Two Tachykinin-Related Peptides with Antimicrobial Activity Isolated from Triatoma infestans Hemolymph

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ABSTRACT: Antimicrobial peptides (AMPs) are molecules that can interact with microbial cells and lead to membrane disruption or intracellular molecule interactions and death. Several molecules with antimicrobial effects also present other biological activities. One such protein group representing the duality of activities is the tachykinin family. Tachykinins (TKs) form a family of neuropeptides in vertebrates with a consensus C-terminal region (F-X-G-Y-R-NH2). Invertebrate TKs and TK-related peptides (TKRPs) are subfamilies found in invertebrates that present high homology with TKs and have similar biological effects. Several of these molecules have already been described but reports of TKRP in Hemiptera species are limited. By analyzing the Triatoma infestans hemolymph by reversed-phase high-performance liquid chromatography, biological assays, and mass spectrometry, two antimicrobial molecules were isolated from the hemolymph of T. infestans. TRP1-TINF is a random secondary structure peptide with 9 amino acid residues. It is susceptible to aminopeptidase degradation and is active mainly against Micrococcus luteus (32 μM). TRP2-TINF is a 10-amino acid peptide with a 310 helix secondary structure and is susceptible to carboxypeptidases degradation. It has major antimicrobial activity against both Pseudomonas aeruginosa and Escherichia coli (45 μM). Neither molecule is toxic to human erythrocytes and both present minor toxicity toward Vero cells at a concentration of 1000 μM. As the first description of TKRPs with antimicrobial activity in T. infestans, this work contributes to the wider comprehension of the insects’ physiology and describes pharmacological relevant molecules.

KEYWORDS: Triatoma infestans, tachykinin-related peptides, antimicrobial peptides, Hemiptera, bioactive molecules

INTRODUCTION

Antimicrobial peptides and proteins (AMPs) are cationic and amphipathic molecules that can interact with molecules present on cell membranes and with intracellular pathways in microbes, leading to cell disruption and death.1

These bioactive molecules were initially described in insects by Stephens2 in 1962, followed by Hink and Briggs3 in 1968, Powning and Davidson4 in 1973, Boman et al.5 in 1974, and Faye et al.6 in 1975. Subsequent studies led to the characterization of the first 2 antimicrobial molecules in 1981 by Steiner and Champagn6-34,43,44,45 identified 2 TKs from the saliva glands of Eledone moschata that was capable of lowering blood pressure, increasing salivation, and stimulating smooth muscle in rabbits and dogs. This substance was named as edelogin and was the first non-mammalian TK described.36,37,41,42 Other TKs from frogs and non-mammalian species were subsequently described and isolated.40,40,43,44

Champagne and Ribeiro46 identified 2 TKs from the salivary glands of the mosquito Aedes aegypti, confirming the presence of peptides related to vertebrate TKs in invertebrates. Following this study, 4 molecules from brain, corpora cardiaca, corpora allata, and subesophageal ganglion extracts of the nervous system of Locusta migratoria, named as locust atachykinins, were found to show 30% homology with vertebrate TKs and up to 45% similarity with fish and amphibian TKs.46-48

After these discoveries in invertebrates, 2 new molecule subfamilies were established: the invertebrate tachykinins (Inv-TKs) and tachykinin-related peptides (TKRPs). The main
difference between these 2 subfamilies is their C-terminal amino acid composition. Tachykinin-related peptides are clearly related to vertebrate TKS, sharing the same C-terminal region (F-X-G-Y-R-NH₂), whereas Inv-TKs differ in their last 2 residues, containing leucine and methionine (F-X-G-L-M-NH₂). However, they still maintain high homology and exhibit similar biological effects.

Several Inv-TKs and TKRP have been described in species such as Agrotis ipsilon, Apis mellifera, Camponotus floridanus, Drosophila melanogaster, Drosophila pseudoobscura, Euschistus servus, Pentatoma rufipes, and Ons et al identified 8 TKRP sequences through neuropeptidome analysis of Rhodnius prolixus. However, they still maintain high homology and exhibit similar biological effects.

Tachykinin-related peptides have been described in other insects in the order Hemiptera, including in Triatoma infestans, which was analyzed in the current study. Neupert et al described 6 TKRPs produced by Nezara viridula, Banasa dimiata, Pyrrhocoris apterus, Oncopeltus fasciatus, Pentatoma rufipes, and Euschistus servus, and Ons et al identified 8 TKRP sequences through neuropeptidome analysis of Rhodnius prolixus.

