# Novel Asparaginyl Endopeptidases/Legumains from the Hard Tick *Haemaphysalis longicornis* and Their Roles in Tick Blood-feeding and Embryogenesis

# （フタトゲチマダニ新規アスパラギンエンドペプチダーゼ/レグマインの吸血および胚発生における役割）

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# Abbreviations

| | |
|---|---|
| **CBB** | **: coomassie brilliant blue** |
| **cDNA** | **: complementary DNA** |
| **DAB** | **: 3', 3'-Diaminobenzidine** |
| **DPE** | **: days post-engorgement** |
| **dsRNA** | **: double stranded RNA** |
| **EST** | **: expressed sequence tag** |
| **Hb** | **: haemoglobin** |
| **HlLgm** | **: *Haemaphysalis longicornis* first legumain** |
| **HlLgm2** | **: *Haemaphysalis longicornis* second legumain** |
| **IgG** | **: immunoglobulin G** |
| **IPTG** | **: isopropyl-$\beta$-D-thiogalactopyranoside** |
| **MCA** | **: a-(4-methyl-coumaryl-7-amide)** |
| **mRNA** | **: messenger RNA** |
| **ORF** | **: open reading frame** |
| **PAGE** | **: polyacrylamide gel electrophoresis** |
| **PBS** | **: phosphate buffered saline** |

| | |
|---|---|
| **PCR** | **: polymerase chain recaton** |
| **PVDF** | **: polyvinylidene difluoride** |
| **REI** | **: reproductive efficiency index** |
| **RFI** | **: reproductive fitness index** |
| **rHlLgm/rHlLgm2** | **: recombinant HlLgm/recombinant HlLgm2** |
| **RNAi** | **: RNA interference** |
| **RT-PCR** | **: reverse transcription-polymerase chain reaction** |
| **S.D.** | **: standard deviation** |
| **SDS** | **: sodium dodecyl sulfate** |
| **TAE** | **: tris-acetic acid-EDTA** |
| **TBS** | **: tris-HCl buffered saline** |
| **Vg** | **: vitellogenin** |

# Unit abbreviations

| | |
|---|---|
| **h** | **: hour(s)** |
| **M** | **: mol/L** |
| **mM** | **: millimol/L** |
| **ml** | **: milliliter** |
| **µl** | **: microliter** |
| **nM** | **: nanomol** |
| **mg** | **: milligram** |
| **µg** | **: microgram** |
| **kDa** | **: kilodalton** |
| **bp** | **: base pair** |

# General introduction

Ticks are arthropods, the origin of which can be traced back to the Late Cretaceous, ~120 million of years ago (17). Ticks are classified within the class, Arachnida; subclass, Acari; order, Parasitiformes; suborder, Ixodida; superfamily, Ixodoidea; family, Ixodidae, Argasidae, and Nuttalliellidae (112, 134). Phylogenetic analysis indicates that the Ixodida are monophyletic (17) and the Ixodidae is traditionally divided into Prostriata (genus *Ixodes*) and Metastriata (Ixodidae other than *Ixodes*) (70). The ixodid ticks (hard-bodied ticks) comprise approximately, 80% (683 spp.) of the world's tick fauna and with the exception of only one species under the family Nuttalliellidae, the remainder are argasid ticks (soft bodied ticks) (183 spp.) (72). Morphologically, the body of ticks is divided into two functional parts, the gnathosoma (capitulum) and the idiosoma. The gnathosoma consists of the segmented palps, chelicerae bearing the sharp denticles, the toothed hypostome and mouth. The idiosoma bears the legs, brain, digestive and reproductive organs (86, 134).

Ticks are cosmopolitan in distribution although, they are not equally common in all parts of the world. The ixodid ticks are exclusively parasitic and extremely stated, they use all potential terrestrial vertebrate hosts including mammals, birds and reptiles and sometimes

amphibians (86, 133). Interestingly, the ixodids are non-nidicolous ticks (86) and live in forests, savannas, grass lands or remain under soil, stones, woods, or in crevices (117, 134). The developmental stages of the ixodids comprise of eggs and three distinct post-embryonic stages-larva, nymph and adult. The ixodids may be one-host, two-host and three-host ticks. The aforesaid life cycle pattern where larvae, nymphs and adults each have to find a host is called a three-host life cycle (86) which in non-nidicolous ticks are characterized by a long periods of diapause in their habitats interspersed with short periods of host seeking or questing (117). Ticks attach to the hosts, feed and drop off the host after repletion. Sometimes, the ability of the ticks to survive off the host is really stupefying and many of them spend more than 95% of their life while off the host (108). Depending on the developmental stages, the engorged ticks either moult into the next stage or the adult females lay eggs and die on the ground. The newly hatched larvae or the newly moulted stages then repeat the process of blood-feeding on a host (117).

Ticks act as direct pests and also cause indirect damage to animals and humans. They are voracious blood suckers and an estimated blood loss ranges from 0.7 ml to as high as 8.9 ml per female in some ixodid ticks (11). Injection of pharmacologically active substances into the hosts can cause severe or sometimes fatal illness such as paralysis and toxicosis, irritation and allergy (132). Infestation of dairy cattle with cattle tick, *Boophilus microplus* and the brown ear tick, *Rhipicephalus appendiculatus* was estimated to cause a loss of 8.9 ml and 9.0 ml milk yield, respectively, per adult engorging (80, 113). Additionally, the wounds that they produce may create sites for secondary infections and diminish the value of the livestock by damaging the hides. Costs to the worldwide cattle industry incurred only for the cattle tick *Boophilus* spp. infestations were estimated at more than US$ 7 billion (57). Ticks are right behind the mosquitoes as disease transmitters (86) and serve as potent vectors of many bacterial, viral,

protozoal, and rickettsial diseases of humans and animals worldwide (11, 22, 23, 134, 147). In Japan, the important tick borne diseases of man and animals include theileriasis, babesiosis, and Q fever which are mainly transmitted by *Haemaphysalis longicornis* Neumann, 1901 (bush tick) (51, 52, 68, 73, 140). *H. longicornis* is also known as a capable vector of Russian spring-summer encephalitis virus (71). *H. longicornis* belongs to the subfamily *Haemaphysaline* under the Metastriata (79). It is a commonly prevalent three-host tick in northeastern parts of Russia, Far East Asia and Australia and is the predominant tick species in Japanese pasture (52, 71). Characteristically, *H. longicornis* shows the strains of both bisexual and parthenogenetic population (50, 71).

The life cycle pattern and other characteristic physiological features of ticks, the most important of which is their blood-feeding and digestion process, make them different from other arachnids and insects. They follow a specialized strategy for feeding. Unlike the mosquitoes and other vessel-feeder insects, ticks are pool-feeders. They do not pierce the host's blood vessels explicitly, rather destroy the tissues and peripheral blood vessels of the dermis with their armed mouth parts and create a blood-pool (84). The cementing substances secreted with tick saliva reinforce the longer time attachment (3–12 days depending on stage and species) and prevent the oozing of the fluid from the blood-pool (84, 86, 134). New cuticle is synthesized during feeding that facilitates accommodation of the ingested blood-meal and the tick body expands enormously (60, 85). On blood-feeding, the weight of the female ixodid ticks may increase 100–200 times. However, the amount of imbibed blood is much bigger as the ticks concentrate the blood-meal returning back excess water and sodium ions to the host via specialized salivary gland cells while feeding (82, 132).

