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Research paper

Rmcystatin3, a cysteine protease inhibitor from *Rhipicephalus microplus* hemocytes involved in immune response

Stephen Lu [a], Tatiane S. Soares [a], Itabajara S. Vaz Junior [b], Diogo V. Lovato [a], Aparecida S. Tanaka [a, *]

[a] Department of Biochemistry, Escola Paulista de Medicina, Universidade Federal de São Paulo, 04044-020 São Paulo, SP, Brazil
[b] Centro de Biotecnologia do Estado do Rio Grande do Sul, UFRGS, Porto Alegre, Brazil

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ABSTRACT

The *Rhipicephalus microplus* tick is responsible for losses in the livestock production estimated in 2 billions USD. Despite its economical importance the knowledge in tick's physiology is sparse. In order to contribute to this scenario we describe the characterization of a cysteine proteinase inhibitor named Rmcystatin-3. Purified recombinant Rmcystatin-3 was able to inhibit cathepsin L (Ki = 2.5 nM), BmCl1 (Ki = 1.8 nM) and cathepsin B (Ki = 136 nM). Western blot and quantitative PCR analysis revealed the presence of Rmcystatin-3 in fat body, salivary gland but mainly in hemocytes. The mRNA levels of Rmcystatin-3 during bacterial challenge are drastically down-regulated. In order to define the Rmcystatin-3 possible role in tick immunity, the cystatin gene was knockdown by RNA interference with and without *Escherichia coli* infection. Our results showed that the Rmcystatin-3 silenced group was more immune competent to control bacterial infection than the group injected with non-related dsRNA. Taking together, our data strongly suggested an important role of Rmcystatin-3 in tick immunity.

1. Introduction

The cattle tick *Rhipicephalus microplus* is responsible for major losses in livestock production in tropical and subtropical regions. Tick parasitism can cause severe anemia, reduction of milk and meat production, diminish the value of skin, and leather production [1,2]. *R. microplus* can also act as vector of bovine pathogens known as the tick-babesia spp.-anaplasma spp complex which is the most important tick–pathogen relation in the world [3]. Tick control is based on acaricides, which lead to the selection of resistant strains and environmental contamination, making necessary the development of alternative control methods [4–6].

Despite the recent advances in invertebrate immunology, the knowledge in tick immunity is still sparse. Tick cellular response is mediated by hemocytes – circulating cells in hemolymph – to which the phagocytic activity is attributed [7–9]. In addition, complement-related molecules such as α-2-macroglobulins were described in several tick species associated with phagocytosis [10–12]. Tick humoral response consist of a vast repertory of antimicrobial peptides (AMPs) such as ixodidin [13], microplusin [14] and the ability to produce AMPs through hemoglobin digestion [15]. The *Drosophila* immune model [16] appears to be a solid base for invertebrate immunity and intermediates from both TOLL and IMD pathways are conserved in other arthropods such as mosquitos [17], crustacean [18] and can also be found in *Ixodes scapularis* genome, suggesting the presence of such pathway in ticks. However, none conclusive data have been shown. Protease inhibitors also participate in tick immunity [19,20] among them, a type-2 cystatin from *Haemaphysalis longicornis* was able to inhibit the *in vitro* growth of *Babesia gibsoni* [21].

The cystatin superfamily is traditionally characterized by *tight-binding* inhibitors of cysteine proteinases, which are largely distributed among live beings [22]. The cystatin family is comprised of three subfamilies classified based on their primary sequence: (i) *Stefins* are 11 kDa inhibitors with absence of signal peptide and disulphide bridges, (ii) *cystatins* present molecular weight from 13 to 15 kDa, with a signal peptide and two disulphide bridges, and (iii) *kininogens* are multi domain proteins with eight disulphide bridges and high molecular weight [23,24]. Although, there is a new classification of the Cystatin family available on MEROPS based on the number of cystatin domains present in the inhibitors [25]. In the last few years, several tick cystatins have been characterized and associated with the parasite–host interface modulating host immunity [26–28], blood feeding [29] and pathogen transmission [30]. A few cystatins have been related to hemoglobin digestion [31] and embryogenesis [32].

* Corresponding author. Tel.: +55 11 55764445, +55 11 55761080.
E-mail address: tanaka.bioq@epm.br (A.S. Tanaka).

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In this study, we describe a new cystatin, named Rmcystatin-3 (*R. microplus* cystatin 3), located in hemocytes of *R. microplus* and related to the tick immune response.

2. Materials and methods

2.1. Animals

R. microplus ticks (Babesia spp.-free) was supplied by Dr. Itabajara da Silva Vaz Junior of Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, RS, Brazil.

