FULL PAPER *Parasitology*

Leucine Aminopeptidase in the Ixodid Tick *Haemaphysalis longicornis*: Endogenous Expression Profiles in Midgut

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ABSTRACT. We previously identified a cDNA from the ixodid tick *Haemaphysalis longicornis* that encodes leucine aminopeptidase, HlLAP. Functionally, recombinant HlLAP effectively hydrolyzed synthetic amino acid derivatives. Here, we investigated the temporal expression profiles of midgut HlLAP in adult *H. longicornis* parthenogenetic ticks from the starting of blood feeding until just before the onset of oviposition. Midgut *HlLAP* transcript expression level was higher during post-engorgement period than that during feeding period. Endogenous HlLAP in the midgut was also observed with higher expression level during post-engorgement period. Histological localization of HlLAP was in the cytosol of midgut epithelial cells, notably the newly differentiated basophilic cells at post-engorgement. Our data suggested that HlLAP was dominantly localized in basophilic cells, where it may play regulatory roles in protein biosynthesis and degradation.

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

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Ticks are second only to mosquitoes as vectors of various disease-causing agents in humans and are the most important arthropod transmitting pathogens (e.g. *Babesia* and *Theileria* protozoa, *Borrelia* bacteria, and hemorrhagic and encephalomyelitis viruses) to domestic and wild animals [16]. The three-host ixodid tick, *Haemaphysalis longicornis* has been distributed mainly in East Asia and Australia [11, 12], where it transmits a wide range of pathogens, including viruses, rickettsia and protozoan parasites [30].

Many studies intending to find an efficient control strategy that would minimize the damages caused by ticks and their transmissible pathogens are currently in progress. As an alternative of chemical acaricide usage, targeting specific molecules which play key roles in tick physiological processes and metabolic pathways, including blood feeding and digestion is one of the useful approaches to interfere with tick survival and thereby control ticks and tick borne diseases [34]. Several proteases relating to blood digestion have been reported from the ixodid tick, *H. longicornis* [4–6, 22], *Rhipicephalus (Boophilus) microplus* [21, 24] and *Ixodes ricinus* [27].

Tick midgut leucine aminopeptidase belonging to the clan MF, M17 cytosolic aminopeptidase family (LAP, EC 3.4.11.1) was isolated from *H. longicornis* (HlLAP; GenBank accession number AB251945). HlLAP was expressed in many organs of adult ticks such as midguts, salivary glands, and epidermis, where it was suggested to catalyze the hydrolysis of amino-acid residues from N-terminus of peptides since recombinant HlLAP expressed in *Escherichia coli* exhibited optimal hydrolyzing activity at pH 8 with a metal divalent cation (such as Mn^{2+}) dependency against fluorogenic amino acid substrates [13]. Especially, HlLAP localizing in midgut tissues was assumed to function as one of the digestive enzymes in the ticks [13, 14]. The blood feeding towards repletion of adult *H. longicornis* takes for around a week, however, the time course profile of endogenous HlLAP expression in the tick midgut has been obscure. In this study, we further investigated mRNA and protein expression kinetics of midgut HlLAP during different phases of blood feeding and post-engorgement periods of pre-oviposition in adult parthenogenetic female ticks.

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MATERIALS AND METHODS

Ticks and experimental animals: The parthenogenetic Okayama strain of the ixodid tick *H. longicornis* maintained at the Laboratory of Parasitic Diseases, National Institute of Animal Health (Tsukuba, Ibaraki, Japan) was bred by feeding on rabbits as described previously [33]. The animals employed for the tick maintenance and for the antibody production were adapted to the experimental conditions for 2 weeks prior to the experiment and were treated in accordance with the protocols approved by the Animal Care and Use Committee, NIAH (Approval nos. 441, 508, 578).

Generation of antibody: One set of oligonucleotide primers derived from the open reading frame of the *HlLAP* gene was used where the sense primer (5'-CGGGATCCGATGCTACTGCGCTCGATC-3') corresponded to the nucleotides 115–132 of HlLAP nucleotide sequence and the antisense

