Tigutcystatin, a cysteine protease inhibitor from *Trypanosoma cruzi* midgut expressed in response to *Trypanosoma cruzi*

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**Abstract**

The insect *Trypanosoma cruzi* is a vector of *Trypanosoma cruzi*, the etiological agent of Chagas disease. A cDNA library was constructed from *T. cruzi* anterior midgut, and 244 clones were sequenced. Among the EST sequences, an open reading frame (ORF) with homology to a cystatin type 2 precursor was identified. Then, a 288-bp cDNA fragment encoding mature cystatin (lacking signal peptide) named Tigutcystatin was cloned fused to a N-terminal His tag in pET-14b vector, and the protein expressed in *Escherichia coli* strain Rosetta gami. Tigutcystatin purified and cleaved by thrombin to remove His tag presented a molecular mass of 11 kDa and 10,137 Da by SDS–PAGE and MALDI-TOF mass spectrometry, respectively. Purified Tigutcystatin was shown to be a tight inhibitor towards cruzain, a *T. cruzi* cathepsin L-like enzyme (*K* i = 3.78 nM) and human cathepsin L (*K* i = 3.29 nM) and human cathepsin L (*K* i = 3.29 nM). Tissue specific expression analysis showed that Tigutcystatin was mostly expressed in anterior midgut, although amplification in small intestine was also detected by semi quantitative RT-PCR. qReal time PCR confirmed that Tigutcystatin mRNA is significantly up-regulated in anterior midgut when *T. cruzi* is infected with *T. cruzi*. Together, these results indicate that Tigutcystatin may be involved in modulation of *T. cruzi* in intestinal tract by inhibiting parasite cysteine proteases, which represent the virulence factors of this protozoan.

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1. Introduction

Cystatins are reversible and tight binding inhibitors of papain-like cysteine proteases, being widespread in plants, animals and microorganisms [1]. Based on sequence similarity, the superfamily of cystatins is divided in (i) type 1, or stefins, which contain about 100 amino acids, (ii) type 2, or cystatins, which are proteins presenting about 120 amino acid residues and two disulfide bridges, and (iii) type 3, or kininogens, which are glycoproteins consisting of three homologous domains of type 2 cystatins [2,3].

Some studies have suggested that cystatins from arthropods can be related to different processes, i.e. control of endogenous proteolysis, the balance of host–vector immune relationships, blood feeding and innate immunity [4–8]. However, the role of cystatins in hematophagous bugs, such as *Triatoma rubrofasciata*, remains unknown.

*Trypanosoma cruzi* belongs to the Triatominae subfamily and is one of the most important vectors of *Trypanosoma cruzi*, a protozoan parasite and etiological agent of Chagas disease [9]. The disease remains prevalent in many Latin American countries, affecting about eight million people [10]. *T. cruzi* colonizes triatomine blood sucking insects’ midgut, which is considered an immune competent tissue [11]. It has been suggested that inducible immune compounds from intestinal tract can modulate parasite development in these insects [12–14].

*T. cruzi* relies on its major papain-like cysteine protease, cruzipain, to survive and infect hosts [15,16]; however, this protozoan is not pathogenic for triatomine bugs [17]. Therefore, cysteine protease inhibitors from vectors of *T. cruzi* are important candidates for studying host–parasite interactions. In this work, we constructed a cDNA library from anterior midgut of *T. cruzi*, and a cystatin DNA fragment was identified by expressed sequence tags (ESTs) analysis. We report for the first time the expression and characterization of a cystatin-like inhibitor (Tigutcystatin) from a triatomine insect whose expression is modulated by infection with *T. cruzi*.

