



Functional and structural characterization of an ecotin-like serine protease inhibitor from *Trypanosoma cruzi*

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ABSTRACT

Ecotin, a serine peptidase inhibitor (ISP), discovered in *Escherichia coli*, inhibit a wide range of trypsin-like serine peptidases, protecting microorganisms from the host's immune response. In eukaryotes, ISPs encoding genes were found only in Trypanosomatidae protozoa, including the genus *Trypanosoma*, which harbors *Trypanosoma cruzi*, the ethiological agent of Chagas' disease. *T. cruzi* encodes the ISP2 Trypanosomatidae orthologous, which in *Leishmania* species present inhibitory activity on mammalian proteases from S1A family suggesting its role in vertebrate-host-parasite interactions. In this study, the structural and biochemical characterization of the recombinant *T. cruzi* ISP2 (rTcISP2), produced in *E. coli* was purified in soluble form and analyzed by circular dichroism, fluorescence spectroscopy, native electrophoresis, dynamic light scattering, low X-ray scattering and homology modeling. The obtained data revealed that rTcISP2 was biologically active and forms homodimers in solution. Furthermore, inhibitory activity of rTcISP2 against human neutrophil elastase (HNE) is the highest among ISP2 orthologous from bacteria and trypanosomatids. The role of NE to control *T. cruzi* parasites through modulation of cellular and humoral innate immune responses in vertebrate hosts, make TcISP2 a key molecular component for parasite infection efficiency, providing a useful basis for investigation of host-parasite interactions and the potential of TcISP2 for biotechnological applications.

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1. Introduction

Despite several advances in medicine, neglected tropical diseases still affect more than one billion people worldwide, especially in places with inadequate infrastructure. Among the several approaches to eradicate these diseases, to know the mechanisms of pathogenicity and more specifically, the virulence factors of the agents that transmit these infections are very useful for both the development of new drugs and for more accurate and accessible diagnostic tools [1,2].

Chagas' disease, caused by the protozoan *Trypanosoma cruzi*, is an important Latin American neglected tropical disease, affecting approximately eight million people [3]. The disease is transmitted mainly by triatomine bugs and other forms of infection correspond to blood and organ transfusions, congenital transmission, contaminated food and beverages, and laboratory accidents. The clinical characteristics of

patients after *T. cruzi* infection vary from absence of symptoms, to acute and chronic diseases and can lead to death, although it is very rare (< 1% of the cases). The chronic phase, which starts two weeks after contact with the parasite, lasts for life. In around 30% of chronic Chagas' disease patients, the disease signs include progressive cardiomyopathy and/or motility disturbances of the gastrointestinal tract. Benznidazole and nifurtimox are the only drugs currently available, and despite its adverse effects, they have the greatest chances of curing the disease, especially during the acute phase. Chagas' disease also has serious socio-economic consequences, because it is a debilitating and disabling chronic illness with little chance of cure [4]. Thus, the search for new drug and treatment targets are of great importance.

The life cycle of *T. cruzi* parasites is complex with differentiation into four principal developmental stages. During the triatomine blood meal on a *T. cruzi* infected mammalian host, the trypomastigotes present in the bloodstream are ingested and in the insect gut they differentiate into a replicative form named epimastigote, which differentiate into metacyclic trypomastigotes in the insect rectum. This form of the parasite can enter into a mammalian host through a wound produced, by scratching, when the infected bug takes a blood meal and defecates. Metacyclic trypomastigotes differentiate into replicative amastigotes

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once they enter the cells in the vicinity of the insect bite. After destroying the host cell, the parasites differentiate in trypomastigotes, which invade new cells through bloodstream, perpetuating the infection. During these processes, a number of proteolytic activities have been described to be essential to the parasite interactions with different cells types and also to escape from the hosts' innate and specific defense mechanisms [5]. Considering the importance in *T. cruzi* infection mechanisms, molecules involved in the proteolytic parasite pathways are targets for Chagas' disease treatment.

There are different approaches currently being adopted in research for a new chemotherapy for Chagas' disease. One approach is to study the virulence factors of microorganism and develop tools to minimize its action and even control the infection [6]. A previous study demonstrated that among several genes found in microbes, ecotin is present in a diversity of pathogens, being a good target as a virulence factor [7,8].

Ecotin is a serine protease inhibitor from the periplasm of *Escherichia coli* bacteria and it was described by Chung *et al* in 1983 [9]. Proteins homologous to ecotin were later found in other bacteria and protozoa. Studies have pointed out that ecotin does not inhibit endogenous proteases in *E. coli*, and is likely to play a role in protecting the bacterium against proteolytic attacks [10]. Ecotin is a potent and promiscuous inhibitor of proteases from S1A family, having great importance in the virulence of pathogens. Trypanosomatids have ecotin-like genes, generally called serine protease inhibitors (ISP). Absence of S1A homologous proteases in trypanosomatids indicates its action on extracellular molecules from host. Besides, the protein derived from ISP encoding genes strongly inhibits mammalian proteases such as trypsin, chymotrypsin, neutrophil elastase (NE), and plasma coagulation factors. The role of this protein in the virulence of trypanosomatids, especially in *Trypanosoma* and *Leishmania* genera parasites have been recognized yet not been fully elucidated [11–13].