primer (5'-CGGGATCCCTACACACGACCACACAC-3') corresponded to the nucleotides 1,675–1,692 of the sequence. The nucleotide sequences of both primers contained a *Bam*HI restriction sites. Amplified product was inserted into the pTrcHis B plasmid vector (Invitrogen, Carlsbad, CA, U.S.A.) after digestion with *Bam*HI. The resultant plasmid (pTrcHis B/ HlLAP) was transformed into *E. coli* (Top10F', Invitrogen). A single bacterial clone carrying pTrcHis B/HlLAP was cultured in LB medium (Becton, Dickinson and Company, MD, U.S.A.). The expression of recombinant protein was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside and followed by 4 hr incubation at 37°C. The recombinant HlLAP fused with a poly-histidine tag (His-HlLAP) was purified using Ni sepharose 6 Fast Flow (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) under denaturing conditions as described in the manufacturer's protocol. The recombinant His-HlLAP was dialyzed against phosphate-buffered saline (PBS) using a Slide-A-Lyzer Dialysis Cassette (Pierce Biotechnology, Rockford, IL, U.S.A.). Protein concentration was determined using micro-BCA reagent (Pierce). To generate polyclonal antisera, recombinant protein was emulsified in complete Freund's adjuvant (Difco, Detroit, MI, U.S.A.) and injected into 5 BALB/c CrSlc mice (SLC Japan, Hamamatsu, Japan) (100 μg per head). The animals were boosted twice at intervals of 10 days with the same dose of the antigen in incomplete Freund's adjuvant, and the sera were collected 1 week after the second booster. The antisera were stored at –20°C until use. To generate the antisera for the loading control of immunoblotting, an ORF of *glyceraldehydes 3-phosphate dehydrogenase* gene from *H. longicornis* embryonic cDNA library (*HlGapdh*, unpublished data) was inserted into *Bam*HI site of pET15b (Merck, San Diego, CA, U.S.A.) and simultaneously transformed into an *E. coli* BL21(DE3) (Merck). Poly-histidine tagged recombinant HlGAPDH (His-HlGAPDH) was expressed and purified protein was used to generate anti-HlGAPDH polyclonal antibody in mice following the methodology described above.

Quantitative reverse transcription (RT)-PCR: Midgut specific *HlLAP* transcriptional kinetics was analyzed by quantitative RT-PCR (qRT-PCR) using dissected midguts collected at different periods: 0 (unfed) to 4 days post-infestation (DPI) and 0 (just after engorgement) to 4 days post-engorgement (DPE). Midguts (30 guts on 0–2 DPI; 5 guts on 3–4 DPI; 1 gut on 0–4 DPE) were dissected and pooled in RNAlater solution (QIAGEN, Hilden, Germany) and then stored at –80°C until RNA extraction. Total RNA from these samples was extracted using RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. Contaminating DNA was removed using TURBO DNA-free™ Kit (Ambion, TX, USA) before synthesis of first strand cDNA (RNA PCR kit, AMV; Takara, Shiga, Japan) as a template of qRT-PCR analyses. LightCycler Fast-StartDNA Master SYBR Green I (Roche Diagnostics GmbH, Nonnenwald, Germany) and LightCycler 1.5 instrument (Roche Instrument Center AG, Roikreuz, Switzerland) were used for qRT-PCR. Gene specific primer sets were indicated as follows, for HlLAP (HlLAP1F, 5'-CGCTAA-GAAGCAGGCTGTCCTA-3'; HlLAP12R, 5'-TCAGAC-CGTAGAAAACTCTGGAC-3') and for tick β-actin (HlActin8F1, 5'-CCCATCTACGAGGGTTACGCTC-3'; HlActin9R1, 5'-CATCTCCTGCTCGAAGTCCAGG-3'). Standard curves for HlLAP and tick β-actin were prepared using 10-fold serial dilutions of known quantities (10^9–10^3 copy for pET15b-HlLAP; 10^9–10^3 copy for pET15b-Hlactin).

Immunoblotting: Groups of 3 to 5 midguts from adult ticks (0–4 DPI and 0–4 DPE, respectively) were suspended in 200 μl of sterile PBS containing protease inhibitors (Roche) and homogenized as described previously [13]. Following sonication, the supernatant was prepared by centrifugation at 25,600 × g, 4°C. Two microliter of midgut protein was diluted as three folds serial dilution and blotted on a nitrocellulose membrane and processed for dot blot analysis using anti-HlGAPDH serum and horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody to determine suitable dilution factor of immunoblotting. Following dot blot result, each diluted sample was electrophoresed on sodium dodecyl sulfate (SDS) 10% polyacrylamide gel and processed for immunoblotting using anti-HlLAP and anti-HlGAPDH sera. Bound antibodies were detected using HRP-conjugated goat anti-mouse secondary antibody. Specific bands were developed using the substrate 3',3'-diaminobenzidine tetrahydrochloride (Sigma Fast® DAB set; Sigma Aldrich, St Louis, MO, U.S.A.). The optical density of each band was measured using ImageJ 1.36b (National Institutes of Health, MD, U.S.A.) and the ratios of the density of respective HlLAP bands relative to that of the HlGAPDH band were compared.

