Characterization of Hlcyst-3 as a member of cystatins from the tick *Haemaphysalis longicornis*

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Abstract The gene encoding cystatin from the tick *Haemaphysalis longicornis* has been reported previously. In the study reported here, we characterized a member of cystatins and designated it as Hlcyst-3 (*H. longicornis* cystatin-3). Its full-length cDNA is 602 bp, and it encodes a putative 129 amino acid protein with an obvious signal peptide. Sequence analysis revealed that it has significant homology with the known secreted cystatin. The recombinant protein was expressed in a GST-fused soluble form in *Escherichia coli* and was purified by affinity chromatography. The inhibitory activity of the recombinant protein against papain and cathepsin L was identified by fluorogenic substrate analysis. Real-time PCR revealed that Hlcyst-3 was mostly expressed in the tick midgut.

Keywords *Haemaphysalis longicornis* · Cystatin · Hlcyst-3

Introduction

Cystatins are inhibitors of papain-like cysteine proteases and are widespread in vertebrates, invertebrates, protozoa, and plants. Based on amino acid sequencing, this superfamily can be subdivided into three closely related families (Rawlings and Barrett 1990; Turk and Bode 1991). The proposed physiological functions of these proteins are the regulation of

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protein turnover, defense against pathogens, and balance of the host-parasite immune relationship (Turk and Bode 1991; Agarwala et al. 1996; Yamamoto et al. 1999; Dainichi et al. 2001; Manoury et al. 2001). Recently, family 1 cystatins (intracellular cystatins) were found in the ticks *Haemaphysalis longicornis, Ixodes scapularis*, and *Rhipicephalus (Boophlus) microplus* (Lima et al., 2006; Zhou et al., 2009), but the biological functions of the tick family 1 cystatin remain unknown. Several tick family 2 cystatins (secreted cystatins) which have also been characterized have functions related to tick innate immunity (Zhou et al. 2006), midgut physiology (Grunclova et al. 2006), and blood-feeding success (Karim et al. 2005; Kotsyfakis et al. 2006, 2007). These studies indicate that the cystatin may be an important molecule in ticks.

Ticks are vectors of a wide variety of disease-causing bacteria, viruses, protozoa, and other pathogenic organisms. The hard tick, *H. longicornis*, is distributed mainly in East Asia and Australia, where it transmits a wide range of pathogens, including bovine theileriosis (*Theileria* spp.), bovine babesiosis (*Babesia ovata*), canine babesiosis (*Babesia gibsoni*), and human rickettsiosis (*Rickettsia japonica*) (Fujisaki et al., 1994; Jongejan and Uilenberg, 2004). Previously, a secreted cystatin named Hlcyst-2 was characterized from *H. longicornis* in our laboratory (Zhou et al. 2006), and an intracellular cystatin named Hlcyst-1 was identified recently (Zhou et al. 2009). In the current study, we characterized a novel secreted cystatin, Hlcyst-3, from the tick *H. longicornis*.

Materials and methods

Ticks and tissue collection

The parthenogenetic Okayama strain of the tick *H. longicornis* has been maintained by feeding on rabbits and mice for several generations in our laboratory (Fujisaki 1978). For tissue collection, adult females of *H. longicornis* were infested on the ears of a rabbit. The ticks were recovered from the rabbit's ears after 4 days, and the tissues were immediately dissected under a microscope (Zhou et al. 2006). Blood cells of the host rabbit were collected as the control samples, and all sample materials were stored at $-80°C$ until used.

Cloning and sequence analysis of the full-length cDNA of Hlcyst-3

A tick midgut full-length cDNA library was made using the vector-capping method described previously (Zhou et al. 2006). A total of 10,000 recombinant transformants from the library were randomly selected for plasmid DNA purification and sequencing. Nucleotide sequences were determined using an automated sequencer (ABI PRISM 310 Genetic Analyzer, USA). The cDNA clone encoding Hlcyst-3 was chosen for further analysis. All other sequence comparisons reported here were done using the BLAST server at the NCBI (http://www.ncbi.nlm.nih.gov/BLAST) and the ClustalW Service at the European Bioinformatics Institute, whereas protein secretion signals were revealed on the SignalP 3.0 server of the Technical University of Denmark.