There are no further reports of TKRP in other Hemiptera species. Thus, the aim of this study was to isolate and characterize the first 2 TKRPs in the T. infestans hemolymph (Hemiptera: Reduviidae).

Materials and Methods

Microbial strains

The microorganisms Alcaligenes faecalis (ATCC 8750), Aspergillus niger (bread isolated), Candida albicans (IOC 4558), Candida parapsilosis (IOC 4564), Candida tropicalis (IOC 4560), Cladosporium sp. (bread isolated), Cladosporium herbarum (ATCC 26362), Cryptococcus neoformans, Enterobacter cloacae b-12, Escherichia coli (SBS363), Bacillus megaterium (ATCC 10778), Bacillus subtilis (ATCC 6633), Micrococcus luteus (strain A270), M. luteus (Nalidixic resistant), Paecilomyces farinosus (IBCC-215), Penicillium expansum (bread isolated), Pseudomonas aeruginosa (ATCC 27853), Saccharomyces cerevisiae, Serratia marcescens (ATCC 4112), and Staphylococcus aureus (ATCC 29213) were provided by the Special Laboratory of Applied Toxinology from their microorganism library (Butantan Institute, São Paulo, Brazil).

Animals

Both male and female T. infestans (Hemiptera: Reduviidae) were provided by the Ecolyzer Group—Entomology Laboratory (São Paulo, Brazil). The insects were maintained under controlled humidity and temperature in the biotherium of the Special Laboratory of Applied Toxinology (Butantan Institute, São Paulo, Brazil) and fed every 2 weeks with human blood (collected from a healthy volunteer donor and stored in citrate buffer [150 mM, pH 7.4]). The study was conducted under exemption from the Institutional Ethics Committee (CEUAIB No. I-1354/15).

Bacteria inoculation and hemolymph collection

Adult T. infestans were injured with thin needles soaked in a pool of culture of Enterobacter cloacae and M. luteus in the logarithmic phase. At 72 hours after induction, hemolymph samples were obtained by excising the metathoracic legs of the insects and pressing gently on the abdomen. Drops were collected with micropipettes and pooled in sterile Eppendorf tubes on ice containing phenylthiourea to avoid activation of the phenol oxidase cascade. The solution was stored at −80°C until use.

Sample fractionation

Intracellular content extraction. To release the intracellular components from the hemolymph, the sample was subjected to acid extraction in the presence of acetic acid (2 M). The supernatant was obtained by centrifugation at 16,000 × g for 30 minutes at 4°C and injected directly into 2 coupled Sep-Pack C18 cartridges (Waters, Milford, MA, USA) equilibrated in 0.1% trifluoroacetic acid (TFA). The sample was eluted with 3 different concentrations of acetonitrile (ACN) in water (5%, 40%, and 80%) and then concentrated in a vacuum centrifuge and reconstituted with ultrapure water.

Reversed-phase high-performance liquid chromatography. Separation steps were performed using high-performance liquid chromatography (HPLC) using a reverse-phase semi-preparative C18 column (Jupiter, 10 × 250 mm; Phenomenex, Torrance, CA, USA) equilibrated in 0.05% TFA. The elution gradient for the fraction eluted with 5% ACN was from 2% solution A (0.05% [v/v] TFA in water) to 20% solution B (0.10% [v/v] TFA in ACN). For the 40% ACN fraction, the elution gradient was 2% to 60% solution B (0.10% [v/v] TFA in ACN). For the 80% ACN fraction, the elution gradient was 20% to 80% solution B (0.10% [v/v] TFA in ACN) in solution A. Each run was performed over 60 minutes at a flow rate of 1.5 mL/min.

Effluent absorbance was monitored at 225 nm, and fractions corresponding to the absorbance peaks were hand-collected, concentrated under vacuum, and reconstituted in ultrapure water.

When necessary, a second chromatography step was performed. The gradient for the second chromatography was determined by evaluation of the molecule’s retention time and performed on a VP-ODS analytic C18 column (Shim-pack; Shimadzu, Kyoto, Japan) at a flow rate of 1.0 mL/min for 60 minutes.