2.2. Rmcystatin-3 oligonucleotide design

Rmcystatin-3 primers were designed based on the predicted sequence obtained from a cDNA library of hemocytes by adding the restrictions sites of *Sac* II and *Xba* I enzymes at the 5′ and 3′ respectively. The sense oligonucleotides was: Rmcys3_FW 5′-CCGCGGGCCAATCCTGTAGGG-3′ and the antisense oligonucleotide: Rmcys3_RV 5′-TCTAGATTAATTTGAAGTGGC-3′.

2.3. Amplification and cloning of Rmcystatin-3 DNA fragment

A Rmcystatin-3 DNA fragment was amplified from a cDNA library of hemocytes by PCR. The reactions were performed in a 50 μL final volume using 1.5 mM $MgCl_2$, 100 μM dNTPs, 10 pmol of each primer, 5 U Taq DNA polymerase (Fermentas), and its corresponding buffer [100 mM Tris–HCl pH 8.8, 500 mM KCl, and 0.8% (v/v)]. The PCR parameters were: pre-denaturation at 94 °C for 10 min; 35 cycles of (94 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min); followed by 5 min at 72 °C. Rmcystatin-3 fragment was separated by agarose gel electrophoresis 1% (w/v) and purified using QIAEX II gel extraction system according the manufacturer's instructions (QIAGEN). Rmcystatin-3 purified fragment and the expression vector pPICZαB were submitted to a double digestion, with *Sac* II and *Xba* I enzymes, in Tango buffer, overnight at 37 °C, followed by the ligation of the digested fragment to the vector. *Escherichia coli* DH5α strain were transformed using the construction pPICzαB-Rmcystatin-3 and plated on Lenox Broth (LB) agar plates (1% tryptone, 0.5% yeast extract, 0.5 NaCl) containing 25 μg/mL of Zeocin. Positive clones for DNA insertion were verified by DNA sequencing and one corrected construction was used in a midi-plasmid DNA preparation using Plasmid Midi Kit from QIAGEN (Hilden, Germany).

2.4. Preparation and transformation of Pichia pastoris yeast

P. pastoris GS115 strain was grown in a yeast extract-peptone-dextrose (YPD) medium and prepared for transformation by electroporation following the manufacturer's instructions. After linearization of plasmid Rmcystatin3-pPICZαB (10 μg) with *Sac I* restriction enzyme, competent *P. pastoris* GS115 were transformed by electroporation. Yeast transformation was performed in a 0.2 cm cuvette in a Gene Pulser (Bio-Rad, Hercules, CA) using the following parameters: 1.5 kV, 25 μF and 400 Ω. The electroporated cells were immediately suspended in 1.0 mL of ice-cold 1.0 M sorbitol and spread on MD agar plate (1.34% YNB, 2% dextrose, 4×10^{-5}% biotin), without histidine. The target gene in the recombinants was detected by PCR reactions using 3′AOX and 5′AOX primers from Invitrogen (Carlsbad, CA, USA) and some positive clones were selected.

2.5. Expression of Rmcystatin-3

One isolated colony of *P. pastoris* GS115 Mut^+ strain containing Rmcystatin3-pPICZαB construction was inoculated in 160 mL of BMGY medium [1.0% yeast extract, 2.0% peptone in 100 mM potassium phosphate buffer pH 6.0, 1.34% YNB, 4×10^{-5} (w/v) D-biotin and 1% (v/v) glycerol] in 1 L sterile flask, and cultivated at 30 °C, 190 rpm until to reach 6.0 in A_{600}. Yeast cells were separated by centrifugation at 3000 *g* for 15 min at 4 °C and resuspended in BMMY medium [1.0% yeast extract, 2.0% peptone in 100 mM potassium phosphate buffer at pH 6.0, 1.34% YNB 4×10^{-5}% (w/v) D-biotin and 0.5% (v/v) methanol] at 1.0 in A_{600}. Expression of recombinant protein was induced by the addition of methanol (0.5% final concentration) every 24 h for the next 96 h at 30 °C and shaking at 190 rpm. By the end of the fourth day, the yeast cells were removed by centrifugation (3000 *g* for 15 min at 4 °C) and the supernatant stored at 4 °C for protein purification.

2.6. Purification of recombinant Rmcystatin-3

Recombinant Rmcystatin-3 was purified from yeast supernatant by an ionic exchange chromatography using a HiPrep Q column connected to a AKTA prime plus (GE Healthcare Life Sciences) previously equilibrated with 25 mM Tris–HCl buffer pH 8.0 and eluted with a crescent gradient of 25 mM Tris–HCl buffer pH 8.0 containing 1 M NaCl. Afterward, Rmcystatin-3 was further purified on a size exclusion chromatography using a Superdex75 column previously equilibrated with 25 mM Tris–HCl buffer pH 8.0 containing 0.2 M NaCl. Collected fractions containing inhibitory activity against cathepsin L were analyzed by SDS-PAGE.

