Developmental Stage- and Organ-Specific Expression Profiles of Asparaginyl Endopeptidases/Legumains in the Ixodid Tick *Haemaphysalis longicornis*

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ABSTRACT. We previously identified two cDNAs from the midgut of adult *Haemaphysalis longicornis* that encode asparaginyl endopeptidases/legumains, HILgm and HILgm2. Functionally, both recombinant HILgm and HILgm2 efficiently digested blood proteins, haemoglobin and bovine serum albumin. Here, we investigated the expression profiles of legumain genes in the developmental stages in the life cycle of *H. longicornis* and in different tissues of adult ticks. Both *HILgm* and *HILgm2* were well expressed in larvae, nymphs and adults. Legumain transcripts were expressed specifically in the midgut and were localized in some digestive vacuoles of gut epithelial cells. Furthermore, expression of either transcript was up-regulated by blood feeding in larvae and nymphs, suggesting the important roles of legumains in blood feeding and blood-meal digestion in ticks.

KEY WORDS: arthropods, enzymes, molecular parasitology, tick, vector biology.

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Ticks are notorious blood sucking ecto-parasites and serve as one of the most important vectors transmitting many bacterial, viral, protozoal and rickettsial diseases from one vertebrate host to another [13, 18]. They solely depend on host's blood protein to get nutrition and energy essential for their development and reproduction [8]. *Haemaphysalis longicornis* is a dominant three host tick species in Far East Asia and Australia [10] that transmits many diseases, in particular, theileriosis [7], babesiosis [21] and Q fever to animals and humans [11]. It is believed that ticks have evolutionarily gained molecules that regulate tick biology. As an alternative of toxic chemical acaricide usages, targeting specific molecules which play key roles in tick physiological processes and metabolic pathways regulation including blood feeding and digestion is one of the useful approaches to thwart tick survival and thereby controlling ticks and tick borne diseases [22]. Different proteases have been reported to function as proteolytic enzymes in the midgut of *H. longicornis* [5, 9, 12, 16], *Boophilus microplus* [15, 19] and *Ixodes ricinus* [20].

Recently, we cloned and partially characterized two cDNAs from the midgut of adult *H. longicornis* that encode asparaginyl endopeptidases/legumains, HILgm [2] and HILgm2 [1]. Recombinant HILgm (rHILgm) and HILgm2 (rHILgm2) digested bovine serum albumin and haemoglobin (Hb) [1, 2]. Previously, we focused on *HILgm* and *HILgm2* expression in the midgut of adult *H. longicornis*. However, since blood feeding is obligatory for every devel-

opmental stage (viz. larva, nymph and adult) of *H. longicornis* and it is very likely that one gene might be expressed in different organs in accordance with its biological relationship, we further investigated each stage and organ specific expression profiles of mRNAs of the two legumain genes in *H. longicornis*.

In this study, the different developmental and feeding stages of parthenogenetic Okayama strain of *H. longicornis* were obtained by feeding on rabbits as described previously [2] and the animals were treated in accordance with the protocols approved by the Animal Care and Use Committee of National Institute of Animal Health, Tsukuba, Japan (Approval nos. 441, 508, 578). To detect the developmental stages and blood feeding phases specific *HILgm* and *HILgm2* mRNA expression profiles, mRNAs were collected from unfed, 24 hr, 48 hr, 72 hr and engorged larvae and nymphs using an RNeasy Mini Kit (QIAGEN) and following the manufacturer's instructions. Reverse transcription (RT)-PCR was performed with a template of 500 ng of total RNA for each 10 µl reaction mixture using a Takara RNA PCR Kit (AMV) Ver.3.0 (Takara, Shiga, Japan) in accordance with manufacturer's protocol. A 500 ng of each cDNA synthesis product and oligonucleotides which were specific for either *HILgm* (forward primer 5'-CGACGAGCAAATCGTAGTCA-3' and reverse primer 5'-ACTTTTCGCTTCCTCCATT-3') or *HILgm2* (forward primer 5'-CCTTCGCAACAAGCTAAAGG-3' and reverse primer 5'-TCAGAAAGTCCTTCGGTG CTT-3') or specific primers for positive control cDNA encoding β -actin were used to perform PCRs in a final volume of 20 µl. PCR products were electrophoresed in 1% agarose gel. The quantitative RT-PCR was carried out in a LightCycler 1.5 instrument (Roche Instrument Center AG, Roikreuz, Switzerland)

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Fig. 1. Expression profile of *HILgm* and *HILgm2* genes in the larvae and nymphs of *H. longicornis*. (A) Reverse transcription (RT)-PCR analysis. The whole body homogenates of larvae and nymphs were subjected to RT-PCR analysis using specific primers for *HILgm* and *HILgm2*. Arrows indicate the level of gene expression. Actin is shown as an internal control. (B) Real-time PCR was performed using the same total RNA and the same primers specific for *HILgm* and *HILgm2* as above. Eng, engorged.

using LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH, Nonnenwald, Germany). A 20 μ l reaction mixture contained 4 mM of $MgCl_2$, 0.5 μ M of each primer (forward and reverse as above), 2 μ l of LightCycler FastStart DNA Master SYBR Green I and 2 μ l of the single-stranded DNA as template. The data obtained were analyzed using LightCycler Software Version 3.5. The results obtained by RT-PCR (Fig. 1A) and quantitative RT-PCR analysis (Fig. 1B) revealed that both *HILgm* and *HILgm2* specific mRNAs were well expressed in both larvae and nymphs. Interestingly, *HILgm2* mRNA expression level was lower than that of *HILgm* mRNA during the different phases of blood feeding. Notably, expressions of *HILgm* and *HILgm2* transcripts were up-regulated by blood feeding process reaching to their peaks at 48 hr both in larvae and nymphs, and thereafter declined. Our previous studies revealed similar expression profiles of *HILgm* and *HILgm2* mRNAs in adult *H. longicornis*, however, expression of the genes was culminated at 72 hr of feeding and then declined [1, 2]. Blood digestion is rapid during the growth phase of feeding which lasts for 1.5 to 2.5 days in *H. longicornis* nymphs [14] and the proteolytic activities reach to the peak at 72 hr of feeding in adult ticks [4]. Blood digestion during initial stage of feeding (preparatory phase) and in expansion phase when ticks feed rapidly to engorge is very low [3]. Feeding behaviours of larvae are almost similar to that of nymphs, and the present results and our previous findings in adults [1, 2] obviously indicate the involvement of the legu-

main in blood protein digestion process in all developmental stages of *H. longicornis*.

To detect the tissue specific expression of *HILgm* and *HILgm2*, individual organs viz. midguts, salivary glands, ovaries, cuticles, tracheae, Malpighian tubules and synganglia were collected from 72 hr fed adult ticks as described previously [1]. RT-PCR of the mRNAs extracted from these organs as above revealed that both legumain genes were specifically expressed in the midgut tissues (Fig. 2). These results are consistent with our previous observations

Fig. 2. Organs specific expression profile of *HILgm* and *HILgm2* in the adult *H. longicornis*. The tissues were dissected from three partially fed (72 hr) adult ticks and subjected to RT-PCR analysis. *HILgm* and *HILgm2* transcripts were expressed only in the midgut tissues (Arrows). *H. longicornis* β -actin mRNA was used as an internal control.

Fig. 3. Intracellular localization of endogenous legumains in the midgut epithelial cells by immunofluorescent staining. The midgut epithelial cells were collected from 72 hr fed adult *H. longicornis*. The cells were fixed in paraformaldehyde and permeabilized with Triton X-100. The cells were incubated with either mouse anti-rHILgm2 serum or preimmune serum and visualized with Alexa Fluor® 488 (green). The nuclei were stained with DAPI (Blue). Scale bar indicates 10 μ m.

on immunohistochemical localization of endogenous legumains using whole tick sections of adult *H. longicornis* probed with mouse anti-rHILgm [1]. Legumain transcript from the hard tick *Ixodes ricinus* (IrAE) has also been reported to be expressed solely in the midgut tissues [20]. We next used the immunofluorescence techniques to visualize the intracellular localization of endogenous legumains in the midgut cells as described previously [17]. The cells isolated from the freshly collected midguts of 72 hr fed adult ticks were attached to a glass slide using the Shandon Cytospin® (Thermo Electron, Waltham, MA, U.S.A.), fixed with 4% paraformaldehyde in PBS (0.1 M, pH 7.4) and permeabilized in PBS containing 0.1% Triton X-100 for 20 min at room temperature. The slides were incubated with mouse anti-rHILgm2 serum (1:200) only, as we observed that polyclonal antibodies generated against one recombinant legumain reacted notably with both legumain isoforms (unpublished data). The slides were then reacted with green fluorescence-labeled secondary antibody (Alexa Fluor®488 goat anti-mouse IgG (H+L); Invitrogen, Carlsbad, CA, U.S.A.). The slides were mounted with VECTASHIELD® mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, U.S.A.) and examined under a fluorescence microscope (Leica, Wetzlar, Germany). Immunofluorescent staining revealed expression of legumains in some digestive compartments of the midgut cells. No reactivity, however, was detected in the cells treated with preimmune mouse serum (Fig. 3). Both rHILgm and rHILgm2 were active at neutral to alkaline pH and the recombinant enzymes digested Hb and bovine serum albumin optimally at pH 7 [1, 2]. In ticks, Hb digestion takes place intracellularly in acidic vacuoles [6]. However, intracellular localization of legumains conceivably suggests their involvement in Hb digestion cascade in midgut cells.

It is plausible that body organs express genes highly correlated with their biological functions. Midgut specific expression of the legumain genes and up-regulation of their

expression levels by blood feeding process suggest their critical roles in blood feeding and blood protein digestion in ticks. Functional characterization of legumains would help to know the prospects of their application as candidate molecules for the control of tick and tick-borne diseases.

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