Liquid growth inhibition assay

All fractions obtained by HPLC and synthetic peptides were evaluated by antimicrobial screening against the microorganism strains (see section “Microbial Strains”) using poor
broth nutrient medium (PB: 1.0 g peptone in 100 mL of water containing 86 mM NaCl at pH 7.4; 217 mM NaNO₃ and Müller-Hinton medium (peptone 5.0 g/L; casein peptone 17.5 g/L; agar 15.0 g/L; Ca²⁺ 20.0-25.0 mg/L; Mg²⁺ 10.0-14.5 mg/L; pH 7.4) for bacteria and poor dextrose broth (1/2 PDB: 1.2 g potato dextrose in 100 mL of H₂O at pH 5.0; 79 mM NaCl) and RPMI 1640 (Roswell Park Memorial Institute medium) medium with MOPS 0.165 mol/L (RPMI without bicarbonate 10.4 g/L; MOPS [3-(N-morpholino)propanesulfonic acid] 34.53 g/L; pH 7.0) at half half-strength.⁵⁹,⁶⁰

Antimicrobial activity was determined in a 5-fold microtiter broth dilution assay in 96-well sterile plates at a final volume of 100 μL. The mid-log phase microbial culture was diluted to a final concentration of 1 × 10⁵ colony-forming units per milliliter.⁵⁹,⁶¹,⁶²

Lyophilized fractions were dissolved in 500 μL ultrapure water, and 20 μL peptide solution was aliquoted into each well containing 80 μL of the bacterial dilution, giving a final volume of 100 μL. To determine the minimal inhibition concentration, 20 μL peptide stock solution was added to each well of the microtiter plate at a 2-fold serial dilution and added to 80 μL of the bacterial dilution. Sterile water and PB were used as growth controls, and streptomycin was used as a growth inhibitor control.

The microtiter plates were incubated for 18 hours at 30°C, and then growth inhibition was determined by measuring the absorbance at 595 nm.⁶³,⁶⁴ To determine the minimal inhibition concentration, the bacterial growth rates were measured after 18 hours of incubation. To determine the minimal bactericidal concentration, the bacterial growth rates were measured at 595 nm after 96 hours.⁶³,⁶⁴ All antimicrobial experiments were performed in triplicate.

**Mass spectrometry**

Active antibacterial fractions were analyzed by liquid chromatography-tandem mass spectrometry on an LTQ-Orbitrap Velos (Thermo Fisher Scientific, Waltham, MA, USA) coupled to an Easy-nLCII liquid nano-chromatography system (Thermo Fisher Scientific). For chromatography, 5 μL of each sample was automatically separated on a C18 pre-column (100 mm I.D. × 50 mm; Jupiter 10 mm; Phenomenex) coupled to a C18 analytical column (75 mm I.D. × 100 mm; ACQUA 5 mm; Phenomenex). The eluate was electro-sprayed at 2 kV and 200°C in positive ion mode. Mass spectra were acquired with a Fourier transform-based mass spectrometer; full scan (MS1) involved using 200-2000 m/z (60000 resolution at 400 m/z) with a mass scan interval in data-dependent acquisition mode. The 5 most intense ions in each scan were selected for fragmentation by collision-induced dissociation. The minimum threshold for selecting an ion for a fragmentation event (MS2) was set to 5000. The dynamic exclusion time was 15 seconds, repeating at 30-second intervals.

**Computational analysis**

Mass spectrometry data were analyzed using Mascot Daemon software (version 2.2.2) through database searching using the Swiss-Prot, NCBInr, Hemipterans, and Triatomineos banks for comparison. Homology searching was performed using the PepBank peptide database, Signal Peptide Database, Vector Base, APD3: Antimicrobial Peptide Calculator and Predictor, and BLAST: Basic Local Alignment Search Tool. The results were considered valid only when they were reproducible by different analyses.

**Primary structure alignment**

Alignment of primary sequences was performed using Clustal W2 version 2 online software with default parameters.

**Solid-phase peptide synthesis**

Peptides were synthesized using the solid-phase method using methylbenzhydrylamine resin (MBHAR) and employing the β-Boc strategy. For synthetic peptide entitled TRP2-TINF, the methionine (second amino acid in the C-terminal region) was changed to norleucine, reducing the molecular weight to 1006 Da. After cleaving the peptides from the resin, the peptides were purified from the lyophilized crude solutions by HPLC on a C18 column. To guarantee high purity and to characterize the peptides, LC-ESI-MS equipment was used.