In this study, we report, for the first time, the cloning, recombinant expression and purification of an ISP-like from *T. cruzi*, highly similar to the ISP2 from *Leishmania* species and thus been named as *T. cruzi* ISP2 (TcISP2). After purification, recombinant TcISP2 (rTcISP2) was analyzed using biophysical and biochemical techniques. Furthermore, we investigated the ability of this protein to inhibit different proteases. The rTcISP2 has potential for biotechnological applications, especially in diagnostics and as a target for new drugs development.

2. Material and methods

2.1. Cloning, recombinant expression and purification of the TcISP2

Plasmid construction was performed following conventional protocols. The TcISP2 encoding gene was amplified by polymerase chain reaction (PCR) employing a thermal cycler, using as a DNA template the genomic DNA of *Trypanosoma cruzi* from Y strain. The DNA encoding the TcISP2 protein was amplified with primers (ISP2r-Tcruzi_Nhe_fow 5' AACGCTAGCATGTGAGAGTTCATTTTC 3' and ISP2r-Tcruzi_Hind_rev 5' TATAAGCTTTTATATGTCAATTCGAATTGG), using the Platinum Taq High Fidelity DNA Polymerase (Invitrogen, USA), producing a fragment of 444 bp flanked in its 5' and 3' boundaries by sequences for the restriction enzymes *NheI* and *HindIII*, respectively. The amplification product was cloned into the *NheI* and *HindIII* restriction sites of the expression vector pET28a(+), which inserts an N-terminal histidine tag (His-tag) into the recombinant protein, and selected by kanamycin resistance after the transformation of the chemically competent *E. coli* Mach-T1 (Thermo-Scientific/Invitrogen, USA). The integrity of recombinant clones were confirmed by PCR, restriction digest and sequencing analysis, by Sanger's method using BigDye Terminator v.3.1 (Thermo-Scientific/Applied Biosystems, USA) and an automated ABI 3100 Genetic Analyzer (Applied Biosystems, USA) following manufacturer's instructions.

The recombinant construct was named pET28-ISP2r-Tcruzi and it was used to transform the chemically competent cells BL21(DE3) (Novagen, Brazil). A total of 1 mL of an overnight *E. coli* BL21(DE3) pET28-ISP2r-Tcruzi saturated culture was inoculated into 1 L of LB medium containing kanamycin (50 µg/mL). The culture was agitated (250 rpm) at 37 °C until attaining an optical density of 0.6 at 600 nm. At this point, the culture was induced with 1 mM of IPTG, incubated overnight at 18 °C and harvested by centrifugation at 5000 ×g for 20 min at 4 °C. The cells were diluted into 20 mM phosphate buffer adjusted at pH 7.4 (buffer A), and disrupted using 4 mg of lysozyme (Sigma, USA) per mL for 20 min at room temperature and then, the same volume of lysis buffer (20 mM phosphate buffer pH 7.4, 50 mM KCl, 1 mM EDTA, 0.5% Tween 20%) was added to the content and incubated for 30 min at 37 °C. The suspension was centrifuged at 5000 ×g for 20 min at 4 °C to separate the cell debris.

The supernatant containing the recombinant ISP2 protein from *T. cruzi* (rTcISP2) was applied into a nickel-affinity column equilibrated with buffer A. After washing out the unbound proteins with ten column volumes of buffer A containing 20 mM and 50 mM of imidazole, the rTcISP2 was eluted from the column with buffer A containing 250 mM imidazole. After exhaustive wash with PBS buffer for the elimination of imidazole, the rTcISP2 protein was concentrated to a final volume of 500 µL using a 10 KDa Vivaspin 2 filter (Sartorius, Germany). Elution was carried out at room temperature with a flow rate of 0.5 mL/min in buffer A and the fractions, of 1 mL each, were analyzed using a 12% SDS-PAGE (Fig. S1).

Purified rTcISP2 was analyzed by denaturing and native 12% SDS-PAGE, after dilution in loading buffer (100 mM Tris-Cl (pH 6.8), 4% SDS, 0.2% bromophenol blue with and without DTT, respectively), and by Western blotting utilizing a monoclonal anti-histidine antibody (Sigma, USA) diluted 1:2000. Colorimetric detection after incubation with IgG anti-mouse alkaline phosphatase conjugate (Sigma, USA, cat. A-3562) and the substrate solution 5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium chloride (NBT-BCIP, Bio-Rad, Hercules, CA, USA) were performed according to manufacturer's instructions.

2.2. Enzymatic evaluation of rTcISP2

The activity inhibition of rTcISP2 was measured by the remaining hydrolytic activity of proteolytic enzymes (bovine trypsin and human neutrophil elastase – HNE) on the specific substrates at pH 8.0 after pre-incubation with the rTcISP2 for 10 min at 37 °C [14]. The concentration of active trypsin was determined by active site titration with *p*-nitrophenyl-*p*-guanidine-benzoate as described by Chase and Shaw [15]. The equilibrium dissociation constants of complexes between the bovine trypsin and HNE with rTcISP2 were determined using the method described by Bieth [16]. Briefly, the serine proteinases were incubated with different concentrations of inhibitors in 0.1 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl and 0.1% Triton X-100 at 37 °C. The residual enzyme activities were measured after addition of the fluorogenic substrates Z-Phe-Arg-AMC (Sigma-Aldrich, USA) for trypsin, and elastase substrate V (Calbiochem, Merck, Germany) for HNE. Apparent *K_i* values were calculated by fitting the steady-state velocities to the equation $(V_i/V_o = 1 - \{Et + It + K_i - [(Et + It + K_i)^2 - 4Et*It]^{1/2}\}/2Et)$ for tight-binding inhibitors using a non-linear regression analysis [17].

