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Characterization of a secreted cystatin from the tick *Rhipicephalus haemaphysaloides*

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Abstract A novel cystatin, designated RHcyst-2, was isolated from the tick *Rhipicephalus haemaphysaloides*. The full-length cDNA of RHcyst-2 is 773 bp, including an intact open reading frame encoding an expected protein of 139 amino acids and consisting of a 23 amino acids signal peptide. Predicted RHcyst-2 mature protein molecular weight is about 13 kDa, isoelectric point is 4.96. A sequence analysis showed that it has significant homology with the known type 2 cystatins. The recombinant protein of RHcyst-2 was expressed in a glutathione S-transferase-fused soluble form in *Escherichia coli*, and its inhibitory activity against cathepsin L, B, C, H, and S, as well as papain, was identified by fluorogenic substrate analysis. The results showed that rRHcyst-2 can effectively inhibit the six cysteine proteases' enzyme activities. An investigation of the RHcyst-2 genes' expression profile by quantitative reverse transcription-PCR demonstrated that it was more richly transcribed in the embryo (egg) stage and mainly distributed in the mid-gut of adult ticks. Western blot analysis confirmed that RHcyst-2 was secreted into tick saliva.

Keywords *Rhipicephalus haemaphysaloides* · Cystatin · Inhibitory activity

Introduction

Ticks are obligate haematophagous ectoparasites considered the principal vectors of disease among animals (Antunes et al. 2015). Aside from the direct damage that ticks inflict on their host, they serve as vectors of a wide variety of pathogens, including protozoa,

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rickettsiae and viruses (Galay et al. 2014). Ticks and tick-borne diseases affect animal and human health worldwide and cause significant economic losses in the animal industry. Functional molecular research is important to understand the biological characteristics of ticks at the molecular level. Enzymes and enzyme inhibitory molecules play very important roles in tick physiology, and the cystatins are tight-binding inhibitors of papain-like cysteine proteases. Cystatins are classified, based on characteristic sequence motifs and the number of conserved cystatin domains, into four subfamilies: the type 1 cystatins (also known as stefins), the type 2 cystatins, the type 3 cystatins (kininogens), and the type 4 cystatin-like proteins (fetuins and histidine-rich proteins) (Rawlings and Barrett 1990). Type 1 cystatins are cytoplasmic proteins that do not have signal peptides; however, the type 2 cystatins are secretion-type proteins containing signal peptides. Cystatins are present in a wide range of organisms, such as vertebrates, invertebrates, and plants, as well as protozoa (Turk et al. 2008; Vray et al. 2002). They are involved in various vertebrate biological processes, such as antigen presentation, immune system development, epidermal homeostasis, neutrophil chemotaxis during inflammation, and apoptosis (Honey and Rudensky 2003; Lombardi et al. 2005; Reddy et al. 1995; Wille et al. 2004). Over the last decade, several cystatins from different hard and soft ticks were identified and biochemically analyzed to determine their roles in the physiology and blood-feeding lifestyle of ticks (Schwarz et al. 2012).

Rhipicephalus haemaphysaloides is a widespread tick species in China and other south Asian countries, where it transmits animal babesiosis and human Kyasanur Forest disease (Zhou et al. 2006a). In this paper, we report on a novel cystatin molecule, named RHcyst-2, identified from *R. haemaphysaloides*. This molecule exhibits significant inhibitory activities against cysteine proteinases. This study provides the basis for biological functionality studies of the RHcyst-2 gene.

Materials and methods

Ticks and animals

The Hubei strain of *R. haemaphysaloides* has been maintained by feeding on rabbits for several generations in our laboratory at the Chinese Academy of Agricultural Sciences (Shanghai, China) (Zhou et al. 2006a). The experimental animals were treated following the approved guidelines from the Animal Care and Use Committee of the Shanghai Veterinary Research Institute.