Expression analysis of Hlcyst-3 in different tissues by real-time PCR

To analyze the abundance of Hlcyst-3 mRNA in different tissues, the salivary glands, midgut, fat body, ovary, and hemocytes were dissected from 4-day-fed adult ticks; the total

RNA was then purified and subjected to real-time PCR. To eliminate the possible contamination of host blood, the total RNA of blood cells from the host rabbit was also subjected to real-time PCR as a control. We used the one-step TaqMan real-time reverse-transcription polymerase chain reaction (R/T RT-PCR) method (Zhou et al. 2006). Gene-specific primers and a fluorogenic probe were designed to target the Hlcyst-3 gene or to control the tick actin gene (accession number: AY254898) using the Primer Express software (Applied Biosystems, USA). The PCR primers for Hlcyst-3 are 5′-CCACAGA AGCAAAGGCGAC-3′ and 5′-TCTCCCAGGGCCTCTCGT-3′, and the TaqMan probe is 5′-FAM-TGCACAGCCGTGGTT-MGB-3′. The PCR primers for tick actin are 5′-CGTT CCCTGGGTATGGAATCG-3′ and 5′-TCCACGTCGCACTTCATGAT-3′, and the Taq-Man probe is 5′-FAM-CACGAGACCACGTACAA-MGB-3′. Samples were amplified by using a program that included a reverse-transcription procedure consisting of one cycle of incubation at 48°C for 30 min and 94°C for 10 min, followed by 45 cycles of a denaturation step at 94°C for 15 s and an annealing/extension step at 60°C for 1 min. We established the standard curves for Hlcyst-3 and actin using serial dilutions (800–50 ng) of total RNA. Amplification and product detection were performed using the ABI PRISM 7900 HT sequence detection system (Applied Biosystems, USA). A positive result was determined by identifying the threshold cycle (C_T) value at which the reporter dye emission could be observed above the background. The relative amount of Hlcyst-3 produced per unit of actin was calculated for each sample. Each analysis was done at least in triplicate.

Expression and purification of Hlcyst-3 in *Escherichia coli*

After removal of the signal peptide sequence, the open reading frame (ORF) of the Hlcyst-3 gene in the pGCAP1 vector was subcloned into the pGEX-4T-3 *E. coli* expression vector (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The resulting plasmid was checked for accurate insertion by sequencing and designated as the pGEX-4T-3/Hlcyst-3 plasmid. The 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 phosphate-buffered saline (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-3 was used to produce the control GST protein, which was expressed and purified in a procedure identical to that conducted for the Hlcyst-3-GST fusion protein. The analysis of recombinant protein expression and purification was carried out by standard SDS-PAGE.

Enzymatic assays

The enzymes used were papain (EC 3.4.22.2, Sigma) and cathepsin L (EC 3.4.22.15, Sigma). The enzymatic assay buffer was 100 mM sodium phosphate containing 1 mM DTT and 2 mM EDTA, adjusted to pH 6.5 for papain and pH 6.0 for cathepsin L. The fluorogenic substrate used was Z-Phe-Arg-AMC (benzyloxycarbonyl-Phe-Arg-7-amido-4-methylcoumarin; Peptide Institute, Osaka, Japan). Protease (0.2 μM) was incubated with different concentrations of GST-fused recombinant Hlcyst-3 or control GST protein and 10 μM of the fluorogenic substrate. The reactions were allowed to proceed at 37°C for

30 min in a black 96-well plate and then were measured by fluorometry with excitation at 355 nm and emission at 460 nm.