2. Materials and methods

2.1. Materials

Bacteria and vector: *Escherichia coli* DH5α (F, endA1, hsdR17, sup E44, thi1, k, recA1, gyrA96, 80 d lacZD15) was used as host for recombinant DNA manipulation. *E. coli Rosetta gami* strain (∆(ara–leu)/7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK

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The cDNA sequence encoding the mature cystatin (lacking signal peptide) was amplified by PCR in a 50-μL reaction volume containing 5 pmol of gene-specific primers, 200 μM DNTPs, 1.5 mM MgCl₂, and 5 U Taq DNA polymerase (Fermenta). PCR conditions were: 94 °C for 5 min, 94 °C for 45 s, 50 °C for 45 s and 72 °C for 45 s × 35 cycles. DNA final extension was carried out at 72 °C for 10 min. The PCR product was purified from 1% agarose gel using a QIAEX II Gel Extraction kit (Qiagen, Hilden, Germany) and cloned using pGEM-T Easy System (Promega). The cDNA encoding Tigutycystatin was digested with NdeI and BamHI restriction enzymes, and sub-cloned into pET-14b vector which was previously digested with the same enzymes above.

2.5. Expression of recombinant Tigutycystatin

Tigutycystatin was expressed by E. coli Rosetta gami strain as a protein fused to a N-terminal 6× His tag. Previously, recombinant Tigutycystatin was produced in 31 LB broth medium containing 200 μg/ml ampicillin, 34 μg/ml chloramphenicol, 20 μg/ml kanamycin, 12 μg/ml tetracycline and one isolated colony of transformed E. coli Rosetta gami. rtTigutycystatin expression was induced with 1 mM IPTG (at OD of 0.7) for 18 h at 37 °C. After, cells were harvested by centrifugation (4000g, 20 min, 4 °C) and suspended in 300 mL of 50 mM Tris-–HCl buffer, pH 8.0 containing 0.3 M NaCl. Cells were lysed using a French press (2000 psi). The culture supernatant was obtained by centrifugation (13000g, 45 min, 4 °C).

2.6. Purification of recombinant Tigutycystatin

The resulting supernatant containing rtTigutycystatin was applied on a Ni–NTA agarose column pre-equilibrated with 50 mM Tris–HCl buffer, pH 8.0, containing 0.3 M NaCl. After a washing step with 50 mM Tris–HCl buffer, pH 8.0, containing 0.3 M NaCl and 20 mM imidazole, protein was eluted with 0.5 M imidazole in the same Tris–HCl buffer (pH 8.0). Solution containing eluted proteins was dialyzed against 50 mM sodium acetate buffer, pH 5.5 and rtTigutycystatin was further purified on a HiTrap SP cation-exchange column connected to an ÄKTA system. The protein was eluted by NaCl linear gradient (0–1 M) in 50 mM sodium acetate buffer, pH 5.5, at a flow rate of 1 mL/min. The elution profile was monitored by measuring absorbance at 280 nm, and the fractions were tested on human cathepsin L for inhibitory activity. All purification steps were analyzed by SDS–PAGE 15% [21].

2.7. Purified rtTigutycystatin processing and mass spectrometry analysis

For mass spectrometry analysis, Tigutycystatin (275 μg) was incubated with 1 U thrombin (Sigma–Aldrich) in PBS pH 7.0 at 37 °C for 6 h to remove His tag. After digestion, released His tag was removed by filtration in a 3-kDa NMWL Ultracel membrane Amicon system. Tigutycystatin without the His tail was applied to a reverse phase chromatography in a Sephasil Peptide C₈ in ÄKTA System (GE Healthcare) equilibrated with 0.1% trifluoroacetic acid (TFA), and the protein was eluted by linear acetonitrile gradient (0–100%) at flow rate of 0.7 mL/min. Molecular masses of the protease inhibitor were analyzed by MALDI-TOF mass spectrometry on Micromass ToFSpec-E spectrometer (Manchester) operating in linear mode and calculated from the m/z peaks in the charge-distribution profiles of the multiple charged ions.