Tick dissection and RNA isolation

The dissection of ticks was carried out as described elsewhere (Gong et al. 2014). Briefly, unfed, 4-day-fed and engorged adult ticks were rinsed and subsequently submerged in autoclaved ice cold phosphate-buffered saline (PBS). Ticks were laid down using a pair of soft-tissue forceps under a dissection light microscope. The dorsal cuticle was cut out with a scalpel blade, and the tissues, including mid-gut, salivary glands, ovaries and fat body were separated from the rest of the tick bodies. Total RNA was extracted from each tissue and whole 4-day-fed adult ticks using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA), according to the manual's instructions.

Cloning the full-length gene by RACE and bioinformatic analysis

Rapid amplification of cDNA ends (RACE) was conducted using a SMARTer RACE cDNA amplification kit (Clontech, San Jose, CA, USA) following the manual's instructions. Cloning was performed using primers from highly conserved regions of cystatin (Rawlings and Barrett 1990). The gene-specific primers used were 5′-CAGGTTGTGGCTGGTACAAACTAC-3′ and oligo-dT Primer. The cDNA template primed by an adapter-linked oligo-dT primer (Clontech) was synthesized from 5 μg of total RNA extracted from ticks that had been partially fed for 4 days. After two rounds of PCR, the PCR fragments were cloned into the pGEM-T plasmid (Promega, Madison, WI, USA) and sequenced. A BLASTx homology search revealed a cDNA encoding a cystatin-like polypeptide. Following contig assembly and singleton identification, gene-specific PCR primers were designed and used to clone the full-length cDNA (Yu et al. 2013).

Expression and purification of rRHcyst-2 in *Escherichia coli*

After removal of the signal peptide sequence, the open reading frame (ORF) of the cystatin gene in the pGEM-T vector was subcloned into the pGEX-4T-1 *E. coli* expression vector (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The accuracy of the insertion in the resulting plasmid was confirmed by sequencing. The cystatin gene was expressed as a glutathione S-transferase (GST)-fusion protein in the *E. coli* BL21 (DE3) strain according to the manufacturer's instructions (Amersham Pharmacia Biotech). The resulting *E. coli* cells were washed three times with PBS, lysed in PBS containing 1 % Triton X-100, sonicated, and then centrifuged at 12,000×*g* for 10 min at 4 °C. Supernatants containing the soluble GST fusion protein were purified with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The purified proteins were dialyzed against PBS for further use. The empty pGEX-4T-1 vector was used to produce the control GST protein, which was expressed and purified using the same procedure as that for the cystatin-GST fusion protein. Recombinant protein expression and purification analyses were carried out by standard SDS-PAGE (Zhou et al. 2009).

SDS-PAGE and Western blot

To determine whether RHcyst-2 was a secreted protein like others type 2 cystatins previously known, serum from rabbits which ticks had repeatedly fed on was used as the anti-saliva antibody to react with rRHcyst-2, and the same process was performed to GST protein as control. The recombinant proteins were mixed with an equal volume of a 2 × SDS gel-loading buffer under reducing conditions. The samples were boiled for 5 min, and each 10 μg of sample was then subjected to 10 % SDS–polyacrylamide gel electrophoresis (PAGE). After SDS-PAGE, the protein bands in the gel were electrically transferred to a membrane (Immobilon transfer membrane; Millipore, Bedford, MA, USA). The membranes were incubated in the primary antibody (1:200 dilutions), and positive signals were detected with peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (1:2000 dilution) in 3,3-diaminobenzidine tetrahydrochloride.

