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Short communication

Characterization of an intracellular cystatin homolog from the tick *Haemaphysalis longicornis*

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ABSTRACT

Cystatins are tight-binding inhibitors of papain-like cysteine proteases. On the basis of amino acid sequencing, cystatins can be subdivided into three closely related families, family 1, family 2 and family 3. Among them, only family 1 cystatins are intracellular. In this report, a gene encoding family 1 cystatin from tick *Haemaphysalis longicornis* (Hlcyst-1) has been identified. Its full-length cDNA is 437 bp, including an intact ORF encoding an expected protein with 98 amino acids. Sequence analysis demonstrated that it has significant homology with the known family 1 cystatin. The recombinant protein was expressed in a GST-fused soluble form in *Escherichia coli*, and its inhibitory activity against papain, cathepsin L, and cathepsin B was identified by fluorogenic substrate analysis. This is the first report of an intracellular cystatin homolog from the tick *H. longicornis*.

1. Introduction

Ticks rank first as arthropod vectors of fungi, protozoa, rickettsiae, bacteria, and viruses, causing diseases in non-human vertebrates, and rank second only to mosquitoes as vectors of pathogens to humans (Bior et al., 2002). A variety of methods have been employed to suppress tick vector populations, including the application of chemical acaricides. However, the development of resistance to acaricides and environmental contamination emphasize the need to develop alternatives for a tick vector control. An anti-tick vaccine is considered to be one of the most promising methods; however, its development still depends on the identification and cloning of key tick molecules and the characterization of their roles in arthropod physiology (Mulenga et al., 2000).

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 contain three domains that are homologous with those of family 2 cystatins. Among them, only family 1 cystatins are intracellular (Turk and Bode, 1991). 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 (Turk and Bode, 1991; Agarwala et al., 1996; Yamamoto et al., 1999; Dainichi et al., 2001; Manoury et al., 2001; Zhou et al., 2006). Recently, several tick family 2 cystatins (secreted cystatins) have been characterized, and their function has been found in tick innate immunity (Zhou et al., 2006), midgut

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physiology (Grunclova et al., 2006), and blood-feeding success (Karim et al., 2005; Kotsyfakis et al., 2006, 2007). Family 1 cystatins (intracellular cystatins) have also been found in tick *Ixodes scapularis* and *Rhipicephalus (Boophlus) microplus*; however, only the *R (B). microplus* family 1 cystatin, named Bmcystatin, has been biochemically characterized (Lima et al., 2006). The biological functions of tick family 1 cystatin remain unknown.

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). Previously, a secreted cystatin Hlcyst-2 had been characterized from *H. longicornis* in our laboratory, and it was found that it was a midgut-specific expressed family 2 cystatin that was involved in tick innate immunity (Zhou et al., 2006). In this study, we report the characterization of an intracellular cystatin Hlcyst-1 from tick *H. longicornis*.

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 (Zhou et al., 2007). The sample materials were stored at −80 °C until used.

2.2. Cloning and sequence analysis of the full-length cDNA of cystatin

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 an intracellular cystatin 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 in the SignalP 3.0 server of the Technical University of Denmark.

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 the control GST protein, which was expressed and purified using a procedure identical to that 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 substrate. 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.

3. Results and discussion

The full-length cDNA of Hlcyst-1 is 437 bp, including an intact ORF encoding an expected protein with 98 amino acids. The sequence of the Hlcyst-1 gene of *H. longicornis* has been submitted to the GenBank database under accession number EU426544. The amino acid analysis using the Signal P program did not reveal the presence of a signal peptide, which is typical of family 1 cystatin, indicating the intracellular localization that is common across the phylogenetic scale. The deduced protein consisted of 98 residues with a calculated molecular weight of 11 kDa and an isoelectric point (pI) value of 5.5. SMART analysis (Schultz et al., 2000) detected the cystatin-like domain in the putative amino acid sequence (position 23–130). BLASTP analysis of the predicted polypeptide sequence against all non-redundant databases accessed through NCBI revealed a significant score with members of the cystatin family 1 of other species. The identities of the putative amino acid of Hlcyst-1 with *R (B). microplus* tick family 1 cystatins (Bmcystatin) and *I. scapularis* cytoplasmatic cystatin are 78.5% and 52%, respectively. The amino acid sequences of Hlcyst-1 include the highly conserved central QxVxG region (x can be one of several amino acids) in various cystatins (Brown and Dziegielewska, 1997) and a conserved glycine in the N-terminal region (Bjork et al., 1996) (Fig. 1).