Ticks get nutrition and energy through digestion of host blood proteins including hemoglobin (Hb) (59). The process of blood digestion in ticks differs greatly from that in haematophagous insects. The ingested blood-meal passes through the large central chamber of the midgut and is further distributed into the diverticulae by peristalsis. Haemolysis of the host erythrocytes occurs in the midgut lumen (2, 5). The midgut digest cells uptake the released Hb by the process of endocytosis (4, 5, 136) and digestion takes place as a slow intracellular process in the lysosomal vacuoles of the midgut digest cells (5, 11, 33, 59, 91). In contrast, blood digestion occurs rapidly in the intestinal lumen in haematophagous insects (5, 33, 67).

As direct pests in their own right and the unique way of interacting as vectors of many deadly diseases, tick infestations warrant a search of strategies for their effective control. Until now, the methods that have been practised for this purpose include the application of chemical acaricides and use of biological control agents. However, the commonly used acaricides for tick control are potentially toxic to humans and animals and also hazardous for the food chain and the environment. Moreover, frequent application of acaricides leads to an enormous problem of widespread multi-acaricide resistance development (15, 157). Immunological approaches through vaccinations seem to be the most promising and sustainable alternatives to the chemical pesticides and other practices to control tick and tick borne diseases (114, 138, 148–150). Vaccination integrated with endosymbiotic approach has also been proposed as a future prospective strategy of tick control (54). However, immunological approaches necessitate searching and targeting specific molecules, playing key roles in tick physiological processes and metabolic pathway regulation including blood-feeding and digestion.

Hb digestion in ticks is performed essentially by the gut-associated proteases (58). Proteases are also known to be involved in digestion of foods in other arthropods (139). Among

different group of proteases, the cysteine proteases are thought to play the key role in blood-meal digestion in haematophagous helminth and malaria parasites (35, 124, 125, 127, 151). It is speculated that cysteine proteases from the ticks might have important roles in host blood-meal digestion and other physio-chemical functions essential for their survival and reproduction. In the present study, I have focused on cysteine proteases, legumains from *H. longicornis*. Legumain is an asparaginyl endopeptidase (AEP, EC 3.4.22.34) which is categorized into clan CD (26) and belongs to the cysteine protease family, C13 (Merops: http://www.merops.co.uk). AEP/legumain shows strict substrate specificity toward the carbonyl side of asparagine residues on the surface of substrate molecules (37, 75). Legumain was first reported from plants as a vacuolar processing enzyme (62) and the name legumain was given by Kembhavi et al. (83) after it was isolated and characterized from the seed of the leguminous plant, *Vigna aconitifolia* (moth bean). Several legumain orthologues have been described in protozoa (92); in blood-feeding helminths, *Schistosoma mansoni* (38) and *Haemonchus contortus* (116); and in mammals (pigs, humans, mice and cattle) (26, 37, 154). Plant legumain is involved in mobilization of storage proteins and post-translational processing of seed proteins (63, 64, 128). Mammalian legumains are known to process the microbial antigen, tetanus toxoid C fragment for class II MHC presentation in the lysosomal/endosomal system (9, 98) and to inhibit osteoclast formation and bone resorption (32). They degrade several biologically active peptides and proteins such as neurotensin, vasoactive intestinal peptide, bovine serum albumin (BSA) and chicken lysosome (37). Legumains from *S. mansoni* (Sm32) (25, 124) and *H. contortus* (116) activate cathepsin B zymogens as a mature enzyme with hemoglobinase activity in the gut of the parasites and Sm32 has been proposed as a candidate in the protease pathway of host Hb degradation (24). However, the exact mechanisms of proteolysis including Hb digestion by legumains are still unclear.

Unraveling the biochemical properties of tick legumains may give an insight into precise molecular basis of blood-feeding and digestion offering novel means of ticks and tick borne diseases control.

In this study, I have identified and characterized two novel cDNAs encoding legumains/AEPs (HlLgm and HlLgm2) from the midgut of adult female *H. longicornis*. The molecular properties and biological functions of these two enzymes in tick physiology have been discussed. Chapter 1 describes the cloning and sequence analyses of the novel legumain genes (*HlLgm* and *HlLgm2*) isolated from *H. longicornis* midgut cDNA library, their deduced motifs, and phylogenetic relationship with AEPs from other sources. Chapter 2 describes the endogenous expression and histological localization of HlLgm and HlLgm2 in ticks' body organs. The relationship of the legumains expression with blood-feeding and digestion has also been discussed. Chapter 3 describes the purification and biochemical profiles of recombinant legumains. The substrate specificity, pH optima, temperature dependency, and metal ion sensitivity of the enzymes have been discussed to clarify the biological function of legumains in ticks. Chapter 4 describes the impacts of knock-down of legumain genes. The potentials of legumains in blood-feeding and digestion, midgut remodelling, and reproduction of ticks have been described by employing RNA interference (RNAi) technique.

Chapter 1:

# Identification of two novel AEP/legumain genes from the hard tick, *Haemaphysalis longicornis*

## 1. Introduction

Proteases (proteolytic enzymes) are ubiquitous enzymes in virtually every biological phenomenon in the living animals. Several proteases in arthropods have been reported to play critical roles in vital body functions including digestion of food materials (16, 21, 96, 110, 111, 135), anti-thrombin effect (76), regulation of the moulting process (69) and synthesis of melanin pigment (78). Among the arthropods, the haematophagous ticks have evolutionarily gained very distinctive biological features especially for blood-feeding and digestion. In ticks, the salivary proteases have been reported to contribute to successful blood-feeding (48, 89, 107). The cysteine and aspartic proteases are believed to contribute to proteolysis in tick midgut (33, 100) and are known to degrade egg yolk protein (94). The proteases from different tick species have been reported to be involved in digesting blood proteins, Hb and BSA (20, 65, 101, 106, 121).

The proteolytic enzymes have also been postulated to play roles in chitin biosynthesis, an essential event during feeding in ticks (74).

Here I describe the isolation of two cDNAs encoding the novel asparaginyl endopeptidases/legumains (HlLgm and HlLgm2) from midgut of *H. longicornis* and identification of these molecules by their sequences, catalytic residues, deduced domain and motif structure, and phylogenetic analyses.

## 2. Materials and methods

### *2.1. Ticks*

The parthenogenetic Okayama strain of *H. longicornis* maintained at the Laboratory of Parasitic Diseases, National Institute of Animal Health (NIAH), Tsukuba, Ibaraki, Japan was bred to obtain its different developmental stages by feeding on rabbits as described previously (50). Briefly, the ears of the rabbits were clipped and the ticks were attached supported by ear bags and a head collar. The ear bags were removed every 24 h and the ticks were collected when engorged or after the indicated period of attachment.