2.3. Structural characterization of rTcISP2

Circular dichroism (CD) spectrum was measured employing a Jasco J-815 spectropolarimeter at 20 °C, over the wavelength range from 195 nm to 270 nm. The rTcISP2 concentration was 10 µM in 20 mM Tris-HCl buffer at pH 8 loaded into a 0.1 cm quartz cuvette. Thermal denaturation was monitored by measuring the ellipticity changes at 215 nm, induced by a temperature increase from 20 °C to 80 °C. Fraction of denatured protein (α): $\alpha = (\theta_N - \theta_{obs})/(\theta_N - \theta_D)$, in which θ_{obs} is the

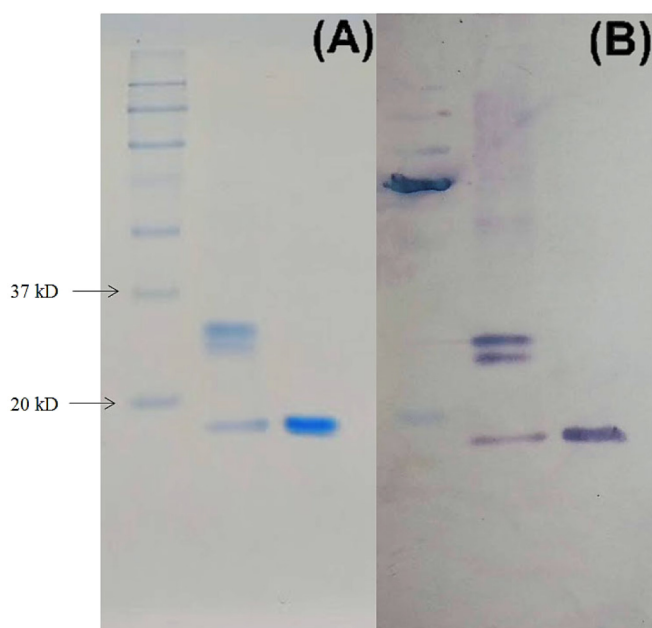


Fig. 1. SDS-PAGE and Western blotting of the Nickel affinity chromatography purified rTcISP2 side by side. (A) The first lane shows the commercial molecular ladder. The second lane shows native rTcISP2 and the third lane shows denatured rTcISP2. (B) Western blotting anti-His-tag antibody and colorimetric detection with the alkaline phosphatase substrate BCIP/NBT.

ellipticity of the protein at a particular temperature; θ_D and θ_N are the values of ellipticity of the denatured and native states.

Fluorescence emissions were measured using a Varian spectrofluorometer (Cary Eclipse, Agilent, USA). The rTcISP2 concentration was 5 μ M in 20 mM Tris-HCl at pH 8 loaded into a 1 cm quartz cuvette. The excitation wavelength measurement was performed at 280 nm or 295 nm. Fluorescence emission spectra were recorded from 300 nm to 450 nm.

The hydrodynamic radius (R_H) was determined by dynamic light scattering technique (Nano-ZS, Malvern, UK). The rTcISP2 concentration was 0.5 mg/mL in 20 mM Tris-HCl at pH 8 loaded into a 1 cm quartz cuvette.

SAXS measurements were performed at the SAXS beamline of National Synchrotron Light Laboratory (LNLS, Campinas, Brazil). The rTcISP2 concentration was 1 mg/mL in 20 mM Tris-HCl at pH 8 loaded into a 1 mm cell made of two thin parallel mica windows. The wavelength of the X-ray beam was 1.48 Å and the sample-to-detector distance was set to 0.95 m and five successive frames of 60 s were recorded for each sample. Buffer scattering was recorded before each sample scattering. The X-ray patterns were recorded using a two-

dimensional CCD detector (MarResearch, USA). The integration of SAXS patterns was performed with the FIT2D program and the curves were scaled by protein concentration. The scattering of water measured on the same sample cell was used to normalize the data to absolute scale. The radius of gyration (R_g) was determined by the Guinier equation. CRYSOLOG program [18] was used to generate theoretical scattering curve from the three-dimensional crystallographic structure. The HHPRED program [19] was used to search for homologs of the ISP2 from *T. cruzi* (Fig. S2). The homology model of the rTcISP2 was built by SWISS model server [20], using as template the available structure of ecotin from *Y. pestis* (PDB 2Y6T). The quality of the homology model was estimated using the index Global Model Quality Estimation (GMQE), which is a number between zero and one, and values close to one indicate higher reliability. All Figures were generated by the PyMol software (www.pymol.org).

3. Results

3.1. Expression and purification of the rTcISP2

The ISP2 nucleotide sequence from Y strain of *T. cruzi* (Genbank: MN538220) obtained by PCR presented 97,1% of similarity to the TcISP2 from Brenner strain (Fig. S3), while the amino acid sequence presented 99,9% of similarity. The ISP2 from *T. cruzi* Y strain coding sequence was successfully cloned into pET28a(+) bacterial expression vector which adds a His-tag at the protein N-terminus. After the BL21 (DE3) *E. coli* strain transformation, growth and IPTG induction, the recombinant protein was purified using a nickel affinity chromatography column under native conditions with a final yield of 1 mg/L of bacterial culture. Denaturing SDS-PAGE electrophoresis of the rTcISP2 showed a single band at the expected size of 18.9 kDa which was confirmed by Western blotting with anti-His-tag antibody (Fig. 1A). Native SDS-PAGE electrophoresis and Western blotting with anti-His-tag antibody showed three different rTcISP2 molecular forms, one with the a molecular mass of 18.9 kDa, corresponding to the protein monomer, and a duplex with an approximately molecular mass of 35 kDa, very likely due to dimerization of the protein (Fig. 1B).

