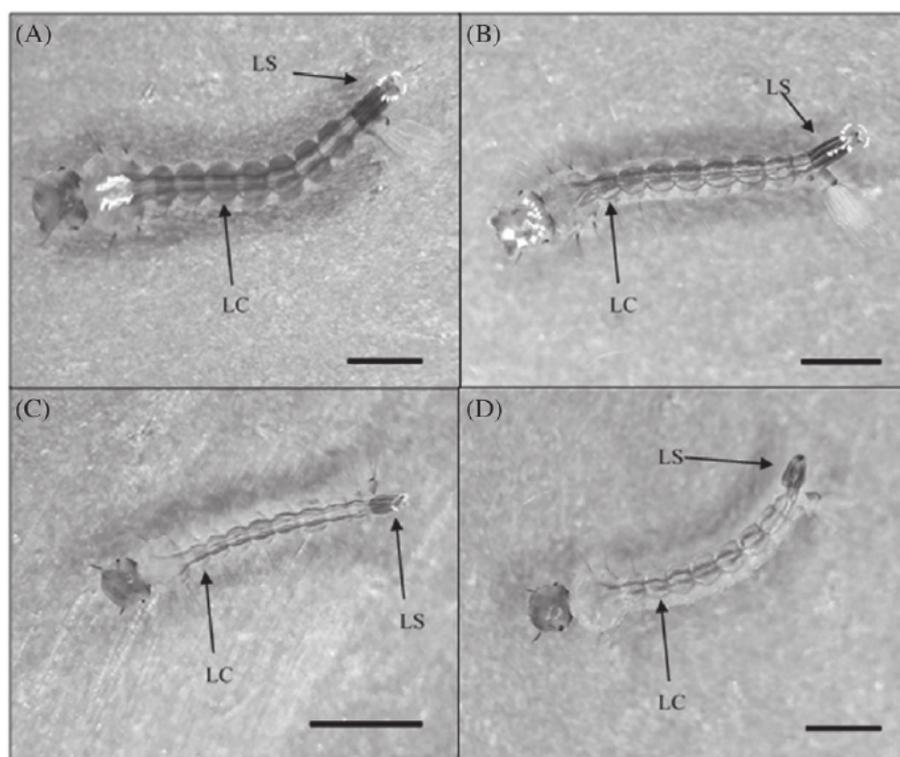|		*Aedes aegypti*								
	Number of individuals 11 days after egg treatment	Number of survivors at different stages						Larval mortality (%)	Time to first mosquito hatching (days)	Percentage delay in development time
Treatment		Larval instars				Pupae	Adults			
		L1	L2	L3	L4					
BSA	76	0	0	0	5	14	57	6.4 ± 9.3^a	7	−12.5
BBI	33	0	0	0	6	7	20	55.4 ± 17.0^b	10	25
LsF5	60	0	0	0	13	17	30	19.4 ± 12.0^a	9	12.5
LsCTI	20	0	0	0	0	13	7	76.9 ± 11.2^b	11	37.5
Control	76	0	0	0	6	14	56	8.0 ± 10.3^a	8	0

Figure 7. External morphology of *Aedes aegypti* larvae after 96 h incubation time in: (A) Distilled water; (B) *Lonchocarpus sericeus* protein fraction (LsF5); (C) soybean Bowman–Birk inhibitor, and (D) LsCTI. All protein samples were used at 0.3 mg mL^{-1}. Protein-exposed larvae (B–D) showed negative effects produced by the proteins in their development, including impaired development of structures such as less thickening of larval cuticle (LC) and reduced growth of larval siphon (LS), indicated by arrows. The bar is 1 mm.

larvae (Figure 7B). In agreement with our results, others authors have reported the ability of protease inhibitors, such as LTI [22] and MoFTI,[39] to delay *Ae. aegypti* larval development.

In our study, BSA treatment apparently provided an additional source of proteins which supplied more nutrients for larval growth and acceleration of their development. In contrast, the chronic ingestion of inhibitors may have impaired protein digestion and uptake of essential nutrients, such as amino acids, and this could have delayed insect development, as suggested by Paiva *et al.*[69] Furthermore, the tight binding of protease inhibitors to insect digestive proteases can trigger an adaptive response, where insects change their protease profile to evade inhibitor action or enzyme overproduction can be elicited to maintain digestive and absorption rates, but sometimes it may decrease the availability of sulfur amino acids.[43]

The mortality rate calculated at the end of 11 days as the ratio of survivors to hatched larvae was not significantly different ($P < 0.05$) among bioactive proteins. Although the protein fraction (LsF5) caused a delay in development, the mortality rate produced by that fraction ($19.4 \pm 12.0\%$) was not significantly different (p < 0.05) compared with BSA ($6.4 \pm 9.3\%$) and distilled water ($8.0 \pm 10.3\%$) (Table 3). LsF5 contains 7% of LsCTI and was able to delay larval development; however, it did not cause as high a mortality rate as the purified inhibitor did. This suggests that LsCTI is the active principle responsible for causing larval death.

Protease inhibitors have frequently been reported to have deleterious effects on insect larvae, such as reducing the growth rate, increasing mortality and causing severe deformations.[69] In our study, we observed all these deleterious effects. Similarly, Sasaki *et al.*[11] reported that the incubation of *Ae. aegypti* larvae with ApTI (0.25 mg mL^{-1}) caused about 80% larval mortality and hypertrophy of the gastric caeca cells; the latter may be related to the overexpression of digestive enzymes. The mortality rate for *Ae. aegypti* larvae treated with LsCTI (0.3 mg ml^{-1}) was higher than those described for trypsin inhibitors from *M. oleifera* flowers and from *C. leiandra*, which caused 47 and 44% mortality, respectively, at the same concentration.[39,21] The insecticidal effect of serine protease inhibitors such as LsCTI is not limited to dipteran insects; there are several studies reporting effects of protease inhibitors on insects belonging to lepidopterans[24,43,56] and, less commonly, coleopterans.[23]

Our findings suggest that LsCTI is a BBI-like inhibitor with promising insecticidal activity against *Ae. aegypti*, as it was able to inhibit insect midgut proteases and to cause high mortality after chronic exposure even at low concentrations. It is also important to point out that interrupting or delaying the insect life cycle, such as that of *Ae. aegypti*, is an important strategy to reduce the vector population.[21] In addition, our results contribute to a better understanding of the insecticidal potential of plant protease inhibitors and in particular suggest that *L. sericeus* may be a source of bioactive proteins. Therefore, LsCTI may be employed as a biotechnological tool in strategies for *Ae. aegypti* control.

Different letters indicate a significant difference by Tukey's test ($P < 0.05$).

ACKNOWLEDGEMENTS

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