Proteinase inhibition assays

To calculate the inhibitory activity of the recombinant protein, the concentration of rRHcyst-2 at which 50 % inhibition of the proteolytic enzymes' activities were achieved

(IC_{50}) and measured. Recombinant protein was preincubated with each enzyme (0.15 μM) in an assay buffer for 30 min. Then, 0.25 mM of the protease-specific substrates was added to each well and residual enzyme activity monitored (Yamaji et al. 2009). The GST protein was used as control. Enzymes used were as follows: cathepsin L, C, B, S and H, as well as papain. All of these enzymes were purchased from Sigma (St. Louis, MO, USA). The assay buffer used consisted of 100 mM sodium acetate, pH 5.5, 100 mM NaCl, 1 mM EDTA, 1 mg/ml cysteine, and 0.005 % TritonX-100. The substrates purchased (Sigma) were as follows: Z-Phe-Arg-AMC·HCl for papain, cathepsin L and cathepsin B; Pro-Arg-4-methoxy-β-naphthylamide acetate salt for cathepsin C; Arg-NMec·HCl for cathepsin H; and Ac-Lys-Gln-Lys-Leu-Arg-AMC for cathepsin S.

Expression analysis of RHcyst-2 at different tick stages and in different tissue distributions

To determine the transcriptional level of RHcyst-2 in different stages and organs of ticks, the total RNA extracted from ticks at different developmental stages (egg, larvae, nymph and adult) and from different tick organs (salivary glands, mid-gut, ovary and fat body) were subjected to qRT-PCR analysis. To normalize the obtained gene expression, the tick elongation factor 1-alpha gene was selected as a housekeeping gene, as described previously (Nijhof et al. 2009). The specific primers used to quantify the cystatin and elongation factor 1-alpha were 5′-CCAGGGAGACGGTCAAGGATA-3′ and 5′-CGCAGTGAAGCAGCCAAGTT-3′ for RHcyst-2, and 5′-CGTCTACAAGATTGGTGGCATT-3′ and 5′-CTCAGTGGTCAGGTTGGCAG-3′ for elongation factor 1-alpha. The amount of mRNA transcripts of the target genes present in the adult samples were considered equivalent to 1 and were used as references for the expression levels in the other stages. Cycling parameters for all amplifications were 5 min at 95 °C followed by 30 cycles of 15 s at 95 °C, 30 s annealing at 60 °C, and extension at 72 °C for 30 s. The qPCR was performed using Platinum SYBR Green qPCR SuperMix kit (Invitrogen) using an Applied Biosystems 7500 thermocycler (Applied Biosystems, Foster City, CA, USA). To ensure primer fidelity, dissociation curve analyses and gel electrophoresis of target gene amplicons were performed for each sample following the qPCR step. All qPCR amplifications were performed in triplicate and repeated twice, with the mean values considered for comparison. To check for genomic DNA contamination, controls lacking reverse transcription were performed.

Results

Cloning and sequence analysis of the full-length cDNA encoding *Rhipicephalus haemaphysaloides* cystatin

We found a novel cystatin gene, RHcyst-2, from the tick *R. haemaphysaloides* using the RACE method. The full-length cDNA of RHcyst-2 is 773 bp, as shown in Fig. 1 A, including an intact ORF encoding an expected protein of 139 amino acids, with a predicted protein molecular weight of ∼13 kDa and an isoelectric point of 4.96. A sequence analysis demonstrated that RHcyst-2 possesses a conserved N-terminal glycine, QXVXG and PW motifs, and two disulfide bridges, which is highly conserved in various type 2 cystatins. SMART analysis detected the cystatin-like domain in the putative amino acid sequence