3.2. rTcISP2 K_i measurement assay

The remaining protease activities as a function of rTcISP2 concentration are shown in the Fig. 2.

Enzymatic inhibitory assays were performed as described in the Methods section. After the calculation, the value of K_i for rTcISP2 against neutrophil elastase was of 1.5 μ M (Fig. 2A) and against trypsin was of 1.9 nM (Fig. 2B). Data from different studies that tested ecotin and ecotin-like proteins against trypsin and neutrophil elastase were compiled to compare the K_i differences among multiple versions of this inhibitor (Table 1).

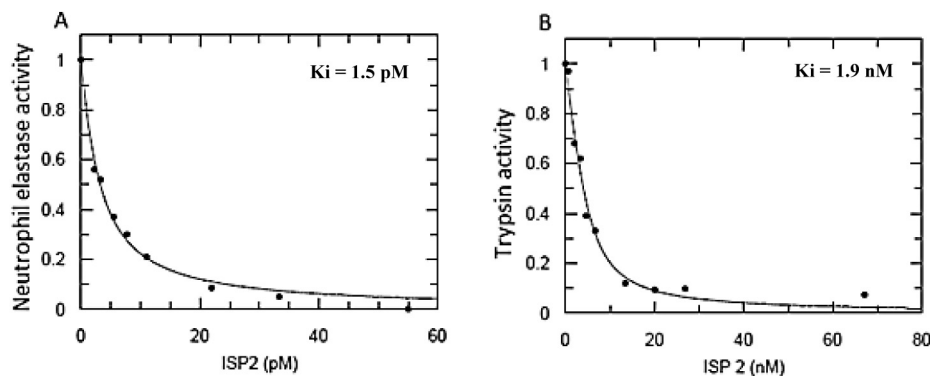


Fig. 2. Enzymatic inhibition assays by ISP2 in a dose-dependent manner. Remaining activity mediated by substrate hydrolysis in the absence and presence of ISP2 for: (A) human neutrophil elastase and (B) bovine trypsin.

Table 1
K_i of ecotin/ISP2 for different organisms against trypsin and neutrophil elastase.

Trypsin	Neutrophil elastase	Organism (strain)	Reference
1.9 nM	1.5 pM	<i>T. cruzi</i> (Y)	Present work
83 nM	7.7 nM	<i>L. major</i>	[13]
<1 pM	12 pM	<i>E. coli</i>	[11]
<1 pM	16 pM	<i>Y. pestis</i>	[11]

3.3. Structural characterization of rTcISP2

The secondary structure of the rTcISP2 was analyzed at pH 8 using circular dichroism (CD) spectroscopy (Fig. 3A). The CD spectrum of rTcISP2 was characterized by a broad negative band with minimum around 215 nm, typically found in proteins with a high content of β -strand structures [21]. Furthermore, the observed positive band with maximum around 236 nm is typically found in proteins rich in aromatic amino acid residues as rTcISP2 containing two tryptophan residues, eleven tyrosine residues and four phenylalanine residues, which correspond to 11.6% of the total number of amino acid residues. The thermostability of the rTcISP2 was monitored at pH 8 following changes in the minimum at 215 nm (Fig. 3B). The minimum at 215 nm remains constant at temperatures below 60 °C. However, a progressive decrease in the minimum was observed above 60 °C indicating loss of regular secondary structure. The melting temperature (T_m) value, defined as the

temperature at which the protein denatures, for rTcISP2 was calculated and resulted in $T_m = 64 \pm 1$ °C.

The tertiary/quaternary structure of the rTcISP2 was analyzed using fluorescence spectroscopy, DLS and SAXS. Fig. 3C shows the fluorescence emission spectrum at pH 8, after excitation at 280 nm, which is characterized by a maximum at 331 ± 1 nm, indicating that, on average, the aromatic amino acids residues are partially exposed to buffer solution. The two tryptophan residues can be selectively excited at 295 nm. In this case, the fluorescence emission spectrum is characterized by a slight blue shift (maximum at 328 ± 1 nm) indicating that the two tryptophan residues are less exposed to the buffer solution. When rTcISP2 was analyzed by DLS at pH 8 the observed profile was characteristic of a monodisperse solution (inset of Fig. 3B). The value determined for the hydrodynamic radius (R_h) was of 2.8 ± 0.1 nm. This value corresponding to an estimated molecular mass of 37 ± 4 kDa, consistent with a homodimer in solution (the molecular mass of the monomer with His-tag is 18,5 kDa), corroborated by native electrophoresis results described above (Fig. 1).