2.8. Dissociation constant (Kᵣ) determination

Proteolytic enzymes, papain, cathepsin L, cathepsin V, cathepsin S, cruzain and cathepsins-L like from supernatant of culture of T. cruzi trypomastigotes and P. falciparum were pre-activated with 1 mM DTT (final concentration) in 50 mM sodium acetate buffer, pH 5.5, containing 60 mM NaCl for 10 min at 37 °C. Afterwards,

rspl. F[laclineall apro] gor522::Tn10 trxB pRARE2 (CamR, KanR, StrR, TetR) was used as a host for protein production, and both strains were purchased from Invitrogen (Carlsbad, CA). Primers (Tigcy5fwd: 5'-GAAATTCATATGGAAGTCGGCCTGATTG-3'; Tigcyrev: 5'-CGGGATCTTTATCTATTACTAT-3'; 18sFwd - 5'-GGCGGGGGGGCCATCCTGATTG-3' and 18srev - 5'-ATCCGGTTGCTGGCATGCTTTATTTGGC-3') were purchased from Sigma–Aldrich. The pET14b expression vector was from Novagen (Madison, WI). Modification enzymes: Restriction enzymes Nde I (Promega, Madison, WI) and BamHI (Fermentas, Hanover, MD); Taq DNA polymerase was purchased from Fermentas (Hanover, MD). Chromatography columns: Ni–NTA agarose resin was obtained from Qiagen (Hilden, Germany). HiTrap SP and Sephasil C8 were from GE Healthcare Life (Uppsala, Sweden). Enzymes and substrates: Recombinant human cathepsin L (EC 3.4.22.15) was expressed in Pichia pastoris as described previously [18], using a plasmid construction kindly provided by Dr. Nagler. Precision Plus Protein Unstained (PPPLUS) and Page Ruler molecular weight markers were purchased from Bio Rad and Fermentas, respectively. Papain (EC 3.4.22.2) and thrombin (EC 3.4.21.5) were purchased from Sigma (St. Louis, MO, USA). Cathepsin S (3. 4. 22, 27), cathepsin V (3. 4. 22. 43) and Z-Phc-Arg-7-amido-4-methylcoumarin were purchased from Calbiochem (San Diego, CA). Cuzain (3. 4. 22) and Plasmodium falciparum culture were supplied by Dr. Luiz Juliano Neto, Dr. Adriana K. Carmona, Dr. Piero Bagnaresi and Dr. Mario A. Izidoro from Department of Biophysics of Escola Paulista of Medicine – Federal University of São Paulo. Culture of T. cruzi was kindly provided by Dr. Sergio Schenkman from Department of Microbiology, Immunology and Parasitology of Escola Paulista de Medicina – Federal University of São Paulo.

2.2. Insects, T. cruzi infections and tissue collection

Adult males T. infestans were reared under controlled temperature (27 ± 2 °C), 12/12 light/dark. Insects starved for 30 days were allowed to feed on anesthetized mice (ketamine 150 mg/kg and xylazine 7 mg/kg) ad libitum and tissues were dissected 24 h after feeding. For infection experiments, insects were infected orally by feeding on anesthetized mice infected with T. cruzi Y strain ad libitum. Mice infection was performed according to Kollien and Schaub [19], and population density (1 × 10⁶ parasites/mL) was determined using Neubauer chamber.

2.3. Construction and screening of a cDNA library of anterior midgut of T. infestans

Total RNA was extracted from anterior midgut of T. infestans adults using TRIzol reagent (Invitrogen, Carlsbad, CA). A cDNA library was constructed using the SMART cDNA system, according to manufacturer’s instructions (Clontech, Palo Alto, CA). The mRNA of T. infestans anterior midgut (1 μg) was used to synthesize first-strand cDNA. cDNA fragments longer than 400 bp were separated by fractionation column and then collected and ligated into pTriPEx plasmid. Subsequently DNA fragments were submitted to pliage packaging reaction using Gigapack Gold III Packaging Extract from Stratagene (La Jolla, CA). About 244 cDNA clones from the amplified library were randomly sequenced. The DNA sequence analysis was performed using the BLAST algorithm tools [20]. Alignment of protein sequences was performed with the ClustalW program version 2.0 and signal peptide was predicted using SignalP program version 3.0.