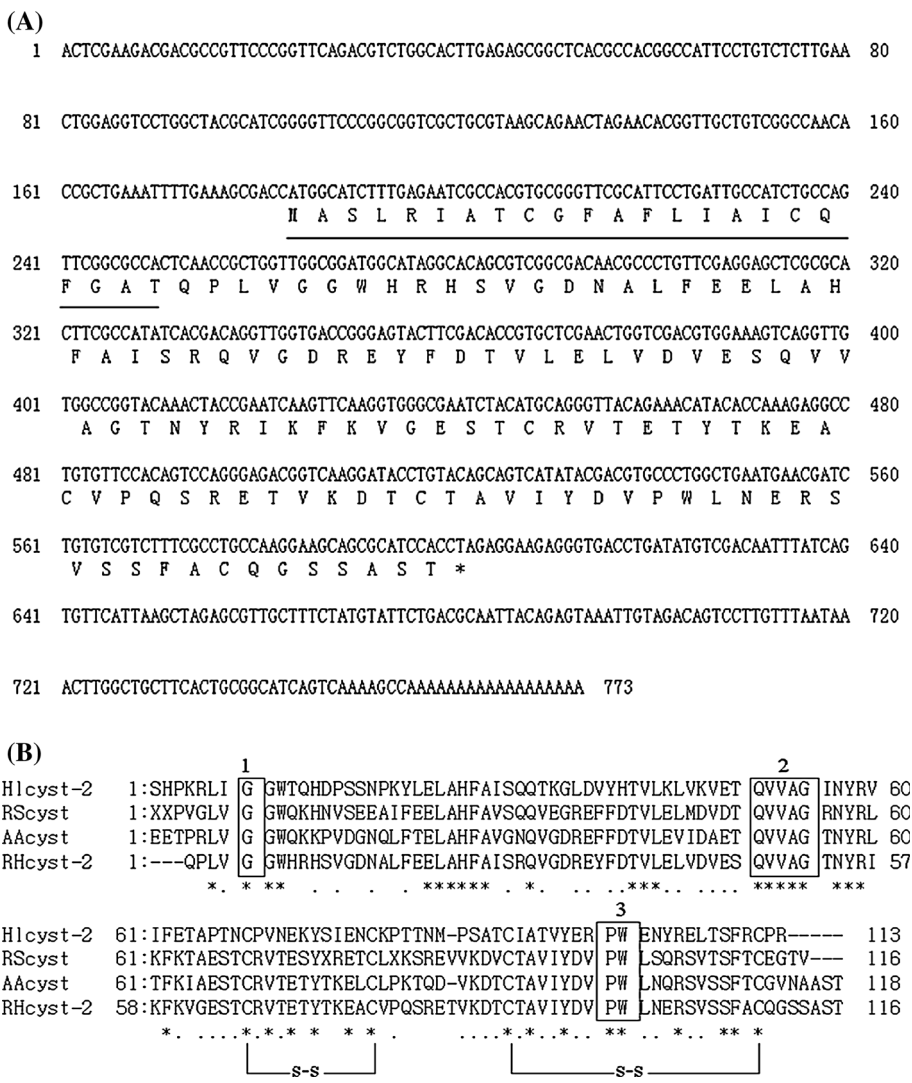

Fig. 1 Analysis of the structure of a novel type 2 cystatin, RHcyst-2, isolated from the tick *Rhipicephalus haemaphysaloides*. The putative signal peptide is underlined with the *thin line*. **a** cDNA and putative amino acid sequences. **b** Putative amino acid alignment of RHcyst-2 with other tick type 2 cystatins: Hlcyst-2 of *Haemaphysalis longicornis* (GenBank accession number: ABC94582); RScyst-2 of *Rhipicephalus sanguineus* (GenBank accession number: ACX53862); and AAcyst of *Amblyomma americanum* (GenBank accession number: AEO35688). The conserved cystatin active sites are boxed (1: N-terminal conserved glycine; 2: QXVXG conserved motif; 3: PW conservative site)

(position 25–133). A BLASTP analysis of the predicted polypeptide sequence against all non-redundant databases accessed through GenBank revealed significant similarity scores with members of the cystatin type 2 family of other species. The identities of the putative RHcyst-2 amino acid sequences with the type 2 cystatin of *Haemaphysalis longicornis* (GenBank accession number: ABC94582), *Rhipicephalus sanguineus* (GenBank accession

number: ACX53862), and *Amblyomma americanum* (GenBank accession number: AEO35688) were 41, 62, and 66 %, respectively, as shown in Fig. 1b. The amino acid analysis using the Signal P program revealed the presence of an obvious signal peptide which the cleavage site was predicted between amino acids 23 and 24. The sequence of the gene encoding RHcyst-2 have been submitted to GenBank under the accession number KM588365.