The SAXS curve for rTcISP2 measured at pH 8 and the associated Guinier plot are shown in Fig. 4. Measurements were performed at 1 mg/mL, the highest concentration achieved since above this concentration value the rTcISP2 aggregated and precipitated. The Guinier plot is linear, indicating good monodispersity. The radius of gyration (R_g) determined using Guinier approximation (Fig. 4B) and the maximum diameter (D_{max}) (Fig. 4C) were 24 ± 1 Å and 90 ± 5 Å, respectively. When compared to proteins with determined three-dimensional structures, rTcISP2 showed the better sequential identity (38%) when compared with ecotin from *Yersinia pestis* [22]. Fig. 5 shows the alignment of the primary sequences of both ecotins, and the two conserved tryptophan residues are highlighted in magenta. Despite the low sequential identity observed, the homology detection server resulted in a high level of confidence (100%), indicating that both ecotins have the same folding. Thus, the homology model of the TcISP2 (Fig.S4) was built using as template the crystal structure of ecotin from *Y. pestis* (PDB 2Y6T). The GMQE for the constructed homology model was of 0,70 which indicated excellent reliability. The homology model showed that TcISP2 has high content of β -sheet secondary structure corroborating with the CD spectroscopic analysis.

Published study reported the crystallographic structure of ecotin from *Y. pestis* in complex with activated bovine chymotrypsin (PDB 2Y6T) [22]. The results showed that ecotin monomer from *Y. pestis* is an elongated protein with a β -barrel fold and compatible with our homology model (Fig.S4), which forms a homodimer shaped like a “butterfly” (Fig. 2D) that provides two clefts into which two chymotrypsin molecules bind [22]. Fig. 4A shows the experimental SAXS curve (open circles) superimposed on the theoretical scattering curve based on the homodimeric high-resolution structure of ecotin from *Y. pestis* (solid line, PDB 2Y6T). The theoretical X-ray scattering curve calculated from the crystallographic structure resulted in an excellent fit ($\chi^2 = 2.6$) to the experimental data. Furthermore, the homodimeric high-resolution structure of ecotin from *Y. pestis* is a molecule with R_g and D_{max} of 23.21 Å and 86.65 Å, respectively. These values are in an excellent agreement with the experimental results described above, evidencing that the rTcISP2 also forms stable homodimers in solution. In addition, the R_g and D_{max} determined for the *Y. pestis* ecotin monomer (PDB 2Y6T) were 20.26 and 76.89 Å, respectively, values lower than those obtained experimentally and not compatible with a homodimer in solution.

4. Discussion

Trypanosomatide ISP2 is a serine protease inhibitor homologous to ecotin, a protein found in diverse species of symbiotic and pathogenic bacteria. Ecotin acts on proteases from the S1A family and it is an important factor associated to protection of bacteria from host-immune system [8,11]. In parasites belonging to the Trypanosomatidae family and the *Leishmania* genus, three genes encoding ISPs were described, the

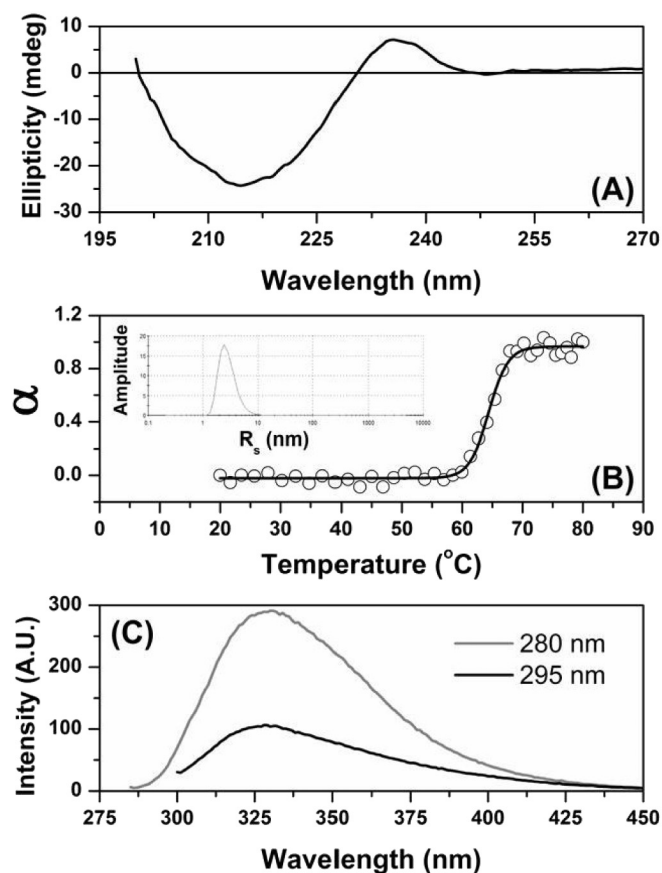


Fig. 3. Structural characterization of the rTcISP2. (A) - CD spectrum of rTcISP2 (B) - Thermostability on the rTcISP2 heated from 20 °C to 80 °C. The inset correspond to the DLS profile at pH 8 (C) - Fluorescence emission spectrum after excitation at 280 nm (gray line) and 295 nm (black line).