**Synthetic peptide concentration**

Peptide concentrations were determined using the Lambert-Beer law using the molar extinction coefficient at 205 nm absorption, obtained using the tool available at http://nickanthis.com/tools/a205.html.

**Peptide stability in serum**

Aliquots of 20 μL peptide solution (10 mg/mL) were added to 1 mL of 25% non-heat inactivated horse serum (Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline (PBS) and incubated at 37°C in triplicate for different time intervals (0, 10, 30, 60, and 120 minutes). During incubation, 100 μL of sample was withdrawn incubated with 10 μL of pure TFA at 5°C for 15 minutes. The resulting mixtures were centrifuged at 300 × g for 5 minutes. A volume of 30 μL of the supernatants was injected in an online HPLC coupled to a mass spectrometer (LC-ESI-MS). Peptide consumption was evaluated using a linear gradient of ACN in acidified water from 3% to 57% in 30 minutes at a flow rate of 0.4 mL/min, followed by the measurement of the area decrease of the peak that corresponds to the peptide in the chromatogram.⁷³
Circular dichroism

Circular dichroism (CD) measurements were performed on a Jasco J-815 circular dichroism spectropolarimeter (Jasco Corp., Tokyo, Japan). For UV measurements (190–250 nm), CD spectra were recorded after 4 accumulations at 20°C using a 0.5-mm pathlength quartz cell between 250 and 195 nm at 50 nm/min with a bandwidth of 0.5 nm. Both peptides were analyzed in 0%, 10%, 30%, and 50% v/v solutions of 2,2,2-trifluoroethanol in water. Fast Fourier transform was applied to minimize background effects.

Cytotoxic assays

The toxicity of the peptides against Vero cells (African green monkey kidney fibroblasts) was evaluated. Cells were obtained from the American Type Culture Collection (ATCC CCL81; Manassas, VA, USA) and maintained at 37°C in T culture flasks (25 cm²) containing 5 mL Leibovitz medium (L-15) supplemented with 10% fetal bovine serum, both from Cultilab (Campinas, Brazil) or in a 96-well microplate. Toxicity was determined using the MTT colorimetric assay. Briefly, the cells were seeded into 96-well plates (2 × 10⁵ cells/well) and cultured for 24 hours at 37°C in a humidified atmosphere containing 5% CO₂. Twelve 2-fold serial dilutions of both peptides were performed with L-15 to give solutions with final concentrations ranging from 1.95 to 1000 μM. Varying concentrations were added and allowed to react with the cells for 24 and 48 hours, followed by addition of 20 μL MTT (5 mg/mL in PBS) and incubation for another 4 hours at 37°C. Formazan crystals were dissolved by adding 100 μL dimethyl sulfoxide and incubation at room temperature until all crystals were dissolved. Absorbance at 550 nm was measured using a microplate ELISA reader (1420 Multilabel Counter/Victor3; Perkin Elmer, Waltham, MA, USA). Cell survival was calculated using the following formula: survival (%) = (A550 of peptide-treated cells/A550 of peptide-untreated cells) × 100.

Hemolytic assay

Human erythrocytes from a healthy donor were collected in 0.15 M citrate buffer (pH 7.4) washed 3 times by centrifugation (700 × g, for 10 minutes at 4°C) with 0.15 M PBS containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄ (pH 7.4) and resuspended in PBS to a final concentration of 3% (v/v). The peptides (serial 2-fold dilutions in PBS) were added to 80 μL erythrocyte suspension to a final volume of 100 μL and incubated for 1 hour at 37°C. Hemoglobin release was monitored by measuring the supernatant absorbance at 405 nm with a Microplate ELISA Reader (1420 Multilabel Counter/Victor3). The hemolysis percentage was expressed in relation to a 100% lysis control (erythrocytes incubated with 0.1% Triton X-100); PBS was used as a negative control.

Results and Discussion

Sample fractionation and peptide purification

The hemolymph was processed and separated on a Sep-Pack C18 column using 5%, 40%, and 80% ACN concentrations to elute the main sample into three different fractions (see section “Sample fractionation”). During purification of the 5% ACN sample by RP-HPLC, the fraction eluted at 67.3 minutes (sample 1; Figure 1A) showed antimicrobial against E. coli, whereas the fraction eluted at 56.2 minutes during 40% ACN sample purification by RP-HPLC (sample 2; Figure 2A) showed antimicrobial activity against M. luteus. Both were completely isolated in the second chromatography step (Figures 1B and 2B).
Peptide identification

After purification, both samples were examined by mass spectrometry and the data were analyzed in MagTran 1.02. We verified that samples 1 and 2 had approximated molecular weights of 951 and 1035 Da, respectively (Figure 3).