2.7. SDS-polyacrylamide electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Laemmli [33]. SDS-PAGE (15%) with 5% stacking gel was used to analyze purified Rmcystatin-3. Proteins were stained by Coomassie Brilliant Blue R-250 solution.

2.8. Rmcystatin-3 gene silencing by RNA interference

Rmcystatin-3 gene silencing was performed by producing double stranded RNA (dsRmcys3) using the T7 *Ribomax Express RNAi system* (Promega) and the Rmcystatin-3 DNA fragment was amplified by PCR using primers conjugated with T7 promoter site: dsRmcys3_FW:

5′-GGATCCTAATACGACTCACTATAGGGCCAATCCTGTAGGGCTCG TG-3′ and dsRmcys3_RV 5′-GGATCCTAATACGACTCACTATAGGGC CAATCCTGTAGGGCTCGTG-3′, and applied in 1% (w/v) agarose gel and purified with QIAEX II kit (QIAGEN). The dsRmcys3 was digested with DNAse (1 μg/μL) and RNAse (1 μg/μL), precipitated with ethanol and resuspended in sterile PBS. The dsRNA quantification was made by absorbance at 260 nm and the quality of the material valued by the 260/280 ratio. It was using preparations with 260/280 above 1.7. Engorged female ticks were divided in groups injected with: dsRmcys3, dsLAC (non-related control), bacterial suspension (1×10^4 CFU/mL) and bacterial suspension plus dsRmcys3, respectively. All groups 24 h post-injection were used to collect hemolymph. Afterward, 2 μL of hemolymph were diluted in 198 μL LB medium and plated in LB agar plates containing 20 μg/mL of kanamycin. The hemocytes were separated from hemolymph by centrifugation at 800 *g* for 10 min and to them were added Trizol and stored at −20 °C. The hemolymph was stored at −20 °C.

2.9. Quantitative real-time PCR

Different tissues – midgut, ovary, hemocytes, fat body and salivary gland – were dissected. All tissues were used for RNA and protein extractions from Trizol solution. RNA preparations were used for cDNA synthesis using the ImProm-II Reverse Transcription System (Promega). The presence of Rmcystatin-3 mRNA in different