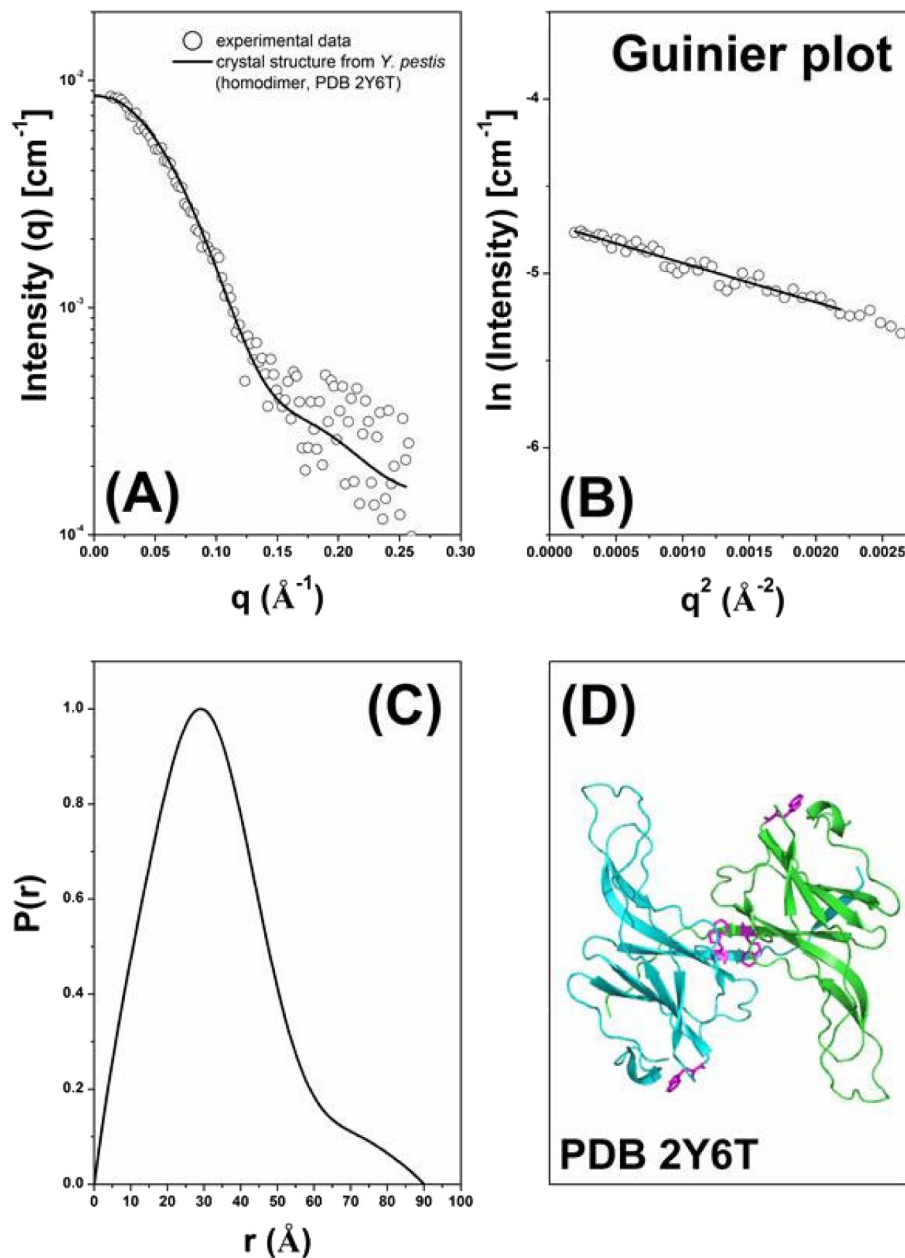


Fig. 4. SAXS measurements. (A) Experimental SAXS curve for rTcISP2 measured at pH 8 compared with the theoretical SAXS curve calculated from the *Y. Pestis* ecotin crystal structure (PDB 2Y6T). (B) Guinier plot. (C) Experimental distance distribution functions $P(r)$ at 25 °C for rTcISP2 at pH 8. (D) - *Y. Pestis* ISP2 homodimer crystal structure (PDB 2Y6T).

ISP1, ISP2 and ISP3. These three genes are conserved among different basal groups of trypanosomatids according to sequences deposited in public databanks. In *Trypanosoma brucei* the ISP3 is absent and in *T. cruzi*, only the ISP2 is present. The ISP2 protein has been shown to play a role in *Leishmania major* invasion and proliferation [12,23] and in *L. donovani*, ISP1 and ISP2 is associated to invertebrate [24], and ISP2 to vertebrate infections promotion [25].

Serine proteases from the S1A family are absent in pathogenic bacteria, and also in trypanosomatids, corroborating the hypothesis of ISP proteins acting as a virulence factor in dealing with host proteases [26,27]. The Chagas' disease protozoan parasite *T. cruzi* belonging to the *Trypanosoma* genus of the Trypanosomatidae family, and genome data published on Genbank from the Brener and Sylvio strains confirmed the presence of a single ISP encoding gene, the TcISP2. Thus, in this study, the biochemical and structural properties of a recombinant *T. cruzi* ISP2 from the Y strain was performed and compared to bacteria and *Leishmania* genus similar proteins.

The rTcISP2 was successfully expressed in *E. coli* and purified by nickel affinity chromatography. Under denaturing conditions rTcISP2 migrated as a monomer (18.9 kDa) (Fig. 1A). However, native electrophoresis showed a mixture of rTcISP2 as a monomer and dimers of two different molecular mass (Fig. 1). This result indicates that rTcISP2 inhibitory mechanism present similarity with bacteria ecotin orthologous that have activity as homodimers [28]. Dimerization of the rTcISP2 was also corroborated by SAXS and DLS results.