Results

The full-length cDNA of Hlcyst-3 is 602 bp, including an intact ORF encoding an expected protein with 129 amino acids. The sequence of the Hlcyst-3 gene of *H. longicornis* has been submitted to the GenBank database under accession number EU426545. The amino acid analysis using the Signal P program revealed the presence of a signal peptide, which is typical of family 2 cystatin. The mature protein consisted of 122 residues with a calculated molecular weight of 11 kDa and an isoelectric point (pI) value of 4.45. SMART analysis (Schultz et al. 2000) detected the cystatin-like domain in the putative amino acid sequence. BLASTP analysis of the predicted polypeptide sequence against all nonredundant databases accessed through NCBI revealed a significant score with members of the cystatin family 2 of other species. The identities of the putative amino acid of Hlcyst-3 with reported cystatin Hlcyst-2 (Zhou et al. 2006) are 47%, and those with other available tick cystatins are in the 38–50% range. The alignment of putative amino acids with several known tick cystatins is shown in Fig. 1. The amino acid sequences of QxVxG (x can be one of several amino acids) are highly conserved in various cystatins (Brown and Dziegielewska 1997). The other conserved regions also include a glycine in the N-terminal region and a PW motif in the second hairpin loop in the C-terminal region (Bjork et al. 1996), all of which are found in the cloned Hlcyst-3 and most reported tick cystatins. In addition, the putative amino acid sequence of the Hlcyst-3 gene contains 4 cysteine residues at the C-terminus; these residues were predicated to form two disulfide bonds in family 2 cystatins (Barrett et al. 1986).

To determine the expression profiles of the Hlcyst-3 gene, total RNA samples from different tick tissues were subjected to real-time PCR. In Fig. 2, the relative amount of Hlcyst-3 mRNA per unit of actin is shown as the relative expression rate (%). The results showed that this gene was expressed most richly in the tick midgut, while only a few copies were detected in the hemocytes, salivary glands, ovary, and fat body. The control
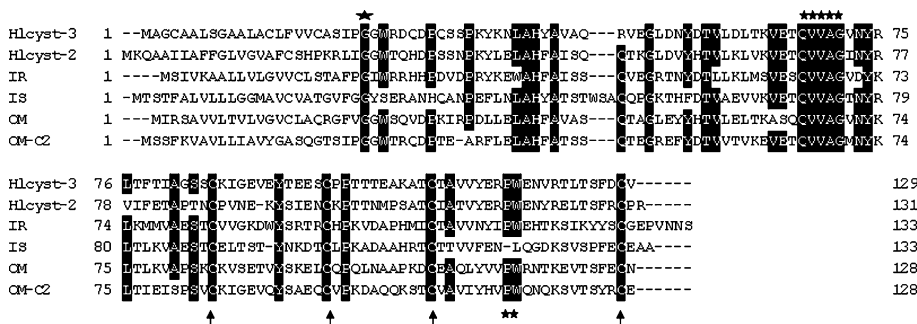

Fig. 1 Alignments of the putative amino acids of Hlcyst-3 with other tick cystatins. The conserved cystatin active sites are labeled with stars, and the cysteine residues forming disulfide bridges are shown with arrows. The sequence of the Hlcyst-3 gene of *H. longicornis* has been submitted to the GenBank database under accession number EU426545. The cystatin gene accession numbers of the ticks *H. longicornis* (Hlcyst-2), *Ornithodoros moubata* (OM and OM-C2), *Ixodes ricinus* (IR), and *Ixodes scapularis* (IS) are DQ364159, AY521024, AY547735, AY547803, and AF483724, respectively
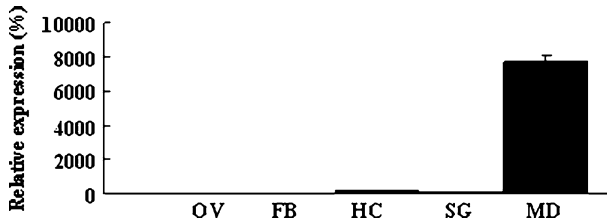
Fig. 2 mRNA expression level of Hlcyst-3 in different tick tissues. SG (salivary glands), MD (midgut), OV (ovary), HC (hemocyte), and FB (fat body). The relative expression (%) is the relative amount of Hlcyst-3 mRNA against each unit of tick actin mRNA. Each *bar* represents the average + standard deviation. Each analysis was done at least in triplicate