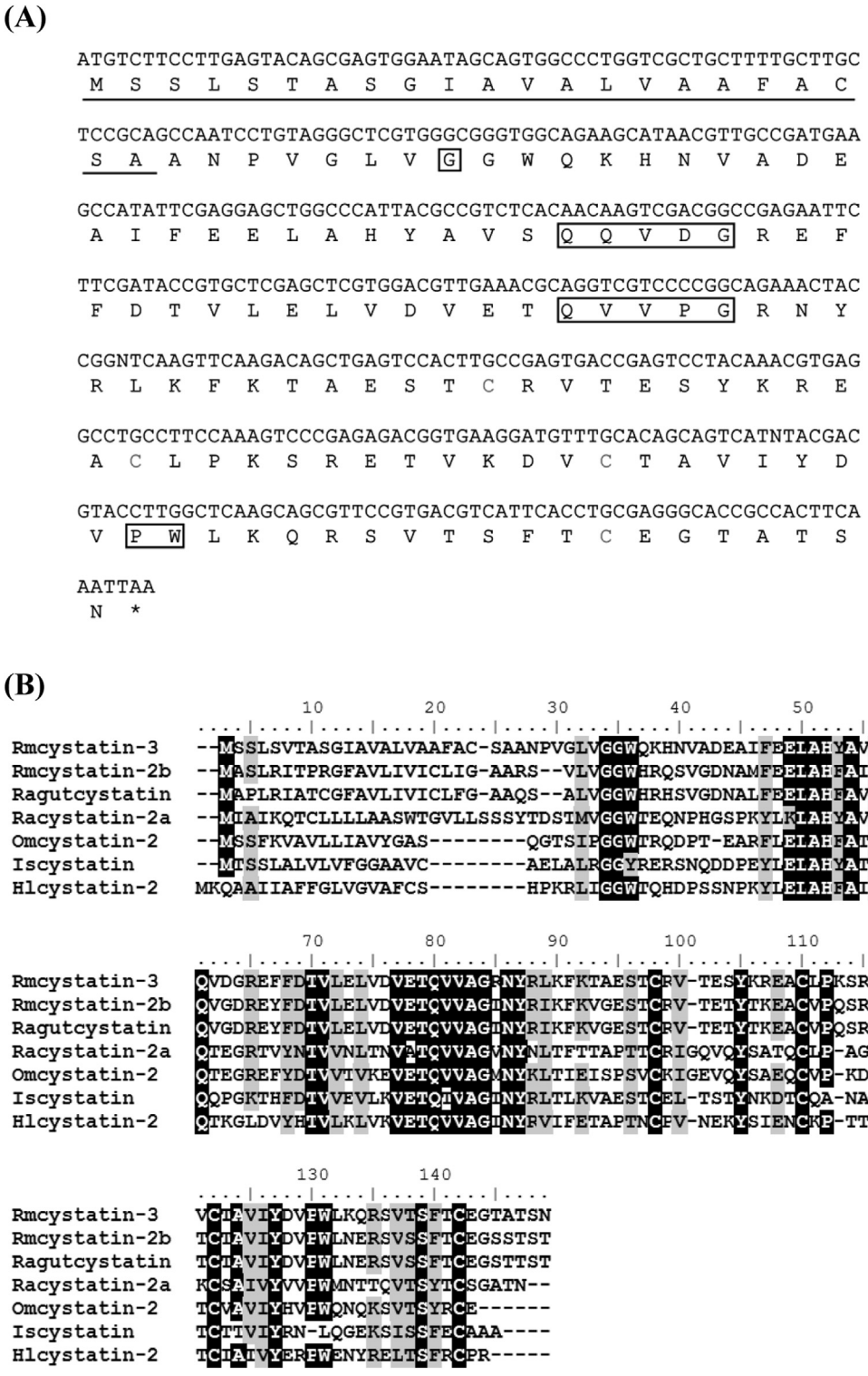

Fig. 1. Analysis of nucleotide and translated amino acid sequence of Rmcystatin-3. (A): The predicated signal peptide (SignalP) is underlined. Cysteine residues are in gray and the conserved motifs of a cystatin are boxed. (B): Alignment of amino acid sequence of Rmcystatin-3 and cystatins among tick species, identical residues are black boxed while similar residues are gray boxed. Rmicroplus-2b (*Rhipicephalus microplus*) GenBank: AGW80658.1; Hlongircornis (*Haemaphysalis longicornis*) GenBank: ABV71390.1; Rappendiculatus (*Rhipicephalus appendiculatus*) GenBank: AGB35873.1.

tissues was determined by quantitative PCR using Rmcys3_FW: 5′-TTA CGC CGT CTC ACA ACA AG-3′ and Rmcy3_RV: 5′-ACA TCC TTC ACC GTC TCT CG-3′. The elongation factor-1alpha (ELF1a) was used as endogenous control [34], using the primers: ELF1a_FW 5′-CGTCTACAAGATTGGTGGCATT-3′ and ELF1a_RV 5′-CTCAGTGGT-CAGGTTGGCAG-3′. The Rmcystatin-3 and ELF1a gene were quantified using SYBR-Green PCR Master Mix in a 7500 Fast Real Time PCR System (Applied Biosystems), according to the following

conditions: 40 cycles of 95 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. For each tissue, 10 ng of cDNA were used as template in two independent experiments. The amount of each mRNA was calculated according to the 2-DDCt method.

2.10. Western-blot analysis

Ticks tissues were used for protein extraction preparation using Trizol manufacturer's instructions. 50 μg of total protein from each tissue were separated on a 12% SDS-PAGE and transfer to a PVDF membrane by electrophoretic transfer system Mini Trans-Blot Cell (BioRad, Hercules, CA) for 45 min using 15 V. PVDF membrane was incubated for 1 h with PBS containing Tween 0.1% and 5% of skimmed milk at room temperature. After blocking, the PVDF membrane was washed three times with PBS containing Tween 0.1% for 15 min, following by incubation with purified anti-serum diluted 1:100 in blocking buffer, overnight at 4 °C. PVDF membrane was washed and incubated with anti-rabbit IgG conjugated with peroxidase (dilution 1:20,000) for 1 h at room temperature. The membrane was stained with SuperSignal West Pico Chemilumiescent Substrate (Pierce) and exposed for 2 min. The image was capture with the image-capturer MR-ChemBIS 3.2 (DNR Bioimaging System).