2.4. Cloning of Tigutycystatin DNA fragment into expression vector pET-14b

The cDNA sequence encoding the mature cystatin (lacking signal peptide) was amplified by PCR in a 50-μL reaction volume containing 5 pmol of gene-specific primers, 200 μM DNTPs, 1.5 mM MgCl₂, and 5 U Taq DNA polymerase (Fermentas). PCR conditions were: 94 °C for 5 min, 94 °C for 45 s, 50 °C for 45 s and 72 °C for 45 s × 35 cycles. DNA final extension was carried out at 72 °C for 10 min. The PCR product was purified from 1% agarose gel using a QIAEX II Gel Extraction kit (Qiagen, Hilden, Germany) and cloned using pGEM-T Easy System (Promega). The cDNA encoding Tigutycystatin was digested with NdeI and BamHI restriction enzymes, and sub-cloned into pET-14b vector which was previously digested with the same enzymes above.

2.5. Expression of recombinant Tigutycystatin

Tigutycystatin was expressed by E. coli Rosetta gami strain as a protein fused to a N-terminal 6× His tag. Previously, recombinant Tigutycystatin was produced in 31 LB broth medium containing 200 μg/ml ampicillin, 34 μg/ml chloramphenicol, 20 μg/ml kanamycin, 12 μg/ml tetracycline and one isolated colony of transformed E. coli Rosetta gami. rtTigutycystatin expression was induced with 1 mM IPTG (at OD of 0.7) for 18 h at 37 °C. After, cells were harvested by centrifugation (4000g, 20 min, 4 °C) and suspended in 300 mL of 50 mM Tris–HCl buffer, pH 8.0 containing 0.3 M NaCl. Cells were lysed using a French press (2000 psi). The culture supernatant was obtained by centrifugation (13000g, 45 min, 4 °C).
Activated enzymes were pre-incubated with different concentrations of His-tag Tigutcystatin for 10 min at 37°C and the substrate Z-Phe-Arg-MCA (50 μM-final concentration) was added. The measurement of absorbance was made at 380 nm excitation and 460 nm emission. Dissociation constant was calculated by fitting the steady-state velocities to the Morrison equation \( \frac{V_i}{V_o} = \frac{1}{C_0} \left( \frac{E_t + I_t + K_i}{C_0} \right) \left( \frac{E_t + I_t + K_i}{C_0} \right)^{1/2} \) for tight-binding inhibitors using a nonlinear regression analysis [22].

### 2.9. Tigutcystatin expression profile after infection with T. cruzi

After feeding (24 h post blood meal), insect tissues (anterior midgut, posterior midgut salivary glands and fat body,) were dissected for semi quantitative PCR of Tigutcystatin for 10 min at 37°C and the substrate Z-Phe-Arg-MCA (50 μM-final concentration) was added. The measurement of absorbance was made at 380 nm excitation and 460 nm emission. Dissociation constant was calculated by fitting the steady-state velocities to the Morrison equation \( \frac{V_i}{V_o} = \frac{1}{C_0} \left( \frac{E_t + I_t + K_i}{C_0} \right) \left( \frac{E_t + I_t + K_i}{C_0} \right)^{1/2} \) for tight-binding inhibitors using a nonlinear regression analysis [22].

### 3. Results

#### 3.1. Nucleotide sequence analysis and cloning of DNA coding for Tigutcystatin

A putative cystatin was found among 244 clones sequenced from a midgut cDNA library. The cDNA fragment encoding a putative cystatin named Tigutcystatin (T. infestans midgut cystatin) was cloned and sequenced. The nucleotide sequence of Tigutcystatin, shown in Fig. 1A, presented a 363-bp ORF encoding a protein with 121 amino acid residues. The amino acid sequence analysis revealed the presence of five cysteine residues, a signal peptide (underlined in Fig. 1A) and a cleavage site between amino acids 25 and 26. The mature protein consisted of 288 bp encoding a protein with 96 amino acid residues with a calculated molecular weight of 11 kDa and isoelectric point (pI) of 8.64. The amino acid sequence of Tigutcystatin showed about 30% of similarity with other insect cystatins, including conserved motifs present in the Cystatin type 2 family (Fig. 1B).