Fig. 3 Enzymatic assay. The rHlcyst-3-GST inhibition of protease activities measured by fluorogenic substrate hydrolysis. Cysteine proteases, papain, and human cathepsin L were incubated with the substrate in the presence of different concentrations of rHlcyst-3-GST. Incubation of proteases with control GST resulted in 100% enzyme activity
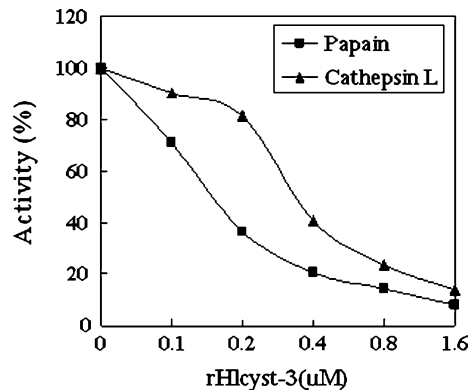
sample of host rabbit blood cells was not detected in any copies by real-time PCR (data not shown), which eliminated the possible contamination of host blood.

The Hlcyst-3 gene was ligated into the bacterial expression vector pGEX-4T-3 and then successfully expressed as a GST-fusion protein. The recombinant Hlcyst-3-GST (rHlcyst-3-GST) was expressed as a soluble form and then was purified by affinity chromatography. The purified rHlcyst-3-GST was assayed for inhibitory activity against cysteine protease papain and human cathepsin L proteases. As measured by Z-Phe-Arg-AMC hydrolysis, both papain and cathepsin L can cause concentration-dependent inhibition effects by rHlcyst-3. As shown in Fig. 3, 0.2 μM of rHlcyst-3-GST strongly inhibits 0.2 μM of papain (>50% inhibition rate), whereas 0.4 μM of rHlcyst-3-GST strongly inhibits 0.2 μM of cathepsin L (>50% inhibition rate).

Discussion

The present study describes the sequence of a novel cystatin Hlcyst-3 from the tick *H. longicornis*, which is different from the cystatin Hlcyst-1 or Hlcyst-2. The characteristics of the putative amino acid sequence of Hlcyst-3 indicate that it is a member of the family 2 cystatins. Various cystatins have been characterized regarding their capacity to inhibit the activity of cysteine proteases. In this study, the GST-fused recombinant Hlcyst-3 cystatin efficiently inhibited the activities of papain and human cathepsin L; similar features have also been found in other tick cystatins (Zhou et al. 2006; Lima et al. 2006;

Kotsyfakis et al. 2007). This inhibition profile demonstrated that rHlcyst-3-GST has a potency similar to that of known cystatins, suggesting that the recombinant rHlcyst-3-GST is produced as a functional correctly folded protein.

Combined with reported Hlcyst-1 and Hlcyst-2, 3 cystatin molecules were identified from the tick *H. longicornis,* this fact shows the cystatin is divergence of the sequence. Hlcyst-3 is different from the Hlcyst-1 that identified as an intracellular cystatin (Zhou et al. 2009), whereas Hlcyst-3 is similar as the Hlcyst-2 that identified as a secreted cystatin (Zhou et al. 2006). Hlcyst-2 had been found to be involved in tick innate immunity and tick midgut physiology (Zhou et al. 2006). In this study, the mRNA of Hlcyst-3 was detected in the midgut but was hardly found in all other tissues examined, which is the same as that of Hlcyst-2. Restricted expression was also found in mammalian and insect cystatins (Agarwala et al. 1996; Frejie et al. 1991; Goto and Denlinger 2002), and the tissue specificity expression suggested a distinct role in different tissues. In the midgut of tick *H. longicornis*, two cathepsin L cysteine proteinases have been identified (Mulenga et al. 1999). Recently, a papain-family cysteine protease, Longipain, has also been identified in the midgut of the tick *H. longicornis* (Tsuji et al., 2008). Hlcyst-3 might be the regulator of tick midgut cysteine proteinases and also involved in tick midgut physiology like Hlcyst-2. On the other hand, Hlcyst-3 might also be the inhibitor of cysteine proteinases that derives from host blood or pathogens, which will be involved in defensive role. The target specificity and their relation of the cystatin Hlcyst-1, Hlcyst-2, and Hlcyst-3 are an interesting topic for future study.

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