Immunofluorescent staining: For precise localization of endogenous midgut HlLAP, the midguts from adult ticks (0–4 DPI and 0–4 DPE) were fixed and embedded in paraffin as described previously [13]. Deparaffinized thin sections (6 μm) were incubated with 3% normal goat serum and then with anti-HlLAP serum (1:100 dilution) for 1 hr at room temperature, followed by Alexa Fluor® 488 conjugated goat anti mouse IgG (1:1,000 dilution, Invitrogen). After washing with PBS, the slides were mounted with VECTASHIELD® mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, U.S.A.), covered with glass cover slips, and then observed under a fluorescence microscope (Leica, Wetzlar, Germany).

RESULTS

Transcriptional kinetics of midgut HlLAP: As shown in Fig. 1, midgut *HlLAP* transcription was observed as a bimodal pattern. *HlLAP* mRNA expression was up-regulated by blood-feeding process reaching to its peak at 2 DPI and thereafter declined. Its expression was re-up-regulated after engorgement reaching to its peak at 2 DPE and thereafter

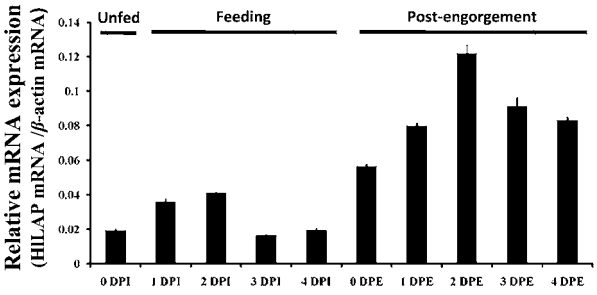

Fig. 1. Midgut *HlLAP* transcription kinetics analyzed by quantitative RT-PCR using midgut total RNA. Relative expression is shown as the ratio of the quantified copy number of *HlLAP* mRNA against that of tick actin mRNA. Stage-specific ticks were recovered from rabbit and dissected at unfed (0 day post-infestation, DPI), feeding (1–4 DPI) and post-engorgement period of pre-oviposition (0–4 days post-engorgement, DPE).

declined slightly. Interestingly, *HlLAP* expression level during the post-engorgement period was relatively higher than that observed during the blood feeding process.

Translational kinetics of midgut HlLAP: Immunoblotting studies using the sera prepared by recombinant His-HlLAP and His-HlGAPDH detected a single band of the expected c.a. 56 and 36 kDa against tick native protein. As shown in Fig. 2A, both endogenous proteins were detected throughout unfed (0 DPI), feeding (1–4 DPI) and post-engorgement periods (0–4 DPE). According to the relative quantification using ImageJ 1.36b (Fig. 2B), relative expression of endogenous HlLAP against HlGAPDH in the unfed (0 DPI) ticks was higher than our assumption. While relative expression of HlLAP was decreased just after infestation on the host (1 DPI), it was up-regulated by progression of blood feeding process (1–4 DPI). During post-engorgement period, relative HlLAP expression was higher than that during the feeding process and was maintained until the onset of oviposition (0–4 DPE).

Localization of midgut HlLAP: Endogenous midgut HlLAP was detected in all samples tested. The midgut epithelial cells displayed diffuse staining indicative of a cytoplasmic localization (Fig. 3). The fluorescence intensity that gradually became stronger was observed in the special type of cells bound directly to the midgut basal membrane during the post-engorgement period (panels 1–4 DPE, Fig. 3A). According to the Hematoxylin-Eosin staining pattern shown in Fig. 3B, these cells were stained strongly with hematoxylin (4 DPE) and were regarded as basophilic cells [1, 19] which were less observed in the midgut epithelium at 0 DPE (Fig. 3B).

DISCUSSION

Cytosolic leucine aminopeptidases play a digestive role in intestinal organ of some organisms, including nematodes, trematodes and arthropods [7, 8, 17, 20, 23, 29]. Since, in ticks, blood digestion in the midgut is entirely on intracellular process [9], we clarified *H. longicornis* cytosolic leucine aminopeptidase (HlLAP) expression kinetics at the level of mRNA and protein, and localization in the midgut tissues, functionally analogous to the vertebrate and invertebrate intestine.

