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A secreted cystatin from the tick *Haemaphysalis longicornis* and its distinct expression patterns in relation to innate immunity

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Abstract

Proteins capable of selective and specific inhibition of cysteine protease have been identified as cystatins and are isolated from a variety of microbes and tissues of animals and plants. The physiological function of these proteins has been proposed to be the regulation of protein turnover and defense against pathogens as well as the balance of the host–parasite immune relationship. Genes encoding cystatins have been found in several species of ticks, but the function of cystatin in ticks is not understood. We cloned a gene encoding cystatin from tick *H. longicornis* and designated it as Hlcyst-2 (*H. longicornis* cystatin-2). Its full-length cDNA is 569 bp, and it encodes a putative 133 amino acid protein with an obvious signal peptide. Sequence analysis demonstrated that it has significant homology with the known cystatin. The recombinant protein was expressed in a GST-fused soluble form in *Escherichia coli* and purified by affinity chromatography. The inhibitory activity of the recombinant protein against papain, cathepsin L, and cathepsin B was identified by fluorogenic substrate analysis. Cystatin was mostly expressed in the tick midgut and hemocyte. Blood feeding induced significantly increased expression in the midgut. Real-time PCR confirmed that LPS-injected adult ticks expressed Hlcyst-2 1.6 more times than the PBS-injected control; *Babesia gibsoni*-infected larvae ticks expressed Hlcyst-2 1.8 more times than normal larvae ticks. The recombinant protein also showed a significant growth-inhibitory effect on *Babesia bovis* cultured in vitro. These results indicated this cystatin Hlcyst-2 is involved in tick innate immunity.

Keywords: Haemaphysalis longicornis; Cystatin; Babesia gibsoni; Innate immunity

1. Introduction

Cystatins are tight-binding inhibitors of papain-like cysteine proteases and are widespread in plants and animals. On the basis of amino acid sequencing, this superfamily can be subdivided into three closely related families (Rawlings and Barrett, 1990; Turk and Bode, 1991). Family 1 cystatins are small polypeptides of about 100 amino acid residues and lack carbohydrate side chains and disulphide bridges. The members of family 2 are secretion-type proteins consisting of about 120 amino acid residues and having two disulphide bridges. Family 3 cystatins are kininogens, which are large multifunctional glycoproteins of blood plasma and synovial fluid. They contain three domains that are homologous with those of family 2 cystatins; two of them inhibit proteinases. The physiological function of cystatins is not well understood. However, the regulation of protein turnover and protection of plants against insects and pathogens have been proposed in plants (Turk and Bode, 1991). Mammalian cystatin C had also been suggested to participate in the defense against the invasion of pathogens (Olsson et al., 1999). Filarial cystatins participate in pathogenicity and are thought to play a key role in the balance of the host–parasite immune relationship (Schierack et al., 2003). In the tick, genes encoding cystatins have also been found in several ixodid ticks (Valenzuela et al., 2002;

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Karim et al., 2005). However, the function of tick cystatins remains unknown.

Ticks are important vectors of a wide variety of disease-causing bacteria, viruses, protozoa, and other pathogenic organisms. Despite the importance of ticks as vectors of disease, very little is known of their basic biology, particularly, immune system. Understanding vector immunity is important in determining the host–pathogen interactions that facilitate or limit disease transmission. The hard tick, *Haemaphysalis 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). In this study, we report the characterization of a secreted cystatin from tick *H. longicornis* and provide evidence that cystatin is involved in tick innate immunity.

2. Materials and methods

2.1. 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 rabbit, ticks were recovered from the rabbit ears after 4 days, and the tissues were immediately dissected under the microscope (You et al., 2001). The sample materials were stored at −80 °C until used.

2.2. Construction of the tick midgut full-length cDNA library by vector capping and cDNA sequencing

A full-length cDNA library was made using the vector-capping method (Kato et al., 2005). Total RNA was prepared from the midgut of partially fed female adult ticks, which had remained attached to the rabbit ears for 4 days. The cDNA was synthesized with 5-μg total RNA by the G-Capping method and ligated into plasmid vector pGCAP1; the resulting plasmid was transformed into electrocompetent cell DH12S (Invitrogen). 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 a secreted cystatin was chosen for further analysis.

2.3. Expression and purification of cystatin in Escherichia coli

The open reading frame (ORF) of a cystatin gene in the pGCAP1 vector was subcloned into the pGEX-4T-3 *E. coli* expression vector (Amersham Pharmacia Biotech, Piscataway, NJ). The resulting plasmid was checked for accurate insertion by sequencing and designated as the pGEX-4T-3/cystatin 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 for producing control GST protein, which was expressed and purified identical to the procedure for the cystatin-GST fusion protein. The analysis for recombinant protein expression and purification was carried out by standard SDS-PAGE.

2.4. Enzymatic assays

The enzymes used were papain (EC 3.4.22.2, Sigma), cathepsin L (EC 3.4.22.15, Sigma), and cathepsin B (EC 3.4.22.1, 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 and cathepsin B. 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 cystatin or control GST protein and 10 μM of the fluorogenic substracte. The reactions were allowed to proceed at 37°C for 30 min in a black 96-well plate and then measured by fluorometry with excitation at 355 nm and emission at 460 nm.

2.5. Real-time quantitative PCR

For the estimation of mRNA abundance, we used the one-step TaqMan real-time reverse-transcription polymerase chain reaction (R/T RT-PCR) method. Gene-specific primers and a fluorogenic probe were designed to target the cystatin gene or control tick actin gene (accession number, AY254898) using the Primer Express software (Applied Biosystems, USA). The PCR primers and TaqMan probes used in this study are listed in Table 1. The one-step R/T RT-PCR reaction was conducted using the TaqMan one-step RT-PCR master mix reagent kit (Applied Biosystems, USA) according to the manufacturer's guidelines. 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