#### 3.2. Expression and purification of recombinant Tigutcystatin

The cDNA fragment encoding to Tigutcystatin was cloned in pET 14b vector and the protein expressed fused to an amino-terminal His tag. Purification of 8 mg of starting protein afforded to obtain 0.9 mg of rTigutcystatin. The recombinant protein was subjected to affinity chromatography on Ni–NTA (Fig. 2A) followed by ion exchange chromatography on Hitrap SP column (Fig. 2B). SDS–PAGE
analysis revealed a major band of His tag Tigutcystatin migrated at 13.5 kDa (Fig. 2C, lane 1, black arrow).

Purified His-Tigutcystatin was digested with thrombin to remove His tag portion. The resulting protein appeared as a single band at 11 kDa in SDS–PAGE electrophoresis (Fig. 3A). Then, the protein was concentrated and applied on a reverse phase chromatography and a protein peak obtained (Fig. 3B, black arrow) was submitted to MALDI-TOF analysis, showing a molecular mass of 10,137 Da (Fig. 3C).

3.3. Tissue-specific expression

Tigutcystatin expression profile analysis using different tissues was performed with cDNA preparation from anterior midgut, posterior midgut, fat body and salivary glands and specific primers for Tigutcystatin cDNA fragment by PCR. The inhibitor expression was highest in anterior midgut, although Tigutcystatin expression was also observed in posterior midgut. Nevertheless, it was absent in salivary gland and fat body (Fig. 4A). 18S ribosomal gene specific primers were used as an endogenous control.

3.4. Quantitative Real time PCR of Tigutcystatin gene expression

cDNA of infected and uninfected insects were used in a quantitative RT-PCR for Tigutcystatin expression analysis. Tigutcystatin expression was significantly up-regulated (4-fold) in infected insects, when compared with uninfected animals (Fig. 4B).

3.5. rTigutcystatin inhibition profile

To investigate Tigutcystatin specificity, inhibition assays towards papain-like enzymes were performed (Table 1). Purified rTigutcystatin was able to inhibit all tested enzymes, but with high selectivity to cruzain from *T. cruzi* (\( K_i = 3.29 \) nM) and human cathepsin L (\( K_i = 3.78 \) nM). However, the inhibitor was comparatively less effective to inhibit cathepsin V (\( K_i = 21.49 \) nM) and cathepsin S (\( K_i = 25.97 \) nM).

4. Discussion

The present study describes expression, purification and characterization of a cystatin-like inhibitor from the Chagas disease vector *T. infestans*. This is the first time a cystatin is characterized in a Triatomine bug. The putative amino acid sequence indicates that this inhibitor belongs to type 2 cystatins (Fig. 1A), which include secretory proteins presenting a signal peptide and a two characteristic disulphide bridges [24]. The alignment of Tigutcystatin amino acid sequence with other insect cystatins showed highly conserved regions (Fig. 1B), including a N-terminal glycine residue, a central motif QxVxG (x represents any amino acid), and the dipeptide PW near the carboxy-terminal region. All these structural features are important cysteine proteases inhibition sites [25,26].

In this study, Tigutcystatin mRNA was detected in small intestine, but it was hardly detected in anterior midgut (Fig. 4A). Moreover, Tigutcystatin showed a significant up-regulation in stomach
Fig. 3. Characterization of purified Tigutcystatin. (A) Analysis of rTigutcystatin thrombin processed by SDS–PAGE (15%). M – molecular weight marker (Page Ruler), (1) Tigutcystatin thrombin processed, (B) Mass spectrometry analysis. The peak obtained from reverse-phase was submitted to mass spectrometry in a Micromass ToFSpec E. (C) Tigutcystatin profile by reverse-phase chromatography. The inhibitor (0.1 mg) was applied on a Sephasil Peptide C8 column previously equilibrated with 0.1% TFA solution in water. Protein was eluted (indicated by a black arrow) with linear gradient of acetonitrile (0–100%) and constant flow rate of 0.7 ml/min.