To determine the amino acid content and perform protein identification, mass spectrometry data were analyzed with Mascot software with the Fingerprint tool and using the Hemiptera database (118,063 sequences). Both samples showed similarities with tachykinin-related peptides I and II (TRP1 and TRP2), respectively, from different insect species (Figure 4).

According to the official TKRP nomenclature, these peptides were named as TRP1-TINF and TRP2-TINF (tachykinin–related peptides I and II from *T. infestans*). As observed for TRP1-TINF, the first match corresponded to an uncharacterized protein from *Acyrthosiphon pisum*. However, this protein showed an expected threshold *P*-value >.05, and thus this result was discarded. Showing 100% coverage with the corresponding sequences, TRP1-TINF is composed of 9 amino acid residues with the sequence GPSGFLGMR and RP2-TINF is composed of 10 amino acid residues with the sequence APAAGFFGMR.

Tachykinins are proteins that comprise a superfamily initially described by Von Euler and Gaddum and have neurotransmitter/neuromodulator activities as their main physiological functions. A subfamily discovered in insects, named as tachykinin-related peptides (or tachykinin-like peptides), has been widely studied. The production of TKRP by species in the order Hemiptera has been described, and all sequences are summarized in Table 1.
Figure 4. Mass spectrometry analysis using MASCOT software: (A) Sample 1 mass spectrometry data compared with a Hemiptera database (118063 sequences). Sample showed similarity with tachykinin-related peptide I from Banasa dimiata, Pyrrhocoris apterus, Pentatoma rufipes, Oncopeltus fasciatus, Nezara viridula, Euschistus servus, and Acrosternum hilare. (B) Sample 2 mass spectrometry data compared with a Hemiptera database (118063 sequences). Sample showed similarity with tachykinin-related peptide II from B. dimiata, P. rufipes, N. viridula, E. servus, and A. hilare.

Table 1. Relationships with TKRPs from Hemipterans.

| SPECIES                  | PEPTIDE                 | FAMILY NAME | PEPTIDE SEQUENCE |
|-------------------------|-------------------------|-------------|------------------|
| Acrosternum hilare      | Tachykinin-related peptide I | TRP1_ACRHI  | GPSGFLGMR        |
|                         | Tachykinin-related peptide II | TRP2_ACRHI  | APAAGFFGMR       |
|                         | Tachykinin-related peptide III | TRP3_ACRHI  | GPSGSGFGMR       |
|                         | Tachykinin-related peptide IV | TRP4_ACRHI  | SPAGSGFGMR       |
|                         | Tachykinin-related peptide V | TRP5_ACRHI  | APLMGFGGVR       |
|                         | Tachykinin-related peptide VI | TRP6_ACRHI  | APMGFGMGMR       |
| Banasa dimiata (Pentatoma dimiata) | Tachykinin-related peptide I | TRP1_BANDI  | GPSGFLGMR        |
|                         | Tachykinin-related peptide II | TRP2_BANDI  | APAAGFFGMR       |
|                         | Tachykinin-related peptide III | TRP3_BANDI  | GPSGSGFGMR       |
|                         | Tachykinin-related peptide IV | TRP4_BANDI  | SPAGSGFGMR       |
|                         | Tachykinin-related peptide V | TRP5_BANDI  | APLMGFGGVR       |
|                         | Tachykinin-related peptide VI | TRP6_BANDI  | APMGFGMGMR       |
| Euschistus servus       | Tachykinin-related peptide I | TRP1_EUSSE  | GPSGFLGMR        |
|                         | Tachykinin-related peptide II | TRP2_EUSSE  | APAAGFFGMR       |
|                         | Tachykinin-related peptide III | TRP3_EUSSE  | GPSGSGFGMR       |
|                         | Tachykinin-related peptide IV | TRP4_EUSSE  | SPAGSGFGMR       |
|                         | Tachykinin-related peptide V | TRP5_EUSSE  | APLMGFGGVR       |
|                         | Tachykinin-related peptide VI | TRP6_EUSSE  | APMGFGMGMR       |

(Continued)
As *Rhodnius prolixus* is phylogenetically close to *T. infestans*, primary sequence alignment was performed to compare their most similar sequences (Figure 5), which revealed a close resemblance.