### *2.2. Cloning and sequencing of* H. longicornis *cDNAs encoding HlLgm and HlLgm2*

HlLgm and HlLgm2 were identified from expressed sequence tags (ESTs) constructed from the midgut cDNA libraries of *H. longicornis* as described previously (20). Briefly, the

plasmids containing legumains gene-encoding inserts were extracted using the Qiagen DNA Purification kit (QIAGEN Sciences, Germantown, MA, USA). The nucleotide sequences of the cDNAs were determined by the big dye terminator method on an ABI PRISM 3100 automated sequencer (Applied Biosystems, Foster City, CA, USA). The GENETYX-WIN DNA analysis software system (Software Inc) was used to deduce the amino acid sequences of HlLgm and HlLgm2 and the BLAST Program (8) for alignment was used to compare these sequences with previously reported AEP sequences available in GenBank (14). The putative signal sequences were analyzed using the prediction server SignalP 3.0 at the Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/service/SignalP/) (13). The asparagine-linked glycosylation sites were determined by using NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc). Theoretical molecular weights (MW) and isoelectric points (pI) were determined using PeptideMass (http://us.expasy.org/tools/peptide-mass.html) (153). Phylogenetic tree was generated from homologies of the legumain amino acid sequences from different sources by the neighbour-joining method, and the confidence of the branching order was verified by making 1000 bootstrap replicates using the program CLUSTALW 1.8. The tree was viewed and converted to graphic format with TREEVIEW (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

## 3. Results

### *3.1. Molecular characterization of HlLgm and HlLgm2*

The nucleotide and deduced amino acid sequence (appears in GenBank accession no. AB279705 and accession no. BAF51711, respectively) revealed that the full-length *HlLgm* cDNA consisted of 2,335 base pairs (bp) which included the complete coding region together with 5′ and 3′ non-coding regions. The in-frame start codon is located at nucleotides 21–23 and there is a stop codon at nucleotides 1,347–1,349. The ORF of 1,329 nucleotides extending from position 21 to position 1,349 is predicted to encode 442 amino acid residues with a calculated molecular mass of 48,835 Da and a isoelectric point (pI) of 6.25. The 5′ non-coding sequence contains 20 bp and the 3′ untranslated region comprises 986 bp. The sequence ends with a 90-bp polyadenylation ($A^+$) tail that begins 19 bp downstream from the polyadenylation signal/processing sequence of AATAAA (Fig. 1). The full-length *HlLgm2* cDNA sequence (GenBank accession no. AB353127) is 1,387 bp long and contains the complete coding region along with the 15-bp and 43-bp untranslated sequences at the 5′ and 3′ end, respectively. The ORF consists of 1,329 nucleotides having the in-frame start codon at nucleotides 16–18 and the stop codon at nucleotides 1,342–1,344 and encodes a polypeptide of 442 amino acids with a predicted MW of 49,630 Da and a pI of 7.05. There is a 20-bp long polyadenylation ($A^+$) tail that begins 16-bp downstream from the polyadenylation signal sequence, AATAAA (Fig. 2). Analysis of the N-termini of the polypeptides with the Signal IP V1.1 program (http://www.cbs.dtu.dk/service/SignalP/) revealed that the HlLgm molecule has a signal peptide predicted to be cleaved at $Ala^{22}$–$Ala^{23}$ (Fig. 1) while the predicted signal peptide of HlLgm2 deduced amino acid sequence (GenBank accession no. BAF95090) consists of the first 20 residues and the polypeptide is predicted to be cleaved between $Gly^{20}$ and $Ala^{21}$ (Fig. 2). The HlLgm preprotein has a predicted molecular weight (MW) of 46,705 Da with a pI of 6.25 and the calculated MW of HlLgm2 preprotein is 47,487 Da with a pI of 6.66. There are two potential

*N*-glycosylation sites in the deduced amino acid sequence of HlLgm, at $Asn^{101}$ (NGSN) and $Asn^{398}$ (NLSK) (Fig. 1). In HlLgm2, there are three sites, viz. $Asn^{86}$ (NSTY), $Asn^{101}$ (NGSN) and $Asn^{430}$ (NVTC), where the asparagines (*N*) are predicted to be glycosylated (Fig. 2). The domain structure analysis using the Scan-Prosite Program (http://us.expasy.org/prosite/) revealed that both the deduced HlLgm and HlLgm2 sequences possess a single peptidase C13 family domain with evolutionarily conserved regions and catalytic residues (data not shown). A BLAST (NCBI, National Institute of Animal Health, http://www.ncbi.nlm.nih.gov.BLAST/) analyses of the translation products deduced from the ORFs revealed that HlLgm and HlLgm2 shared high sequence similarity with arthropods, mammals, plants and blood flukes (schistosomes) legumains. GenBank™ analyses showed that the deduced amino acid sequences of HlLgm and HlLgm2 shared the highest similarity (75% identical) with each other. HlLgm and HlLgm2 sequences also shared a very high sequence similarity with that of *Ixodes ricinus* legumain (GenBank™ accession no. AAS94231), 70% and 64% identical, respectively. Alignment with mammalian legumain sequences revealed that the levels of HlLgm and HlLgm2 amino acid homologies were 51% each for human (*Homo sapiens*) legumain (GenBank™ accession no. CAA70989); 52% and 50%, respectively for bovine (*Bos taurus*) legumain (GenBank™ accession no. BAB69947); 51% and 52%, respectively for mouse (*Mus musculus*) legumain (GenBank™ accession no. CAA04439), and 53% and 51%, respectively for rat (*Rattus norvegicus*) legumain (GenBank™ accession no. BAA84750). The identities of *Schistosoma japonicum* (GenBank™ accession no. P42665) hemoglobinase precursor (antigen Sj32), *H. contortus* legumain (GenBank™ accession no. CAJ45481) and *Canavalia ensiformis* (GenBank™ accession no. P49046) legumain precursor (asparaginyl endopeptidase) with the deduced amino

acid sequences for HlLgm and HlLgm2 were 44% and 42%, 43% and 40%, and 40% and 39%, respectively.

The active catalytic residues were identified as $His^{155}$ and $Cys^{196}$ for both HlLgm and HlLgm2 (numbered according to HlLgm) (Fig. 3). Apart from the active site cysteine residues, the position of all other cysteine residues in both the sequences coincides exactly with the position of cysteine residues of other legumain sequences excepting the *S. japonicum* (GenBank™ accession no. P42665) and *C. ensiformis* (GenBank™ accession no. P49046) (Fig. 3), indicating that the position of the cysteine residues is well-conserved in legumains. There is a block of four predominantly hydrophobic residues N-terminal to active site $His^{155}$ and $Cys^{196}$ in both HlLgm and HlLgm2 sequences. Between residues 128–130 (numbered according to HlLgm), an RGD (Agr-Gly-Asp) motif has been identified in mammals and a KGD (Lys-Gly-Asp) motif in schistosomes (26) and it is likely that there is a QGK (Gln-Gly-Lys) motif in both the HlLgm and HlLgm2 sequences. Also the *I. ricinus* legumain possesses the same QGK motif (Fig. 3), indicating that this motif is conserved in tick legumains. Alignment analysis also showed that, in common with human legumain and other legumains of animal, plant and helminth origin, HlLgm and HlLgm2 encode the putative N-terminal prodomain of 13 and 15 residues, respectively and the C-terminal extension of 114 residues for each (Fig. 3). Furthermore, several highly conserved regions, the most important of which are seven-residue-long sequences (52-NYRHQAD-58 and 194-EACESGS-200) and a six-residue-long sequence (80-MYDDIA-85) (numbered according to HlLgm sequence) are also seen in both HlLgm and HlLgm2 sequences. It will be interesting to explore the significance of these highly conserved regions in the legumains structures and their functional involvement.

### 3.2. *Phylogenetic analysis of known legumains*

A phylogenetic tree was constructed using amino acid sequences of legumains from different sources by the neighbour-joining method, and the confidence of the branching order was verified by making 1000 bootstrap replicates with the CLUSTALW program (Fig. 4). The neighbour-joined trees revealed that HlLgm and HlLgm2 grouped together with the arthropod and mammalian orthologues and interestingly represented a separate group from haematophagous helminth legumains. Furthermore, within the mammalian-arthropod subgroup, HlLgm and HlLgm2 are most closely related to the hard tick, *I. ricinus* legumain.