2.11. Determination of dissociation constant (K_i)

Cysteine proteinases, cathepsin L, BmCl1 and papain were pre-incubated in 50 mM sodium acetate buffer pH 5.5 containing 1 mM DTT at 37 °C for 10 min. Activated enzymes were pre-incubated with different concentrations of purified recombinant Rmcystatin-3 followed the addition of Z-Phe-Arg-MCA substrate (2 mM) for papain, cathepsin L and BmCl1, and the substrate Z-Arg-Arg-MCA (2 mM) for cathepsin B. The residual activity was measured at 380 nm excitation and 460 nm emissions. The dissociation constant calculated by fitting steady-state velocities to Morrison equation ($V_i/V_o = 1 - \{E_t + I_t + K_i - [(E_t + I_t + K_i)^2 - 4E_tI_t]^{1/2}\}/2E_t$) for *tight-binding* inhibitors [35].

3. Results

3.1. Rmcystatin-3 nucleotide and amino acid sequences analysis

The nucleotide sequence of Rmcystatin-3 (*R. microplus* cystatin-3) was identified in a hemocyte cDNA library of bovine tick. The sequence showed a 426-bp-ORF and an amino acid sequence characteristic of the family 2 cystatins: a signal peptide with cleavage site between amino acid residues 24 and 25 and four cysteine residues. Rmcystatin-3 primary structure reveals a mature protein of 142 aa (Fig. 1A) with a theoretical molecular weight of 13 kDa and isoelectric point (pI) of 5.39. The multiple sequence alignment with other tick cystatins reveals the conserve motifs among cystatin family, the Gly residue on N-terminal, the motif QxVxG and the pair proline–tryptophan at the C-terminal (Fig. 1B).

3.2. Cloning, expression and purification of rRmcystatin-3

The DNA fragment encoding for Rmcystatin-3 was cloned in the pPICZαB vector and expressed using the yeast expression system with *P. pastoris* GS115 Mut$^+$ strain. The protein expression yield was 35 mg/L. The recombinant protein (rRmcystatin-3) was purified by an ionic exchange chromatography using a HiPrep Q column (Fig. 2A). Fractions were screened for inhibitory activity against cathepsin L and submitted to a size exclusion chromatography in a Superdex75 column (Fig. 2B), and them analyzed by SDS-PAGE revealing a major protein around 15 kDa (Fig. 2C).