Recent studies have shown that a few components of the vertebrate tachykinin family have antimicrobial activity against some bacterial strains such as *S. marcescens* and *P. aeruginosa*.77-79 Although none of the TKRP sequences mentioned above were
evaluated in antimicrobial assays, urechistachykinin I and urechistachykinin II, which are tachykinin-related neuropeptides isolated from the Echiuroid worm and share the C-terminal region, presented antimicrobial activity against different Gram-positive and Gram-negative bacteria and different fungi.80

As a main characteristic of the peptide family, all sequences share the same C-terminal region (F-X-G-Y-R-NH2); thus, it is expected that they have similar biological effects.

**Characterization of isolated TKRPs**

**Bioassays.** TRP1-TINF and TRP2-TINF were artificially synthesized. For synthetic TRP2-TINF, the methionine (second amino acid in the C-terminal region) was changed to norleucine, reducing the molecular weight to 1006 Da. For comparison, the synthetic peptides were also tested against *M. luteus* and *E. coli*. Both peptides were active against the two microorganisms, indicating the antimicrobial potential of the synthetic molecules.

The synthetic peptides were tested against a broad range of bacterial, yeast, and fungal species and their antimicrobial activities are reported in Tables 2 and 3. The initial concentration of TRP1-TINF was 128 µM and that of TRP2-TINF was 164 µM.

Both TRP1-TINF and TRP2-TINF could partially or completely inhibit the growth of several bacteria and fungi.

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**Table 2.** Synthetic TRP1-TINF antimicrobial activities against bacterial and fungal strains.

| MICROORGANISMS | MIC, µM (MG/ML) | MBC, µM (MG/ML) |
|----------------|-----------------|-----------------|
|                | MH              | PB              |
| Gram-positive bacteria |
| *Micrococcus luteus*  | 128 (0.20) 32 (0.05) |
| *Staphylococcus aureus*  | NA NA |
| *Micrococcus luteus BR2*  | NA NA |
| *Bacillus megaterium*  | NA NA |
| *Bacillus subtilis*  | NA NA |
| Gram-negative bacteria |
| *Alcaligenes faecalis*  | NA NA |
| *Enterobacter cloacae*  | NA NA |
| *Serratia marcescens*  | NA NA |
| *Pseudomonas aeruginosa*  | 64 (0.10) 64 (0.10) |
| *Escherichia coli*  | 100 (0.10) |
| Yeasts |
| *Candida albicans*  | NA NA |
| *Candida parapsilosis*  | NA NA |
| *Cryptococcus neoformans*  | NA 128 (0.20) NA 128 (0.20) |
| *Saccharomyces cerevisiae*  | NA NA |
| *Candida tropicalis*  | NA NA |

(Continued)
Table 3. Synthetic TRP2-TINF antimicrobial activities against bacterial and fungal strains.

| MICROORGANISMS | MIC, μM (MG/ML) | MBC, μM (MG/ML) |
|----------------|----------------|-----------------|
|                | RPMI | PDB | RPMI | PDB |
|Filamentous fungi |     |     |     |     |
| Cladosporium sp. | NA  | NA  | NA  | NA  |
| Penicillium expansum | 128 (0.20) | 128 (0.20) | 128 (0.20) | 128 (0.20) |
| Aspergillus niger | NA  | 128 (0.20) | NA  | 128 (0.20) |
| Paecilomyces farinosus | NA  | NA  | NA  | NA  |
| Cladosporium herbarum | NA  | NA  | NA  | NA  |

Abbreviations: MBC, minimal bactericidal concentration values obtained by liquid growth inhibition assay; MH, Müller-Hinton medium; MIC, minimal inhibition concentration; NA, not active at a concentration of 128 μM; PB, poor broth nutrient medium; PDB, potato dextrose broth medium; RPMI, Roswell Park Memorial Institute medium.

Diniz et al
Neither peptide showed specific activities, although both could disrupt bacterial cells at a lower concentration than that required for disrupting fungi cells. For instance, TRP1-TINF was active against Gram-positive and Gram-negative bacteria (M. luteus with 32 µM and P. aeruginosa with 64 µM), and TRP2-TINF has predilection toward Gram-negative bacteria, presenting activity with lower concentrations toward P. aeruginosa and E. coli.