## 4. Discussion

Asparaginyl endopeptidase/legumain has been reported from diverse sources, such as plants (62), mammals (26, 37, 154) and parasites (38). However, little is known about legumains and their biological functions in the haematophagous ticks. In this chapter the isolation and identification of cDNA clones that encode legumains (HlLgm and HlLgm2) from the midgut of *H. longicornis* have been discussed.

Sequence and phylogenetic tree analyses revealed that HlLgm and HlLgm2 are homologous to arthropod, mammalian, plant and helminth legumains and belong to the arthropod-mammalian legumain subfamily. Legumain is a cysteine endopeptidase which belongs to the peptidase family C13. The catalytic residues of all known cysteine peptidases are a dyad of cysteine and histidine. In legumain, this dyad occurs in the motif His-Gly-spacer-Ala-Cys in the amino acid sequence (29). The HlLgm and HlLgm2 sequences conserve the same

catalytic motif. The conserved known catalytic residues ($His^{155}$ and $Cys^{196}$) and other cysteine residues in asparaginyl endopeptidases (29, 154) are also evolutionarily well conserved in HlLgm and HlLgm2 sequences. The presence of conserved block of four predominantly hydrophobic residues N-terminal to active site $His^{155}$ and $Cys^{196}$ is consistent with other proteinases of this class (29). However, in the motif N-terminal to active site histidine ($His^{155}$), one hydrophobic residue has been replaced by the hydrophilic amino acid, $Asn^{151}$ in both HlLgm and HlLgm2 sequences. There are two and three potential *N*-linked glycosylation sites in HlLgm and HlLgm2 amino acid sequences, respectively. Legumains from other sources such as the mammalian legumain (26) and several plant legumains (7, 83) have been reported to be glycosylated. These characteristic findings suggest that HlLgm and HlLgm2 are cysteine peptidases of the legumain family C13 (29).

The proposed QGK (Gln-Gly-Lys) motif in both HlLgm and HlLgm2 corresponds to the RGD (Arg-Gly-Asp) and KGD (Lys-Gly-Asp) motifs in mammalian and schistosome legumains (26), respectively. According to Chen et al. (26), the interaction of the RGD motif of mammalian legumain with cell membrane components may help retain mammalian legumain in the lysosomal acidic environment of cells. In cell-adhesive proteins such as fibronectin (43,123) and disintegrins (56) the RGD sequences are responsible for the binding of the proteins to their cell-surface receptors. It is pertinent to speculate that the QGK motif might have important roles for binding of legumains to the cell receptors and help them take part in intracellular haemoglobin digestion process. However, the precise function of the QGK motif in tick legumains is yet to be determined. Both the sequences were shown to possess N-terminal propeptide and C-terminal extension. The asparaginyl endopeptidases mature to the active form by removing a short N-terminal and a long C-terminal propeptide through a cleavage after

aspartic and asparagine residues, respectively (93). The N-terminal cleavage sites, $Asp^{25}$ for human legumain is missing in the HlLgm2 sequence and both HlLgm and HlLgm2 sequences are lacking the C-terminal cleavage site, $Asn^{323}$ for human legumain. It is speculated that the activating N-terminal cleavage site for HlLgm2 is located between the nearest $Asp^{51}$ and $Asn^{52}$. The theoretical C-terminal cleavage sites predicted for HlLgm and HlLgm2 C-terminal extension using the server 'NetAEP: Predicting Asparaginyl Endopeptidase Specificity' (http://theory.bio.uu.nl/kesmir/AEP/) were at $Asn^{365}$ and $Asn^{364\text{-}365}$, respectively. The C-terminal autocleavage site for *I. ricinus* legumain (IrAE) was reported to be $Asn^{345}$ (131).

Two novel genes, *HlLgm* and *HlLgm2* have been identified for the first time from the midgut of the haematophagous tick *H. longicornis*. Results obtained from sequences analyses and homology search, and also the phylogenetic belongings of HlLgm and HlLgm2 suggest that these novel genes might encode the functional asparaginyl endopeptidases.

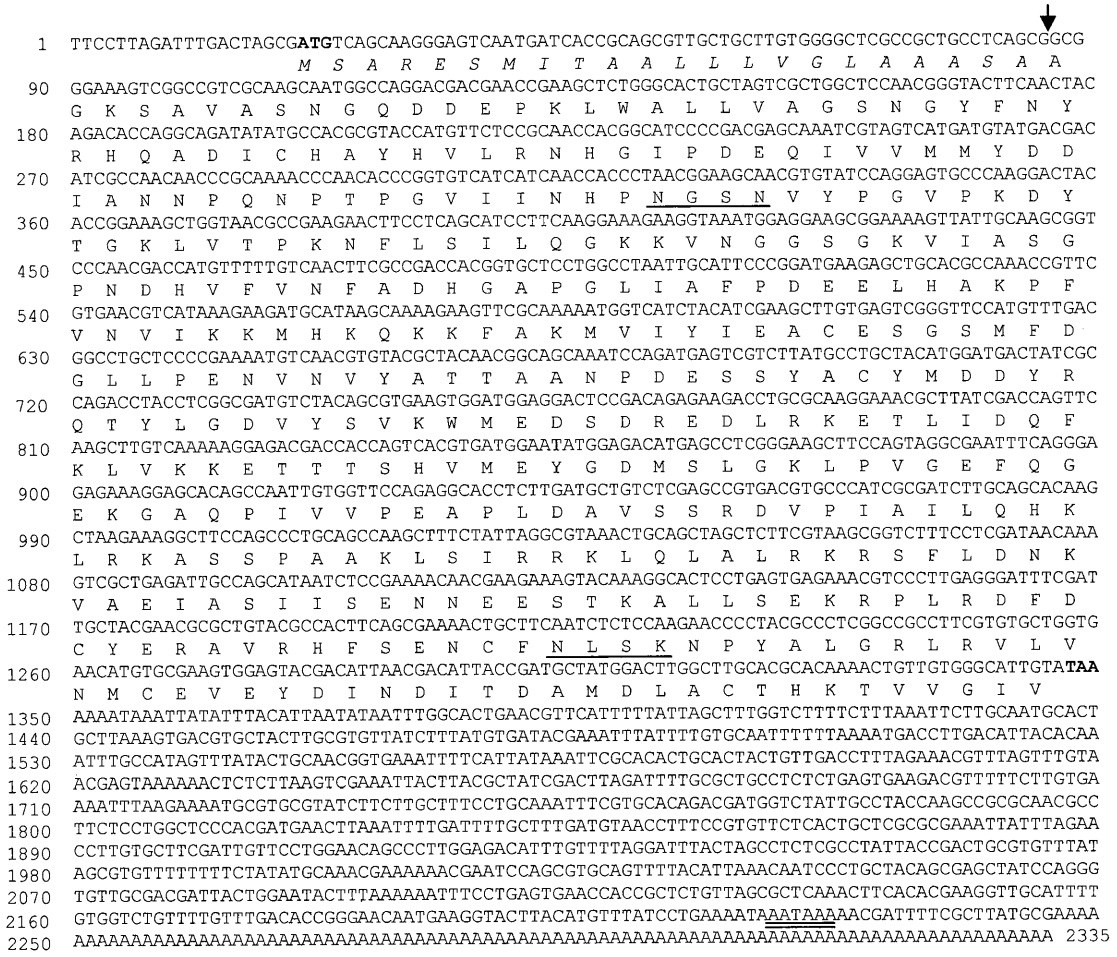


**Fig. 1. The nucleotide and deduced amino acid sequence of *H. longicornis* legumain (HlLgm) full-length cDNA**

The initial codon ATG and termination codon TAA are shown in bold. The eukaryotic consensus polyadenylation signal AATAAA is double underlined. The putative signal peptide of the deduced amino acid sequence is indicated by italicized letters. The *N*-linked glycosylation sites are underlined. The nucleotide and amino acid orders are indicated by numbers put on the left and right, respectively.