Expression and purification of rRHcyst-2

The gene encoding RHcyst-2 was ligated into the bacterial expression vector pGEX-4T-1, and the recombinant was successfully expressed as GST-fusion protein with an expected size of 39 kDa (Fig. 2). The rRHcyst-2 was expressed in a soluble form and then purified by affinity chromatography using Sepharose 4B columns according to the manufacturer's instructions. The purity of rRHcyst-2 was more than 95 % as estimated by SDS-PAGE analysis.

Western blot analysis

To determine whether the RHcyst-2 protein was secreted into tick saliva, serum from rabbits which ticks had repeatedly fed on was used as the anti-saliva antibody to react with rRHcyst-2. Western blot analysis showed that the anti-tick saliva serum was able to recognize rRHcyst-2 (Fig. 3), suggesting that RHcyst-2 is secreted into the host during feeding.

Proteinase inhibition assays

To investigate the efficiency of RHcyst-2 in inhibiting its overlapping target enzymes, purified recombinant cystatin was used to test the inhibitory activity against cysteine proteases. The results showed that: rRHcyst-2 can effectively inhibit cathepsin L, B, C, H, and S, as well as papain, enzyme activities, as shown in Fig. 4 and Table 1.

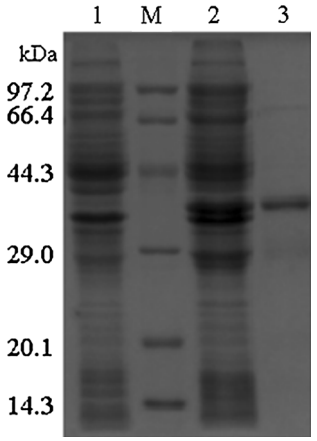

Fig. 2 Expression and purification of the recombinant protein of RHcyst-2, a novel type 2 cystatin isolated from the tick *Rhipicephalus haemaphysaloides*, in a GST-fused soluble form, rRHcyst-2-GST, in *Escherichia coli*. A total of ~10 μg recombinant protein or bacterial lysate was electrophoresed in a 12 % SDS–polyacrylamide gel, and then stained with Coomassie blue. Lane 1: The supernatant of uninduced cell lysate; lane 2: The supernatant of induced cell lysate; and lanes 3: purified RHcyst-1 recombinant protein

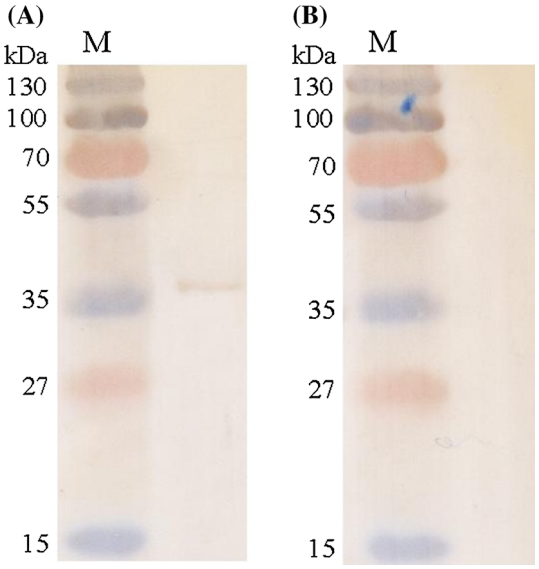

Fig. 3 Western blot analysis of rRHcyst-2. In total, 10 μg of purified rRHcyst-2 were electrophoresed in SDS–PAGE and then transferred to a membrane for Western blot analysis with rabbit serum (**a**) exposed to repeated tick bites and normal rabbit serum (**b**) as control