To check if the protein was functional, enzymatic inhibitory assays were performed and the results showed that rTcISP2 inhibit bovine trypsin and human neutrophil elastase (HNE) in the nanomolar and picomolar range, respectively. In comparison to ecotin and ecotin-like proteins from *E. coli*, *Y. pestis*, and *Leishmania* species, it showed different dissociation constant (K_i) for Trypsin and HNE (Table 1). The dissociation constant of rTcISP2 in picomolar range for HNE was ten times lower when compared to the *E. coli* and *Y. Pestis* ecotin and 1000 times lower than to the K_i obtained with *L. major* ISP2 on HNE. Trypsin

<i>T. cruzi</i>	13	APYPEATPNQKRYVIFLEEKDYDSELEEYKLQLIPGRVMKVDPVNRCFIAGSVKEKTIPG	72
<i>Y. pestis</i>	09	APYPQAEKGMSRQVIFLEPQKDESR---FKVELLIGKTLNVD-CNRHMLGGNLETRTLTG	64
<i>T. cruzi</i>	73	WGYNYVVE-MGPMATTIMAVSPGSPVRKFVP--MAERPFIPYNSKLPVVVYMPKEGEL	129
<i>Y. pestis</i>	65	WGFDYLVMDKISQPASTMMACPEDSKPQVKFVTANLGDAAMQRYSRLPIVVYVPQGVVEV	124
<i>T. cruzi</i>	130	HYRVW	134
<i>Y. pestis</i>	125	KYRIW	129
sequential identity (38%)			
confidence homology (100%)			

Fig. 5. Alignment of the amino acid sequences of *T. cruzi* and *Y. pestis* ISP2 protein. It presents 38% sequential identity and 100% confidence homology. Identical residues are highlighted and aromatic residues are colored in violet and magenta.

inhibition K_i was lower in bacteria by the order of 1000 times. These activity differences indicate evolution divergences of biological roles among bacteria and trypanosomatids ecotin-like. Initially, *E. coli* ecotin high inhibitory activity against gut serine peptidases including trypsin, chymotrypsin and HNE was associated to the host colon environment [29] and its activity on serum serine peptidases was assumed as non-physiological [30]. However, identification of ecotin orthologous in pathogenic bacteria such as *Y. pestis* [22], *Pseudomonas aeruginosa*, *Burkholderia pseudomallei* [8], and in Trypanosomatidae parasites revealed potent inhibitory activity of these proteins on human serine peptidases involved in cellular and humoral innate immunological pathways [31], consequently, acting as anti-microbicidal molecules being recognized as virulence factors. Neutrophil elastase (NE) is an important serine peptidase of the chymotrypsin family acting in innate immunological defense mechanisms.

The NE is found in the primary (azurophil) granules of polymorphonuclear neutrophils (PMNs), acting as a microbicidal molecule to degrade ingested pathogens or that captured by the neutrophil extracellular trap (NET) produced by inflammation recruited PMNs [32]. NE also participates in the activation of Toll Like Receptors (TLRs) and in the modulation of Tumor Necrosis Factors (TNFs) and Interferons (INFs) expression, in PMN and macrophages, triggering different immunological mechanisms dependent and independent of oxidative stress pathways to eliminate pathogens [33]. Thus, ecotin-like proteins produced by bacteria and trypanosomatids interacts in different ways with NET and others NE associated mechanisms in order to infect and to survive in vertebrate hosts cells. The role of NE in defense of vertebrates to Gram-negative bacteria infection is well described in the literature and involves degradation of the outer membrane protein A (OmpA), a mechanism controlled by the infecting bacteria through ecotin-like proteins [34,35]. Considering the highest dissociation constant of rTcISP2 on HNE comparatively to others ecotin-like orthologous and the absence of OmpA similar proteins in trypanosomatids, probably, the TcISP2 is acting on different mechanisms associated to HNE.

The NET composed of DNA and histones decorated with NE and peptides with anti-microbial activity was described for the first time in 2004, as an innate immune system response capable to kill bacteria [36]. Afterwards, several studies revealed a controlled mechanism of extracellular traps (ET) formation induced by pathogens or molecules expressed under physiological and pathological conditions, and a specific degranulation process (ETosis) triggered by different factors including complement factor 5a, platelets, singlet oxygen, cytokinins, Fc receptors, etc. [37,38]. ET/ETosis is an ancient innate defense mechanism associated to neutrophils and others granulocytes, including macrophages, being conserved from fishes to mammals and NE exerts essential role in this processes [39–41], including histones degradation

for chromatin deconsensation and as an anti-microbial molecule [33]. Species of the trypanosomatids from genera *Leishmania* and *Trypanosoma* infect a diversity of vertebrates from fishes to mammals and present differential interactions with ET/ETosis. Promastigotes of the viscerotropic *L. donovani* scape from NET by producing lypophosphoglycan (LPG) [42] while the dermatropic *L. amazonensis* is killed by NET through action of histones and NE [43]. The infectivity of *T. cruzi*, is affected by NET and more specifically by the NE and its association to production of specific cytokines after activation of TLR2 and TLR4 [44]. Also, *T. cruzi* escape from ET stimulated by trypomastigotes in neutrophils and others granulocytes of domestic dogs and the opossum *Didelphis marsupialis* which are decorated mainly with NE [45]. These data suggest an essential role of NE in *T. cruzi* infection associated to ET/ETosis, corroborating the adaptation of the parasites through selection of an ISP2 with a more specific and higher inhibitory activity when compared to ISPs from others pathogens.