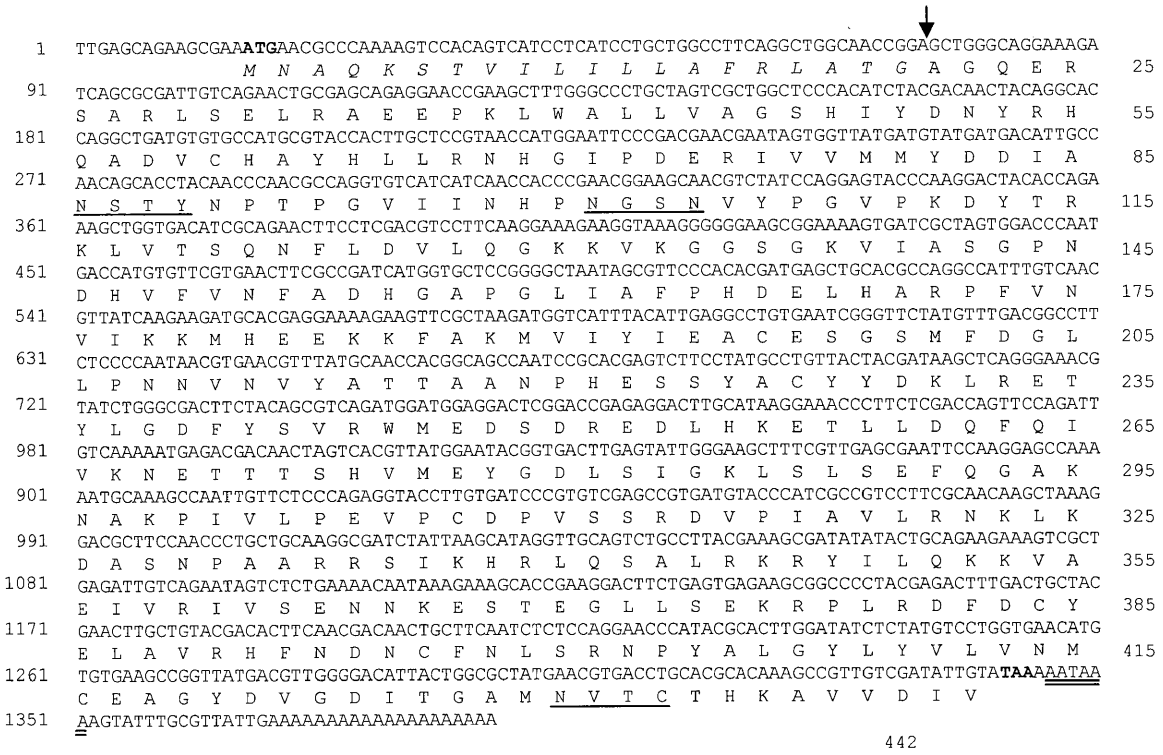


**Fig. 2. The nucleotide sequence of full length second legumain cDNA from *H. longicornis* (HlLgm2) and its deduced amino acid sequence**

The start codon ATG and stop codon TAA are shown in bold. The polyadenylation signal AATAAA is double underlined. The putative signal peptide of the deduced amino acid sequence is indicated italicized letters. The *N*-linked glycosylation sites are underlined. The nucleotide and amino acid orders are indicated by numbers put on the left and right, respectively.

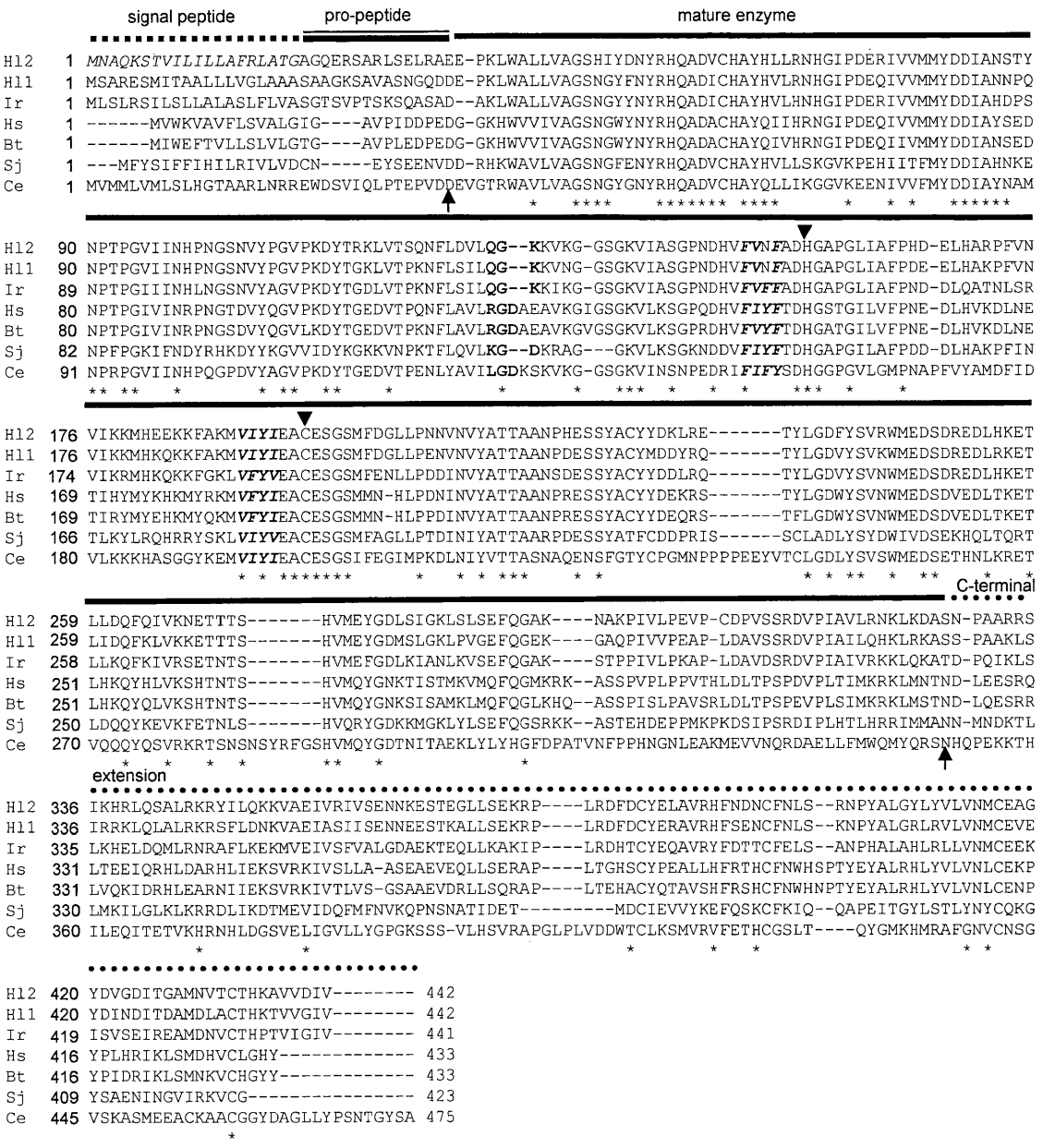


**Fig. 3. Alignment of amino acid sequence of *H. longicornis* legumains with those of other homologous legumain molecules from various sources**

The identical residues are marked with asterisks. Gaps, marked by hyphens, were inserted for better alignment. The square dots indicate signal peptides, double lines indicate the N-terminal pro-peptide, the bold line indicates the mature enzyme and the round dots indicate the C-terminal extension. Arrowheads indicate the conserved His and Cys residues forming the catalytic dyad. QGK and the corresponding motifs are shown in bold and the blocks of four hydrophobic residues N-terminal to the catalytic His and Cys residues are shown in bold and italics. Arrows indicate the cleavage sites of the N- and C-terminal pro-peptides experimentally determined for human legumain. Hl2 (HlLgm2, GenBank accession no. AB353127), Hl1 (HlLgm, BAF51711), Ir (*I. ricinus*, AAS94231), HS (*H. sapiens*, CAA70989), Bt (*B. taurus*, BAB69947), Sj (*S. japonicum*, P42665) and Ce (*C. ensiformis*, P49046).