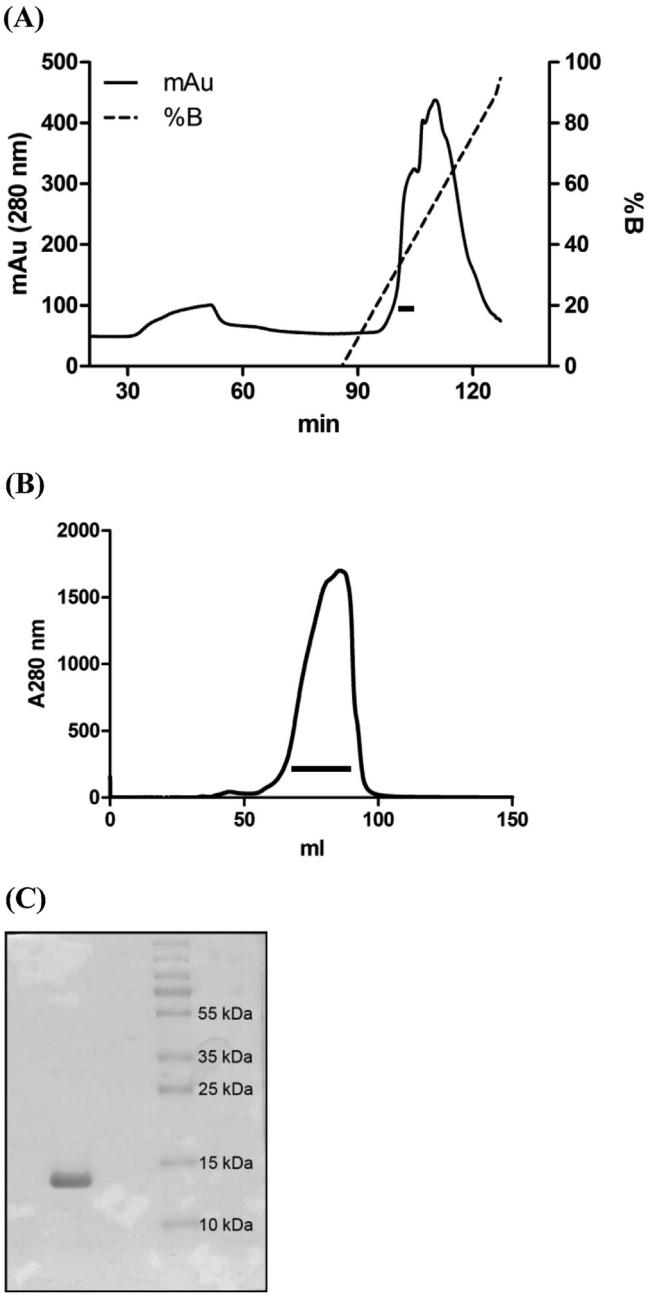

Fig. 2. Purification of rRmcystatin-3: (A): The supernatant of *Pichia pastoris* GS115 strain culture containing Rmcystatin-3 was submitted to an ionic exchange chromatography on a Hiprep Q column, proteins were eluted with a linear gradient of NaCl (0–1 M), the dash indicates the region containing inhibitory activity for cathepsin L. (B): Size exclusion chromatography in a Superdex75 column. The dash indicated the fractions containing inhibitory activity for cathepsin L. (C): Rmcystatin-3 analysis by SDS-PAGE (15%) sample pre-incubated with reducing agent (1) and (2) the marker *PageRuler*.

3.3. Characterization of rRmcystatin-3 inhibitory activity

To determine rRmcystatin-3 inhibitory specificity, purified inhibitor was assayed with different cysteine proteases: papain, cathepsin L, *Boophilus microplus* cathepsin L-1 (BmCl-1) and cathepsin B (Fig. 3). Table 1 summarized dissociation constant data for different cysteine proteases; rRmcystatin-3 strongly inhibited human cathepsin L ($K_i = 2.45$ nM), BmCl1 ($K_i = 1.8$ nM), cathepsin B ($K_i = 136$ nM) but not papain.

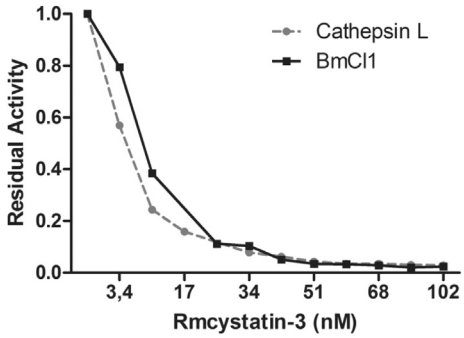

Fig. 3. Dissociation curves of rRmcystatin-3 for cathepsin B and BmCl-1. The values of the dissociation constant (K_i) were calculated by fitting steady-state velocities to Morrison equation $(V_i/V_o = 1 - \{E_t + I_t + K_i - [(E_t + I_t + K_i)^2 - 4E_tI_t]^{1/2}\}/2E_t)$ for *tight-binding* inhibitors.

3.4. Localization of Rmcystatin-3 in different tissues of R. microplus

Rmcystatin-3 expression profile was analyzed by quantitative real time PCR using cDNA preparation from different tissues – midgut, ovary, hemocytes, fat body and salivary gland – of *R. microplus*. Rmcystatin-3 mRNA was detected in hemocytes, fat body and salivary glands (Fig. 4A). Despite the localization of Rmcystatin-3 mRNA among tick tissues, Western blot analysis using a purified polyclonal antibodies anti-Rmcystatin-3 revealed the presence of the native inhibitor only in hemocytes and fat body (Fig. 4B).

3.5. Rmcystatin-3 knockdown by RNA interference

The profile of Rmcystatin-3 mRNA during RNA interference was analyzed by quantitative real time PCR and western blot. The tick group injected only with PBS showed a decreased in the Rmcystatin-3 expression. As expected the injection of dsRmcystatin-3 (3.5 μg/μL) successfully knocked down the Rmcystatin-3 expression while the injection of the double-strand non-related gene (dsNR – 3.5 μg/μL) revealed a similar effect of the PBS injection. The *E. coli* infected ticks also showed down-regulated expression of Rmcystatin-3 (Fig. 5A). The protein level of Rmcystatin-3 knockdown in infected hemocytes was also confirmed by Western blot analysis (Fig. 5B). At 24 h post-injection, hemolymph was collected and spread on LB agar plates and incubated overnight at 37 °C. The CFU in each plate were counted and compared. The Rmcystatin-3 silenced group presented 8.3 ± 1.8 CFU per plate whereas the non-related gene group showed 64 ± 22 CFU per plate, a reduction of approximately 78% (Fig. 5C).

4. Discussion

In this study, we describe a novel cysteine protease inhibitor named Rmcystatin-3. The nucleotide sequence of Rmcystatin-3 is composed of 426 bp encoding a 142 amino acids protein with predicted signal peptide of 23 aa. The mature protein presents a theoretical molecular weight of 13 kDa, isoelectric point of 5.39 and

Table 1
Dissociation constant (K_i) of Rmcytatin-3 for different cysteine proteinases.