Tick blood digestion pattern supported by previous

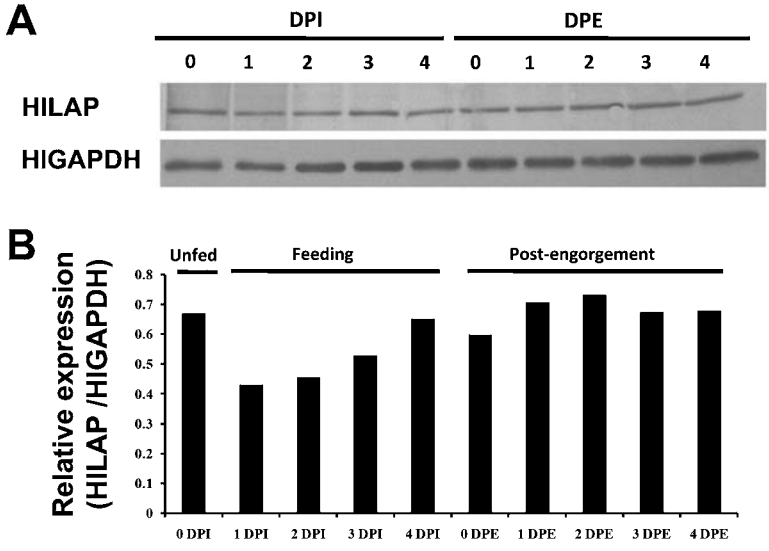

Fig. 2. Endogenous HlLAP expression profile in the adult *H. longicornis* midgut. A) Midgut soluble protein extracts from the adult ticks at different stages (0–4 DPI and 0–4 DPE) were resolved on reducing SDS-polyacrylamide gels (10%), transferred onto nitrocellulose membranes, and probed with anti-HlLAP and anti-HlGAPDH sera. (B) Relative expression is shown as the ratios of the density of each HlLAP band relative to that of HlGAPDH band analyzed by ImageJ software.