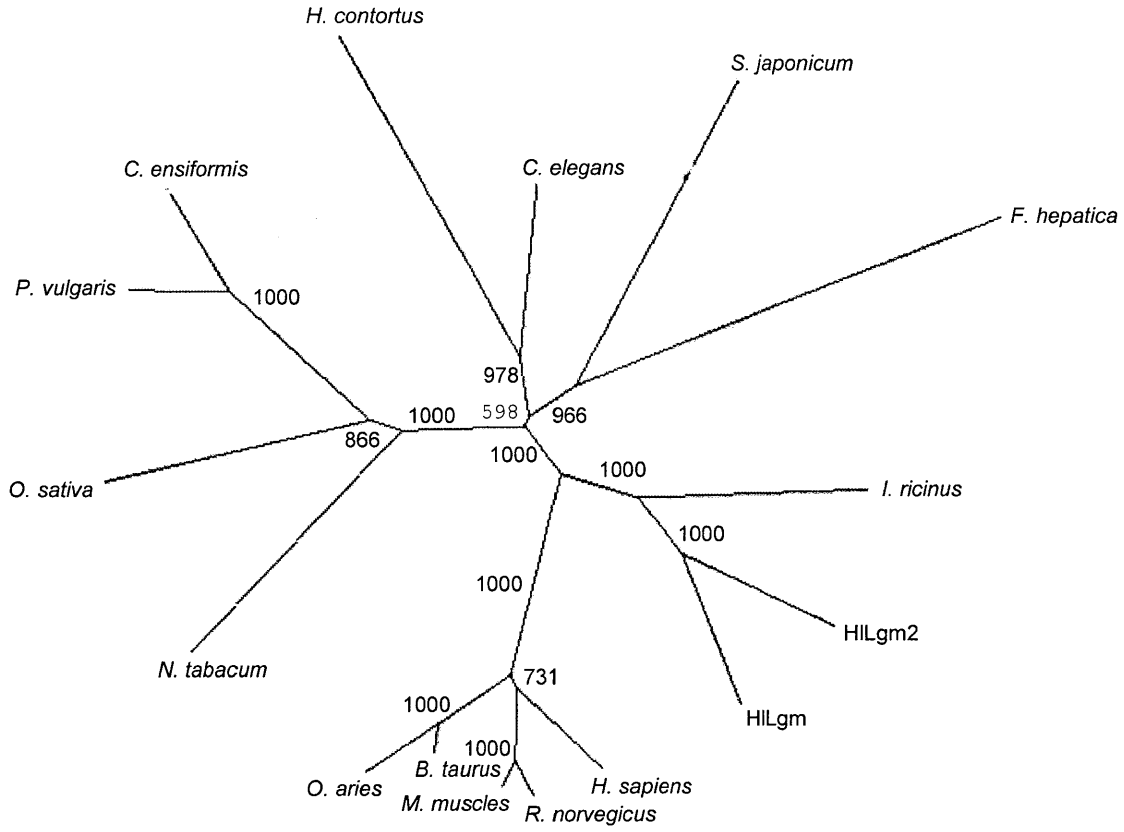


**Fig. 4. Phylogenetic tree of the legumains from *Haemaphysalis longicornis* and other sources**

The sequences shown are those from (GenBank accession numbers are indicated in parenthesis): HlLgm (*H. longicornis* first legumain, BAF51711), HlLgm2 (*H. longicornis* second legumain, BAF95090) *I. ricinus* (AAS94231), *H. sapiens* (CAA70989), *R. norvegicus* (BAA84750), *M. musculus* (CAA04439), *B. taurus* (BAB69947), *Ovis aries* (ABB02307), *Nicotina tabacum* (CAB42650), *Oryza sativa* (BAA84650), *Phaseolus vulgaris* (CAB17079), *C. ensiformis* (P49046). *H. contortus* (CAJ45481), *Caenorhabditis elegans* (AAF21773), *S. japonicum* (P42665), *Fasciola hepatica* (CAC85636). The bar indicates the number of substitutions per site. Unrooted neighbour-joining trees were generated from homologies of legumain sequences and the confidence of the branching order was verified by making 1000 bootstrap replicates using the CLUSTALW program. The tree was viewed and converted to graphic format with TREEVIEW.

Chapter 2:

# Molecular characterization of endogenous HlLgm and HlLgm2 protein in *H. longicornis* and their expression profiles during blood-feeding process

## 1. Introduction

Chapter 1 describes the isolation and identification of two novel genes encoding the asparaginyl endopeptidases/legumains, HlLgm and HlLgm2 from the ESTs constructed from the midgut cDNA libraries of *H. longicornis*. The sequence analyses revealed that both HlLgm and HlLgm2 nucleotide and deduced amino acid sequences contain a single C13 family catalytic domain and other structural properties of legumain family. Blood-feeding is obligatory for every active developmental stage (viz. larva, nymph and adult) of the ticks and it is very likely that one gene might be expressed in different organs/tissues in accordance with its biological relationship. Until recently, legumains have been identified in different tissues/organs of plants (62, 63, 83, 128), animals (26, 27, 37, 154), blood sucking helminths (38, 116), protozoa (92), and in arthropods (131). To speculate the biological roles of legumains in ticks, the expression profiles of legumain transcripts (*HlLgm* and *HlLgm2*) during blood-feeding process in larvae, nymphs

and adults and tissues of partially fed (72 h) adult *H. longicornis* were investigated. The expression and localization of translated endogenous legumains (HlLgm and HlLgm2) in unfed and partially fed larvae, nymphs and adult ticks have also been discussed in this chapter.

## 2. Materials and methods

### *2.1. Experimental animals*

All animals employed in this study were acclimatized to the experimental conditions for 2 weeks prior to the experiment. Animal experiments at NIAH were conducted in accordance with the protocols approved by the NIAH Animal Care and Use Committee (Approval nos. 441, 508, 578).