Enzyme	K_i (nM)
Cathepsin B	136 ± 16.70
Cathepsin L	2.48 ± 0.08
BmCl-1	1.80 ± 0.214
Papain	N.I

N.I = Not inhibited.

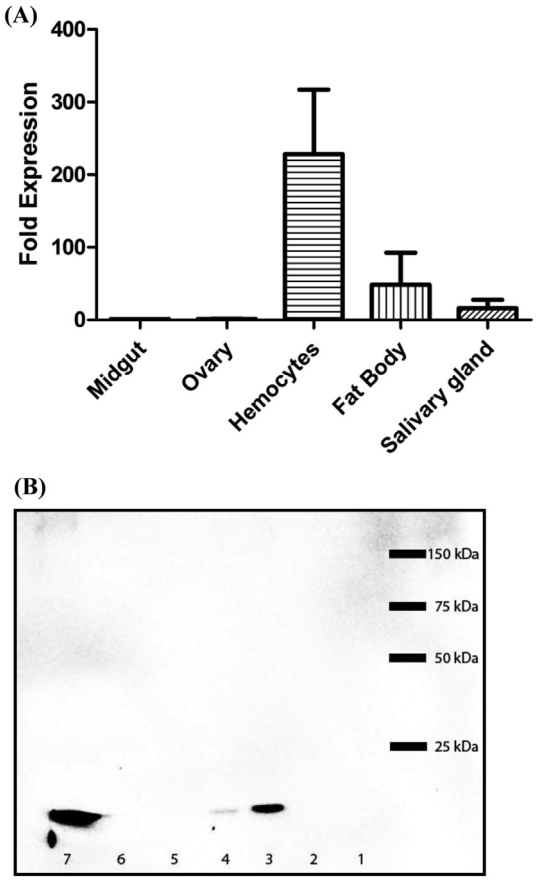

Fig. 4. Localization of Rmcystatin-3 in *R. microplus* tick. (A): Rmcystatin-3 gene expression profile in different tissues of engorged females by quantitative PCR. All data were normalized by expression level of ELF1a (elongation factor 1-alpha) as endogenous control. The error bars correspond to the standard error of the mean from two independent experiments. (B): Protein extract from tick tissues (1) midgut, (2) ovary, (3) hemocytes, (4) fat body, (5) salivary gland, (6) hemolymph and the (7) recombinant Rmcystatin-3 were used in Western-blotting assays with an anti-Rmcystatin-3 purified polyclonal antibody.

highly conserved domains of the cystatin family: a glycine residue on the N-terminal region followed by a hairpin loop composed of QxVxG and a second hairpin formed by the pair PW. Interestingly, Rmcystatin-3 possesses a second QxVxG motif (Fig. 1A) which may contribute to its inhibitory specificity. Also the absence of the SND domain [36] suggest that Rmcystatin-3 inhibitory activity against legumain will be unfavorable.

Rmcystatin-3 transcript was present in several tick tissues – hemocytes, fat body and salivary gland – but with dominant presence in hemocytes (Fig. 4A and B) which are related to immune response [37]. In ticks there are 3 subpopulations of hemocytes that has been describe [38]: plasmatocytes, granulocytes I and granulocytes II, associated with phagocytosis of bacteria and yeast [7], moreover in *R. microplus* the phagocytic activity to *Saccharomyces cerevisiae* was related to the production of reactive oxygen species [8]. To establish the role of Rmcystatin-3 within tick immunity, RNA interference assays were performed in ticks infected with gram-negative bacteria (*E. coli*). The Rmcystatin-3 gene knockdown was evaluated by quantitative PCR (Fig. 5A) and Western blot (Fig. 5B), revealing that Rmcystatin-3 silencing was successful although not completely 24 h after the injection. It is important to note that the PBS injection was enough to decrease the expression of Rmcystatin-3 to 50% and the injection of *E. coli* to almost 10%, suggesting that Rmcystatin-3 takes part in tick response to physical