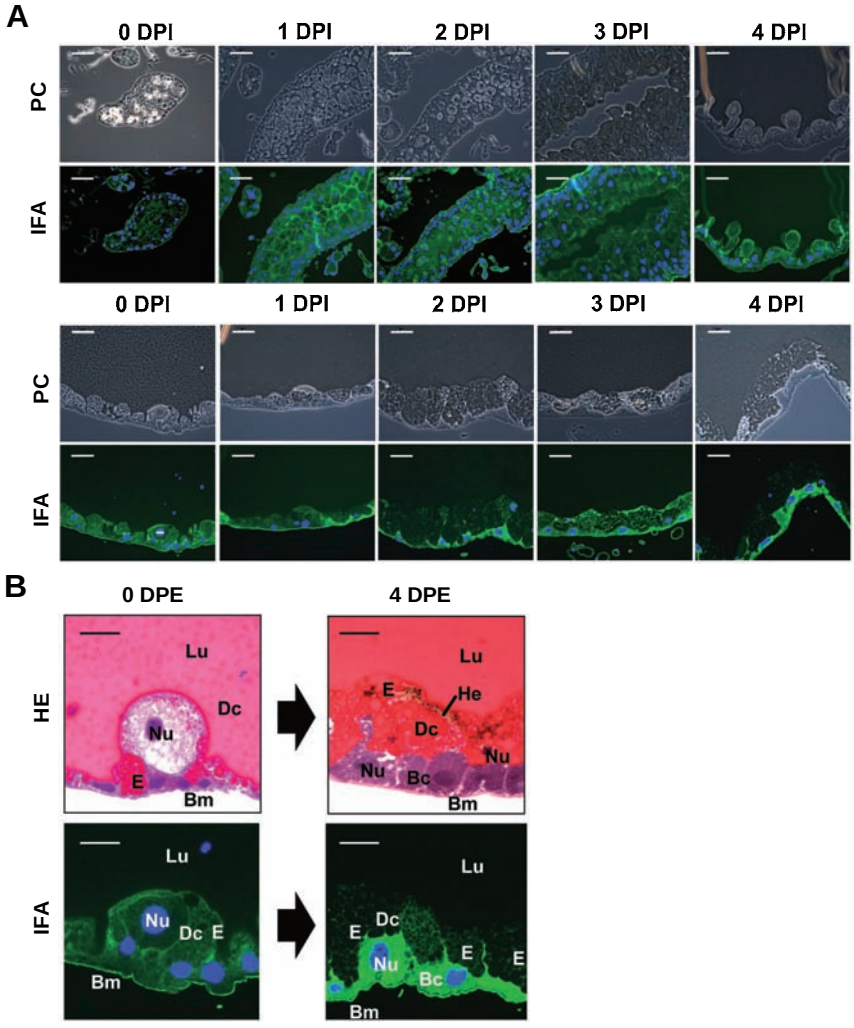

Fig. 3. Immunofluorescent staining of endogenous HlLAP on the sections of midguts prepared at different feeding and post-feeding stages of adult *H. longicornis*. (A) Midgut sections (0–4 DPI and 0–4 DPE) were incubated with anti-HlLAP serum followed by visualization with Alexa Fluor® 488 (green). Nuclei were stained with DAPI (blue). PC, phase contrast. IFA, immunofluorescent antibody. Scale bars were shown as 50 μm. (B) Hematoxylin-Eosin (HE) and IFA staining pattern of midgut epithelial cells at 0 and 4 DPE. Bc, basophilic cell; Bm, basal membrane; Dc, digestive cell; E, endosomes in digestive cell; He, hemosomes in digestive cell; Lu, lumen; Nu, nucleus. Scale bars were shown as 25 μm.

ultramorphological observation of lysosome in midgut digestive cells consists of three distinct phases: phase-1, slow feeding process but rapid digestion activity in midgut digestive cells; phase-2, rapid feeding process but slow digestion in the cells, which starts at 12–36 hr before engorgement; and phase-3, continuous digestion phase during post-engorgement period [2, 18, 28, 32]. Consistently, midgut HlLAP mRNA expression was quite synchronized with the tick blood digestion pattern. A bimodal expression profile of HlLAP transcript as observed during feeding and pre- and post-engorgement processes, including a relatively higher expression level of the transcript at post-engorgement period, suggests that HlLAP may play more active role in digestion and degradation of blood proteins. These findings are strongly supported by previous reports on tick legumains, belonging to the C13 asparaginyl endopeptidase family, which localize mainly in midgut as blood protein digestive enzymes [3–5].

At the translational level, an unexpected high expression of HlLAP was observed at 0, 3 and 4 DPI. This kind of discordance between transcriptional and translational expression level might be caused by the shift of mRNA turnover- and/or stability-rate [31] during unfed and feeding period. Post-engorgement blood digestion was suggested to contribute mostly to the acquisition of nutrient for egg production [9]. Indeed, steady translational expression level of HlLAP

during post-engorgement was achieved in this study and was higher than that during unfed and feeding periods, further suggesting that HlLAP might be functionally important during post-engorgement period.

Midgut epithelium is classified as around three types of cells (digestive, secretory and stem cells) during feeding and two types of the cells (digestive and basophilic cells) during post-engorgement [28]. To discuss further the midgut HlLAP function, localization of the endogenous HlLAP in midgut epithelium was clarified. A constitutive HlLAP expression in the midgut epithelium was achieved throughout unfed to post-engorgement periods of pre-oviposition. Interestingly, during post-engorgement period, abundant expression was observed in the special type of cells bound to the basal membrane, named basophilic cells [1, 19]. These cells have large amounts of rough endoplasmic reticulum, contributing cell basophilic feature, and a well developed basal labyrinth, characteristic of cells engaged in synthesis of large amounts of secretory proteins [10]. Some literatures also suggested that basophilic cells are one of the sites producing vitellogenin, the egg yolk protein precursor [25]. It is assumed that, at the same time of protein synthesis, proteolytic process by the ubiquitin-proteasome system selectively and efficiently degrades denatured and/or misfolded proteins that arise as a result of mutations, immaturity, or post-translational environmental stress [15, 26] and also degenerate nascent peptide chains, co-translationally [35]. Therefore, HlLAP in basophilic cells may catalyze post-proteasomal peptide degradation to amino acids, the source of the de novo synthesis of protein. Coons and co-workers [9] also observed that basophilic cells take up electron-dense materials, believed to contain the digested blood meal components from adjacent digestive cells via receptor mediated endocytosis along its lateral plasma membrane as well as dense membrane bound bodies, probably of lysosomal origin for proteolytic activity. Morphologically, digestive cells during post-engorgement periods are filled with large amount of endosomes. They have small cytosolic space and have little areas of direct contact with the basal membrane, while basophilic cells have enormous attachment area to the membrane and hemocoel [9, 19]. Hence, it is reasonable to think that some parts of final degradation of peptides from blood protein may be regulated by HlLAP in basophilic cells during post-engorgement period.

In summary, from the understanding of midgut HlLAP temporal expression, the functional importance of HlLAP was dominantly mapped in newly differentiated basophilic cells at post-engorgement. Therefore, HlLAP is assumed to play key roles in protein or peptide catabolism in these cells after engorgement. Elucidating the temporal cascade of blood digestive enzymes in ticks would contribute to better understanding of the mechanisms of blood meal digestion, leading to the development of a novel control strategy for both ticks and tick-borne pathogens.

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