### *2.2. Detection of* HlLgm *and* HlLgm2 *transcripts in larvae, nymphs and adult* H. longicornis *during blood-feeding*

To detect developmental stages- and blood-feeding phases-specific *HlLgm* and *HlLgm2* mRNA expression profile, the whole unfed, partially-fed (24 h, 48 h and 72 h) and engorged larvae and nymphs and the midgut tissues from unfed, partially-fed (24 h, 48 h, 72 h and 96 h) and engorged adult ticks were used for total RNA extraction. For collection of midgut tissues from adults, the ticks were washed with gentle agitation in 70% ethanol and rinsed with PBS prior to dissection under a microscope. Immediately after collection, the midgut tissues were submerged in RNA*later* RNA Stabilization Reagent (QIAGEN Sciences, German Town, MA,

USA). The total RNA from the whole larvae and nymphs and from the midgut samples of adults was extracted by using an RNeasy Mini Kit (QIAGEN) as described by the manufacturer and either used immediately or stored at –80 °C until used. Reverse transcription (RT)-PCR was performed with a template of 500 ng of total RNA for each 10 µl reaction using a Takara RNA PCR Kit (AMV) Ver.3.0 (Takara, Shiga, Japan) and following the protocol provided by the manufacturer. PCRs were carried out using 500 ng of each cDNA synthesis product and oligonucleotides specific for either HlLgm (forward primer 5′-CGACGAGCAAATCGTAGTCA-3′ and reverse primer 5′-ACTTTTCCGCTTCC TCCATT-3′) or HlLgm2 (forward primer 5′-CCTTCGCAACAAGCTAAAGG-3′ and the reverse primer 5′-TCAGAAG TCCTTCGGTGCTT-3′) or oligonucleotides specific for control cDNA encoding β-actin in a final volume of 20 µl. PCRs were performed for 5 min at 95 °C and for 35 cycles of 30 s at 95 °C, 30 s at 55 °C, and 1.5 min at 72 °C followed by elongation at 72 °C for 5 min. The PCR products were subjected to electrophoresis in a 1% agarose gel in tris-acetic acid-EDTA (TAE) buffer to check the size of the amplified fragments comparing with DNA molecular weight marker (100 bp DNA ladder) (Promega, Madison, WI, USA). The quantitative RT-PCR was performed in a LightCycler 1.5 instrument (Roche Instrument Center AG, Roikreuz, Switzerland) using LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH, Nonnenwald, Germany). The reaction mixture of 20 µl contained 4 mM $MgCl_2$, 0.5 µM each primer (forward and reverse as described above), 2 µl of LightCycler FastStart DNA Master SYBR Green I and 2 µl of the single-stranded DNA template. The data obtained were analyzed by using LightCycler Software Version 3.5.

### 2.3. *Detection of tissue-specific HlLgm and HlLgm2 mRNA expression in adult* H. longicornis

To detect the tissue specific expression of *HlLgm* and *HlLgm2* mRNA, individual organs viz. midguts, salivary glands, ovaries, cuticles, tracheae, Malpighian tubules and synganglia were collected from 72 h fed adult ticks, stored in RNA*later* RNA Stabilization Reagent (QIAGEN) and were subjected to RT-PCR as described above.

### *2.4. Generation of mouse polyclonal antibodies*

Polyclonal antibodies against recombinant HlLgm (rHlLgm) and recombinant HlLgm2 (rHlLgm2) were generated separately in female BALB/c mice (Japan SLC, Inc., Hamamatsu, Japan) by subcutaneous injection of 50 μg of rHlLgm or rHlLgm2 emulsified with Freund's complete adjuvant (DIFCO, Detroit, MI, USA) followed by another two booster immunizations 2 weeks apart by the same route emulsifying with Freund's incomplete adjuvant (DIFCO). The mice were bled 1 week after the last immunization. The mouse anti-rHlLgm and anti-rHlLgm2 sera were stored separately at –20 °C until used.

### *2.5. Preparation of tick antigens and SDS–PAGE analysis*

The extracts from unfed and partially fed (48 h) adults, nymphs and larvae were prepared as described previously (156). Briefly, the ticks were homogenized under liquid nitrogen followed by sonication in phosphate buffered saline (PBS) containing a protease inhibitor cocktail (Roche Biochemicals, San Diego, CA, USA) on ice and centrifugation at 26,300 × *g* for 30 min at 4 °C. The supernatants were collected and stored at –80 °C until used. Electrophoresis was performed on sodium dodecyl sulfate polyacrylamide gel electrophoresis

(SDS–PAGE) gels under reducing conditions. The proteins were either stained with Coomasie Brilliant Blue (CBB) or transferred onto nitrocellulose membranes for immunoblotting.

### *2.6. Immunoblot analysis*

Immunoblot analysis was performed as described previously (156). Briefly, tick extracts separated by SDS–PAGE were transferred onto nitrocellulose membranes, and the membranes were incubated for 1 h with 5% skim milk. For the detection of endogenous HlLgm and HlLgm2, mouse anti-rHlLgm serum and mouse anti-rHlLgm2 serum, respectively, were used separately at a dilution of 1:250. The membranes were washed with Tris buffered saline–Tween (TBS–T) and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) (ZYMED, San Francisco, CA, USA) as a secondary antibody. After the membranes were washed with TBS–T, the proteins bound to the secondary antibody were visualized with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (BCIP/NBT, Promega).

### *2.7. Cross-reactivity check of the polyclonal antibodies generated against the legumain isoforms*

The amino acid sequence similarity of both the legumain isoforms is apparent (75% identical). It is very likely that the polyclonal antibodies generated against one recombinant legumain would react with the other recombinant legumain. To show the specificity of the antibodies (mouse anti-rHlLgm and mouse anti-rHlLgm2) against the recombinant isoforms (rHlLgm and rHlLgm2), SDS–PAGE and immunoblot analyses were performed using 40 µg

each of rHlLgm and rHlLgm2 one next to another and probed with mouse anti-rHlLgm or mouse anti-rHlLgm2 at a dilution of 1:250 separately.

### *2.8. Immunohistochemistry*

The immunohistochemical study was performed as described previously (155). Briefly, unfed and partially fed (48 h) adult ticks were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) overnight, embedded in paraffin, and used to make thin flat sections. The sections on glass slides were blocked for 1 h in 1% $H_2O_2$ in PBS containing 10% ethanol to inactivate endogenous peroxidase. The slides were blocked in PBS containing 10% goat serum (Wako, Osaka, Japan) for 1 h at room temperature and then incubated overnight at 4 °C only with mouse anti-rHlLgm serum diluted 1:600 in pBS/*E. coli* lysate as it was observed that polyclonal antibodies generated against one recombinant legumain reacted notably with both legumain isoforms. Subsequently the slides were rinsed thoroughly with PBS and reacted with peroxidase-labeled anti-mouse IgG secondary antibody and the substrate 3′,3′-diaminobenzidine tetrahydrochloride (Fast™ DAB set; Sigma, St. Luois, MO, USA). After colour development, the slides were dehydrated in a graded series of alcohol, cleared in xylene and then covered with cover slips and examined under a microscope (DM 4000B, Leica).

### *2.9. Immunofluorescent staining*

To visualize the intracellular localization of legumains, the midgut cells from 72 h fed adult ticks were prepared by teasing freshly collected midguts through a stainless steel mesh in

PBS containing complete proteinase inhibitors. The cells were washed three times, 10 min each time in PBS at 1,700 × g at room temperature to remove cell debris and host's erythrocytes and then attached to a glass slide using the Shandon Cytospin® (Thermo Electron, Waltham, MA, USA). The cells were fixed with 4% paraformaldehyde in PBS (0.1 M, pH 7.4) for 20 min at room temperature and permeabilized in PBS containing 0.1% Triton X-100 for 20 min at room temperature. After the slides were washed with PBS and blocked with 10% goat serum (MP Biomedicals, Irvine, CA, USA) for 30 min at room temperature, the slides were incubated for 1 h at room temperature with mouse anti-rHlLgm2 serum (1:200) only. The cells were washed three times with PBS, and then reacted with green fluorescence-labeled secondary antibody (Alexa Fluor®488 goat anti-mouse IgG (H+L); Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature. The cells were washed thoroughly with PBS and the slides were mounted with VECTASHIELD® mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA), covered with cover glasses and then photographed with a fluorescence microscope (Leica Microsystems, Wetzlar, Germany) using appropriate filter sets. Images were collected by using Leica FW4000 software.