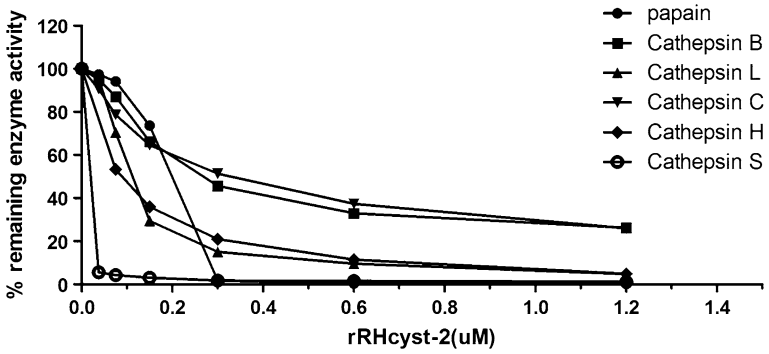

Fig. 4 Inhibition of protease activities by the recombinant protein of RHcyst-2, a novel type 2 cystatin isolated from the tick *Rhipicephalus haemaphysaloides*. Cathepsin L, B, C, H, and S and papain were incubated with each of the substrates in the presence of different concentrations of rRHcyst-2. All of these enzymes concentration is 0.15 μM. Incubation of cathepsins without rRHcyst-2 resulted in 100 % enzyme activity

Expression analysis of RHcyst-2 at different tick stages and in different tissue distributions

To determine the expression profile of RHcyst-2, total RNA samples from four different tick developmental stages and organs were subjected to real-time PCR. RHcyst-2 mRNA transcripts were detected in eggs (embryos), larvae, nymphs, and adults. RHcyst-2 was most highly expressed in the embryo stage, as shown in Fig. 5a. The tissue distribution of RHcyst-2 in fed, unfed and engorged ticks is shown in Fig. 5b. The results showed that this gene was expressed most richly in the tick mid-gut and a few copies were detected in salivary glands, ovary, and fat body. However, the expression of RHcyst-2 in all organs of fed ticks was significantly up-modulated compared with unfed ticks. It was suggested that RHcyst-2 play a key role in tick blood-feeding process.

Table 1 Protease inhibition assays

Enzyme	Enzyme concentration (nM)	RHcyst-2 IC_{50} (nM) (95 % confidence interval)
Papain	150	174.4 (154.1–197.3)
Cathepsin B	150	316.3 (228.8–437.3)
Cathepsin L	150	111 (89.1–138.4)
Cathepsin C	150	331.6 (286.4–384.1)
Cathepsin H	150	85.8 (80.8–91.1)
Cathepsin S	150	0.1 (0–0.4)

The concentration of a novel type 2 cystatin, RHcyst-2, isolated from the tick *Rhipicephalus haemaphysaloides*, at which 50 % of the proteolytic enzymes' activity is inhibited (IC_{50})

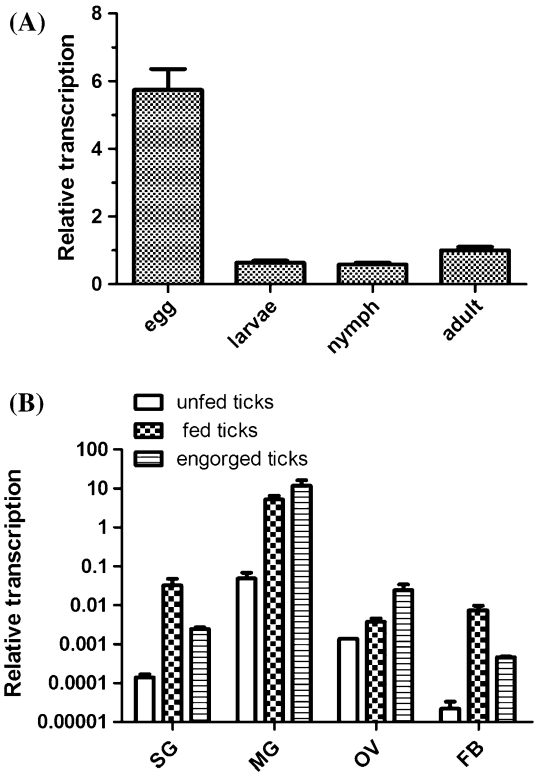

Fig. 5 Gene expression patterns of a novel type 2 cystatin, RHcyst-2, isolated from the tick *Rhipicephalus haemaphysaloides* in different developmental stages (**a**) and in different tissues from unfed, fed and engorged adult ticks (**b**). *SG* salivary glands, *MD* mid-gut, *OV* ovary, and *FB* fat body