Besides ET/ETosis, NE can activate TLRs to trigger innate immunological defense mechanisms in PMN and macrophages in response to *Leishmania* and *Trypanosoma* parasites infection, and parasites ISP proteins are differentially involved in the modulation of these mechanisms. *L. major* ISP2/ISP3 knockout parasites were more susceptible to macrophage killing [12] and unable to sustain prolonged infections in C57BL6 mouse in response to increased Nitric Oxide Synthase 2 (NOS-2) expression in monocytes and by promoting inflammation [27]. Controversely, ISP2 defective *L. donovani* strains, revealed dependence of NE activity for infection establishment in macrophages and to parasite burden in liver [26]. The molecular mechanism associated with NE dependency requires TLR4 and INF- β which is significantly reduced upon expression of *L. donovani* ISP2. These data are corroborated by the occurrence of null mutation in ISP2 encoding gene from two different human isolated *L. donovani* strains [26]. Studies on BalbC and C57BL6 mice strains, the susceptible and resistant models for *T. cruzi* infection, revealed trypanocidal effect of neutrophils from C57BL6 mouse strain on infected macrophages, through induction of TNF α , NO and NE. Neutrophils from the *T. cruzi* resistant mouse produces two to three times more elastase than neutrophils from the susceptible Balb C [46]. Thus, NE is involved in several immune defense processes associated to *T. cruzi* infection in vertebrate host, and the lowest dissociation constant of rTcISP2 show to be necessary for parasite evasion and replication in macrophages.

The ISP2 from *Leishmania* species and *T. cruzi* are expressed in the invertebrate promastigotes and epimastigotes, respectively. Verma et al. study showed that *L. donovani* ISP2 is important for the optimal adaptation and survive of the parasite in the insect midgut, whose gut serine proteases might differ from the bovine trypsin and chymotrypsin, which could explain differences found between the K_i

of the ecotin and ISP2 activity on these proteases [24,47]. The role of rTcISP2 in triatomines susceptible to *T. cruzi* needs to be investigated. Considering the life cycle of the parasites it is possible to wonder that evolutive selection for a higher HNE inhibitory activity in *T. cruzi*, compared to *Leishmania* parasites, is due to the presence of a motile blood stage parasite in the former, which is frequently exposed to neutrophils.

Comparison of amino acid sequence of TcISP2 with the ISP2 from *Leishmania* parasites showed 54% of identity (Fig. S1). The amino acid identity of rTcISP2 with the *Y. pestis* ecotin was 38%, however, the Ki values of rTcISP2 is closest to the *Y. pestis* ecotin than to the *L. major* ISP2. Also, as showed by structural evaluation, the rTcISP2 present 100% of similarity with the tridimensional conformation of the *Y. pestis* ecotin by homology modeling, indicating a biological role associated to the molecular structure of these molecules.

The ISP2 encoding gene from *T. cruzi* Y strain presented only one different amino acid residue synonymous substitution (Fig. S5), when compared with the ISP2 encoding gene of *T. cruzi* from CL Brenner strain, demonstrating a high intraspecific conservation of ISP2 in *T. cruzi*.

Within the advent of anti-pathogens drug resistance, specific virulence factors are an excellent alternative target to develop new treatments [48]. Chagas' disease has only two highly toxic anti-parasitic drugs, so the control of virulence factors could be a good solution or a parallel method to deal with the disease [4]. Ecotin inhibits proteases from the human immune system, having the potential to elicit specific antibodies. These could lead to a new approach into the diagnostics and prognostics of Chagas' disease.

5. Conclusions

In this study, we report for the first time the biochemical and structural characterization of an endogenous protease inhibitor, from the causative agent of Chagas' disease, whose similar molecules are recognized as a virulence factor of bacteria and trypanosomatids. Structural characterization of ISP2 from *T. cruzi* exhibited its homodimerization in solution and a strikingly similarity to the *Y. pestis* ecotin tridimensional homology modeling. NE is an important molecule associated to innate defense mechanisms associated to *T. cruzi* infection and rTcISP2 presented the highest inhibitory biochemical activity against HNE when compared to others bacteria and trypanosomatids ecotin-like proteins. The findings described here may influence the development of a molecular diagnostic using ISP2 as antigen and as a possible drug target to diminish *T. cruzi* virulence.

CRedit authorship contribution statement

Felipe Baena Garcia: Project administration, Data Curation, Methodology, Writing - review & editing. **Aline Diniz Cabral:** Data curation, Formal analysis, Methodology, Writing - review & editing. **Max Mario Fuhlendorf:** Writing - review & editing. **Geomar Feitosa da Cruz:** Methodology, Writing - review & editing. **Juliete Vitorino dos Santos:** Methodology, Writing - review & editing. **Graziele Cristina Ferreira:** Methodology, Writing - review & editing. **Bernard Robin Carneiro de Rezende:** Methodology, Writing - review & editing. **Carla Moreira Santana:** Data curation, Writing - review & editing. **Luciano Puzer:** Formal analysis, Writing - review & editing. **Sérgio Daishi Sasaki:** Formal analysis, Writing - review & editing. **Wanuis Garcia:** Investigation, Methodology, Conceptualization, Writing - review & editing. **Márcia Aparecida Sperança:** Project administration, Resources, Supervision, Funding acquisition, Methodology, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Declaration of competing interest

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2020.02.186>.

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