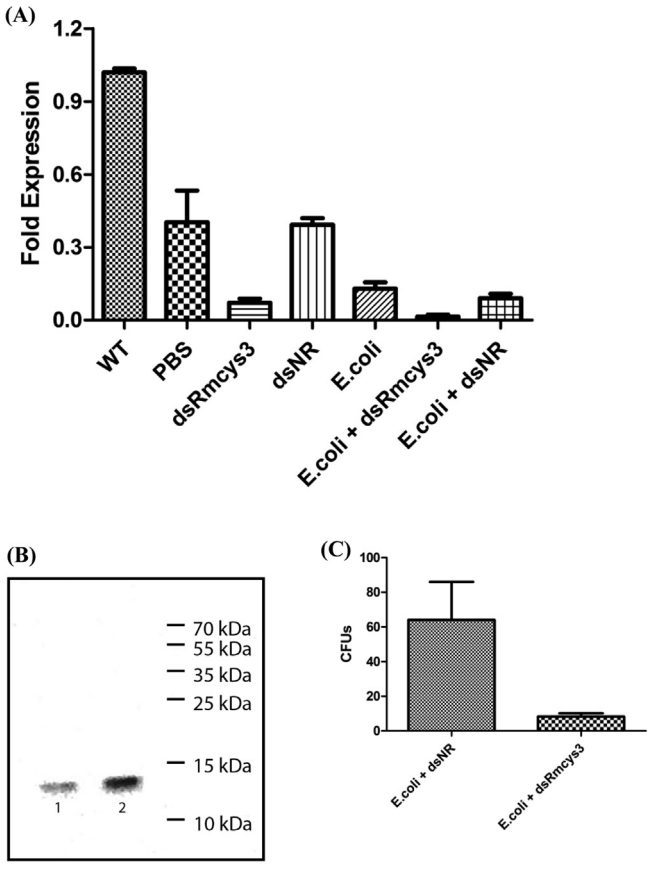

Fig. 5. Rmcystatin-3 gene silencing though RNA interference. (A): Analysis of Rmcystatin-3 mRNA in silenced and non-silenced ticks by quantitative PCR. The error bars correspond to the standard error from the mean of two independent experiments. (B): Protein analysis in hemocytes of silenced ticks by Western blot, (1) hemocytes from ticks injected with dsRmcystatin-3 and (2) hemocytes from ticks injected with dsLac (non-related control). (C): Effect of Rmcystatin-3 silencing upon gram-negative bacterial infection. Fully engorged females were injected with *E. coli* suspension (1×10^4 CFU/mL) and with dsRmcystatin-3 (3.5 μg/μL) or dsLac (3.5 ug/μL). 24 h post-injection hemolymph was collected and added to LB agar plates containing kanamycin (20 μg/mL). 48 h post-injection, number of bacteria (CFU) on each plate were obtained and compared. The error bars correspond to the standard error from the mean of two independent experiments.

stress and bacterial challenge. The silencing of Rmcystatin-3 in infected ticks drastically reduced the number of bacteria (CFU) survive in the hemolymph in comparison to the non-related silenced ticks (Fig. 5C) indicating that Rmcystatin-3 may be a negative modulator of tick immune response.

Several invertebrate cystatins have been associated with different functions [39–42]. In ticks, cystatins were related to parasite–host interface. The sialostatin L, a cystatin from the salivary gland of *I. scapularis*, displays an anti-inflammatory and immunosuppressive activity on host immunity, is capable of inhibiting lymphocyte proliferation and modulating cytokines levels [26,27]. To date only one tick cystatin has been related to tick immunity, Hlcys-2 from *H. longicornis* expressed in midgut and hemocytes, and it was up-regulated during LPS challenge and infection with *B. gibsoni*. In addition, the recombinant inhibitor was able to inhibit *B. gibsoni* growth *in vitro* [21]. In contrast, Rmcystatin-3 expression was down-regulated during bacterial infection suggesting that this inhibitor acts as a negative modulator of tick immune response, for example, controlling cysteine proteases in the production of antimicrobial peptide, in a similar manner described in midgut [15].

Kinetics assays with recombinant Rmcystatin-3 revealed a high affinity for cathepsin L (K_i = 2.5 nM). The relationship between cathepsins and immunity is better understood in vertebrates where cathepsins are actively involved in MHC class II antigen presentation [43,44]. A proteomic approach for hemocytes-like cell culture of *Drosophila* reveal the presence of several cysteine proteases but it's role is still unclear [45]. In the Chinese shrimp *Fenneropenaeus chinesis* a cathepsin L like proteases were up-regulated during WSSV (White Spot syndrome Virus) and bacteria (*Vibrio)* challenge [46], also the transcriptome shows that several cysteine proteases are up-regulated during infection [47]. Taking all our data together with other studies [48–50] reinforce the role of cysteine proteases and probable their inhibitor such as cystatin in invertebrate immunity.

In this work, we characterize a novel type-2 cystatin inhibitor name Rmcystatin-3 identified in hemocytes of *R. microplus* ticks; together with the RNA interference, our results strongly suggest the role of Rmcystatin-3 in tick immune response during bacterial challenge. We hypothesized that Rmcystatin-3 act as a negative modulator of tick immunity through inhibition of a cysteine protease in tick hemocytes, which physiologically control a proteolytic pathway decreasing the production of immune effectors.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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