Fig. 4. Tissue specific and immune challenge expression profile of Tigutcystatin. (A) Semi-quantitative PCR analysis of Tigutcystatin expression in different tissues of *T. infestans*. Electrophoresis on agarose gel (1%) of PCR products using cDNA preparation of: (1) anterior midgut; (2) posterior midgut; (3) fat body; (4) salivary glands. (B) Quantitative Real time PCR. Relative amount of mRNA induced by *T. cruzi* infection. Adult insects infected with *T. cruzi* and uninfected *T. infestans* were used for analysis. All data were normalized to ribosomal 18S, representing the mean (n = 3) of identical triplicates ± standard deviation. Two-tailed unpaired t-test (P < 0.05) was performed for statistical analysis. Significant differences are represented by two asterisks.
of T. infestans T. cruzi – infected (Fig. 4B). These results suggested more than one role of this molecule in T. infestans. Since triatomine insects use cathepsins for protein digestion in posterior midgut [27], Tigutcystatin could be also involved in regulation of endogenous cysteine proteases in stomach, thus controlling undesired proteolysis in this compartment. On the other hand, cystatins are related to innate immunity in insects [4]. Tigutcystatin might also have a defensive role when T. infestans is infected with T. cruzi by inhibiting cruzipain, a parasite cysteine protease used to invade host cells [28].

Regarding inhibition properties, kinetic assays revealed that Tigutcystatin binds tightly to cruzain, the recombinant cruzipain truncated at C-terminal [28] and cathepsins L-like present in T. cruzi trypomastigotes and P. falciparum (Table 1). Tight inhibition of cruzipain by other recombinant type 2 cystatins (human cystatin C and chicken cystatin) has also been demonstrated by Stoka, Nycander, Lenarcic et al. [29]. Together, these data support the evidence for the defensive role of Tigutcystatin against protozoan parasites. Tigutcystatin was less effective to inhibit cathepsins V and S, indicating that this inhibitor might be able to discriminate cathepsins L-like of parasites from other cathepsins.

In spite of the protozoan cathepsins L-like functions, cruzipain has also been associated to T. cruzi survival in hosts [30]. The expression of a cysteine protease inhibitor in T. infestans midgut is noteworthy, which is colonized by T. cruzi. Thus, Tigutcystatin might be able to modulate T. cruzi colonization inside the insect midgut. Tigutcystatin may also be involved in interaction of T. infestans–Trypanosoma rangeli, since this protozoan parasite is transmitted by species from the genus Rhodnius (via hemolymph invasion and salivary glands establishment), though it is not transmitted by species from the genus Rhodnius (via hemolymph invasion); however differences in susceptibility of Triatoma and Rhodnius are involved in the ability of T. rangeli to establish inside the insect midgut.

Tigutcystatin may also be involved in interaction of T. infestans–Trypanosoma rangeli, since this protozoan parasite is transmitted by species from the genus Rhodnius (via hemolymph invasion and salivary glands establishment), though it is not transmitted by Triatoma species [31]. It has been suggested that proteases are involved in the ability T. rangeli demonstrates to cross midgut membranes and invade hemolymph and salivary glands [17]. Even though differences in susceptibility of Triatoma and Rhodnius genus remain unknown, in Triatoma species T. rangeli seems to be restricted to the digestive tube [32]. Since rangelipain, the main cathepsin L-like from T. rangeli, shares high identity with cruzipain [33], we hypothesize that Tigutcystatin might be involved in limitation of T. rangeli inside the digestive tract by inhibiting rangelipain. However, further studies are needed to demonstrate the interactions between Tigutcystatin and T. rangeli cysteine proteases.

In summary, Tigutcystatin was able to inhibit parasite cysteine proteases, being up-regulated in response to T. cruzi infection. From our results, we can suggest that Tigutcystatin might be involved on immune processes concerning T. infestans–T. cruzi interactions. Our future studies may also help elucidate these vector–parasite relationships.

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