Hemolysis percentages of 0.9% and 0.6% were observed when red blood cells were incubated with 300 µM of TRP1-TINF and 360 µM of TRP2-TINF, respectively. Thus, the peptides showed no significant hemolytic activity (P < .05). In contrast, when incubated Vero cells with the highest tested concentration of both peptides (1000 µM), cell viability was approximately 84% and 90% after exposure to TRP1-TINF and TRP2-TINF, respectively (Figure 6). The selectivity index was not calculated, as no median lethal dose values were found at the maximum evaluated concentrations.

To investigate the physicochemical dynamics of the peptides, we performed tridimensional structure analysis and degradation analysis in plasma assays. In CD analysis, the structural conformation of asymmetric molecules was obtained from the patterns of absorption and refraction of the absorbance of right- and left-handed circularly polarized light as a hygroscopic liquid was added to the original solution.

As shown on the CD spectra, TRP1-TINF (Figure 7A) tended to assume a random coil conformation, whereas TRP2-TINF (Figure 7B) tended to assume a Poly (Pro) II (PPII) or helix-3, conformation.

TRP1-TINF showed a random secondary structure because of 2 main factors. It is formed of less than 10 amino acid residues and nearly half of these residues have small and neutral side chains (3 glycine residues and 2 proline residues), lowering the possibility of forming a stable secondary structure.

Tachykinin-related peptides are present in different insect tissues because of hemolymph circulation, thus it is crucial to determine their degradation rate in the plasma to develop improve the understanding of the peptide bioavailability. The percentages of available peptides in the plasma after different incubation times are shown in Figure 8.

Within 15 minutes, the percentage of TRP1-TINF decreased to 69% peptide remaining; this process occurs gradually, with only 16% of the whole peptides remaining in solution at 120 minutes.

TRP2-TINF was more susceptible to degradation. In 15 minutes, the peptide concentration decreased to 34% and in 30 minutes decreased to 14%. By 60 minutes, 2.3% of the peptide remained, and no peptide was detected at 120 minutes.

The molecular weight of each peptide and the degradation curves were analyzed by mass spectrometry to determine the amino acid loss (Table 4).

By observing the amino acid loss at each time point, it is possible to determine if the sequence was degraded by aminopeptidases, carboxypeptidases, or endopeptidases. After

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**Figure 6.** TRP1-TINF and TRP2-TINF toxicity assays. Cytotoxicity of TRP1-TINF (Δ) and TRP2-TINF (•) over Vero cells in a 2-fold series dilution at a maximum concentration of 1000 µM.

**Figure 7.** TRP1-TINF and TRP2-TINF CD spectra: (A) Diffraction pattern of polarized light of TRP1-TINF, corresponding to a random structure. (B) Diffraction pattern of polarized light of TRP2-TINF, corresponding to a PPII structure.
15 minutes of incubation, 3 molecular weights were detected, and 903, 846, and 749 Da were also registered. These molecular weights correspond to the entire sequence and loss of G and G-P, respectively. Additional amino acid losses continually occurred until 120 minutes. At the last time point, 5 molecular weights corresponding to the full sequence without G (846 Da), G-P (749 Da), G-P-S (661 Da), G-P-S-G (604 Da), and G-P-S-G-F (457 Da) were observed. These losses indicate the susceptibility of the peptides to degradation by aminopeptidase enzymes.85

The degradation profile of TRP2-TINF is compatible with degradation by a carboxypeptidase. Within 15 minutes, fragments with molecular weights of 850 and 736 Da were detected, corresponding to the entire sequence without R and RN. In agreement with this, at 30, 60, and 120 minutes RNG loss was observed (681 Da). This degradation is indicative of carboxypeptidase activity.86

**Conclusions**

These results increase the understanding of triatomines physiology and represent a pharmacological relevant study object, providing the first description of TKRP molecules produced by T. infestans and demonstrating the antimicrobial potency and physicochemical features of the isolated molecules.

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**Author Contributions**

LCLD was responsible for the development of all experiments and writing the manuscript; PIdS Jr participated during the purification experiments, mainly HPLC, and all of the mass spectrometry analysis; AdM participated during peptide synthesis, in plasma degradation, circular dichroism, and mass spectrometry analysis; and FLA participated during circular dichroism experiments. All the authors approve the entire manuscript content and ensure the accuracy and integrity of any part of the work.

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