Discussion

The present study describes the sequence of a novel cystatin, RHcyst-2, from the tick *R. haemaphysaloides*. The characteristics of the putative RHcyst-2 amino acid sequence indicate that it is a member of the type 2 cystatins.

The capacities of various cystatins to inhibit the activity of cysteine proteases have been characterized. In this study, the GST-fused recombinant cystatin efficiently inhibited the

activity of cathepsin L, B, C, H, and S, and papain. The rRHcyst-2 effectively inhibited cathepsin S, and it also had quite a high inhibitory efficiency against cathepsin L. In recent studies, cathepsin L and S were considered to be effectively controlled targets of tumor cells, because they play key roles in the invasion and migration of tumor cells (Chang et al. 2007; Yang and Cox 2007). The SV-cystatin from snake venom was found to inhibit the invasion and metastasis of mouse melanoma cells and human gastric carcinoma cells (Xie et al. 2011). Thus, based on this information, RHcyst-2 may be an important candidate molecule for anti-cancer drug research and development in the future.

Cystatins were considered to involve in many important physiological processes of ticks. Hlcyst-2 (a type 2 cystatin from *H. longicornis*, GenBank accession number: ABC94582) experimental evidence showed its role in tick innate immunity, since increased Hlcyst-2 transcript levels were detected in *Babesia gibsoni*-infected larval ticks and the protein inhibited *Babesia* growth (Zhou et al. 2006b). The transcription of two cystatins named HlSC-1 and Sialostatin L2(HlSC-1: a type 2 cystatin from *H. longicornis*, GenBank accession number:BAI59105; Sialostatin L2: a type 2 cystatin from *Ixodes scapularis*, GenBank accession number: AAY66685) was highly upregulated in these tick tissues during feeding (Kotsyfakis et al. 2007; Yamaji et al. 2009). Vaccinating hosts against Sialostatin L2 and Om-cystatin 2 (a type 2 cystatin from *Ornithodoros moubata*, GenBank accession number: AAS55948) as well as silencing of a cystatin gene from *Amblyomma americanum* significantly inhibited the feeding ability of ticks (Karim et al. 2005; Kotsyfakis et al. 2007; Salat et al. 2010). Additionally, Om-cystatin 2 and Sialostatin L (another type 2 cystatin from *I. scapularis*, GenBank accession number: AAM93646) possessed strong host immunosuppressive properties by inhibiting dendritic cell maturation due to their interaction with cathepsin S (Kotsyfakis et al. 2006; Salat et al. 2010). Other cystatins, such as Hlcyst-1 (a type 1 cystatin from *H. longicornis*, GenBank accession number: ABZ89553) or Om-cystatin 2 are assumed to be involved in regulating blood digestion (Salat et al. 2010; Yamaji et al. 2010; Zhou et al. 2009). Only for Bmcystatin (a type 1 cystatin from *R. microplus*, GenBank accession number: ABG36931) was a role in tick embryogenesis suggested (Lima et al. 2006). The expression of mRNA for Hlcyst-2 was detected at the four different developmental stages, indicating the important physiological role of this molecule throughout the tick life cycle (Zhou et al. 2006b).

In this paper, a novel secreted cystatin, RHcyst-2, identified from *R. haemaphysaloides*. RHcyst-2 exhibited significant inhibitory activities against cysteine proteinases but its functional study is still to be needed.

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