### *2.10. Subcellular localization of endogenous legumains in* H. longicornis *midgut epithelial cells*

The midgut tissues were dissected from the partially fed (72 h) adult *H. longicornis* under a microscope and disrupted in PBS. The tissues were then subjected to stepwise extraction of subcellular fractions using a ProteoExtract® Subcellular Proteome Extraction Kit following the manufacturer's instructions (Calbiochem, EMD Biosciences, Inc., La Jolla, CA, USA). The

proteins were separated by SDS–PAGE and blotted onto nitrocellulose membranes. The membranes were incubated with mouse anti-rHlLgm serum (1:250) to determine the subcellular localization of endogenous legumains in midgut tissues.

## 3. Results

### *3.1. Expression of* HlLgm *and* HlLgm2 *genes and their kinetics in developmental stages of* H. longicornis *during different phases of feeding*

RT-PCR and quantitative RT-PCR were performed with total RNA extracted from the unfed, partially fed and engorged larvae, nymphs and adult *H. longicornis* using the specific primer sets for the *HlLgm* and *HlLgm2* genes. RT-PCR products were subsequently electrophoresed in agarose gels to analyze the expression patterns of the two paralogues in *H. longicornis* during different phases of blood-feeding. Expression of *HlLgm* and *HlLgm2* transcripts consistent with their expected sizes was detected in unfed, partially fed and engorged ticks of all developmental stages, and was further up-regulated by the blood-feeding process (Fig. 5A). The kinetics of mRNA expression of both *HlLgm* and *HlLgm2* as determined by quantitative RT-PCR also clearly showed the up-regulation of their expression during specific phases of feeding (Fig. 5B). Both transcripts were expressed most strongly at 48 h of feeding in larvae and nymphs and at 72 h of feeding in adults and thereafter their expression declined sharply. It was noted that *HlLgm2* transcript expression level was lower compared with that of *HlLgm* during different phases of blood-feeding in all developmental stages. More interestingly, unlike *HlLgm*, *HlLgm2* gene expression became completely undetectable in engorged adult ticks

(Fig. 5A, B) suggesting that these two paralogues are involved in blood-protein digestion during different phases of blood-feeding especially in adult ticks. RT-PCR of the total RNA extracted from different organs revealed that both the legumain genes were expressed specifically in the midgut tissues (Fig. 6).

### *3.2. Up-regulation of endogenous legumains by the blood-feeding process*

The expression levels of endogenous HlLgm and HlLgm2 at various developmental stages as well as in unfed and partially fed (48 h) *H. longicornis* were examined by immunoblot analysis. SDS–PAGE and immunoblot analyses using antigens from unfed and partially fed (48 h) ticks and mouse anti-rHlLgm and anti-rHlLgm2 sera revealed that endogenous HlLgm and HlLgm2 of apparent molecular mass of 38 kDa were expressed in all developmental stages and their expression was augmented in partially fed larvae, nymphs and adults (Fig. 7A, B). Interestingly, the polyclonal antibodies generated against one recombinant protein reacted notably with both the isoforms (data not shown).

### *3.3. Localization of endogenous legumains*

Immunohistochemical analysis performed on unfed and 48 h fed adult tick sections using mouse anti-rHlLgm antibody showed that endogenous legumains were intensively localized in the midgut epithelial cells (Fig. 8A). No antibody reactivity, however, was observed in other tissues, including salivary glands, cuticle, trachea, muscles, fat bodies or the genital organs of the ticks, indicating the midgut-specific expression of the endogenous legumains (HlLgm and

HlLgm2). Notably, more intense reaction was observed in 48 h fed tick sections than in their unfed counter parts. Furthermore, there was no reactivity either in the internal or external tissues when the sections were reacted with preimmune mouse serum. Immunofluorescence microscopy of the midgut epithelial cells from 72 h fed adult *H. longicornis* with mouse anti-rHlLgm2 revealed a strong fluorescence reaction in some digestive vacuoles in the epithelial cells. No reactivity, however, was detected in cells treated with preimmune mouse serum (Fig. 8B).

### *3.4. Subcellular localization of HlLgm in tick midgut tissues*

The subcellular fractions of midgut tissues obtained from 72 h fed adult female *H. longicornis* were prepared and analyzed by immunoblotting using mouse anti-rHlLgm serum. An immunoreactive band of 38 kDa was detected in the fraction of membranes and membrane organelles (Fig. 9). These results indicate that *H. longicornis* legumains are membrane-bound proteins.

## 4. Discussion

*HlLgm-* and *HlLgm2-* specific mRNAs were well expressed throughout the *H. longicornis* developmental stages (larvae, nymphs and adults) while feeding on the host and in unfed conditions as revealed by RT-PCR and quantitative RT-PCR analyses. The endogenous HlLgm and HlLgm2 expression was detected in soluble fractions of whole larvae, nymphs and adult homogenates by immunoblotting. RT-PCR and immunohistochemistry analyses showed

that *HlLgm* and *HlLgm2* transcripts and endogenous proteins were strictly localized in the midgut of ticks. Furthermore, the legumains specific transcripts and the endogenous proteins expressions were up-regulated by blood-feeding process. The midgut specificity and the expression profiles of legumain transcripts during blood-feeding process clearly suggest some critical relationships between legumains and blood-meal digestion. The legumain transcript from the hard tick *I. ricinus* (IrAE) has also been reported to be expressed solely in the midgut tissues (131). In the parasitic nematodes, *H. contortus* (116) and *S. mansoni* (45), legumains were expressed in the gut epithelia and were reported to contribute to Hb digestion, perhaps directly and/or by *trans*-processing through cleavage of the pre-pro-region of other gut-expressed peptidase zymogens (Cathepsin L, B, C and D) involved in the complete breakdown of Hb (34, 41, 124). However, it is difficult to explain the puzzling fact of the lower level of *HlLgm2* mRNA expression than that of *HlLgm* during various phases of blood-feeding in all developmental stages. Furthermore, the reasons for complete absence of detectable *HlLgm2* transcript in engorged adult ticks, as revealed by both RT-PCR and quantitative RT-PCR analyses are unclear. In ixodid ticks, blood-meal digestion appears to occur in the midgut cells in three distinct phases: 1) a continuous digestion phase, also known as the preparatory phase during the slow feeding period (1-3 days post-attachment). There is an increase in the number of the digestive cells in the midgut epithelium which become much more active as digestion proceeds; 2) reduced digestion in the rapid feeding period for a further 2 days due to a decrease in the number of active digestive cells and the fact that new digestive cells with less activity replace the sloughed-off, spent digestive cells; 3) a second phase of continuous digestion throughout the post-feeding period of pre-oviposition and oviposition caused by a further increase in the number of active digestive cells in the midgut epithelium (Tarnowski and Coons,

(136) for *Dermacentor variabilis* adults; Agyei and Runham, (3) for *Boophilus microplus* and *Rhipicephalus appendiculatus* adults; Koh et al., (88) for *H. longicornis* nymphs). In unfed adults, the midgut is lined with empty digestive cells, and sometimes secretory cells with lysosomal activity and some stem cells (3). According to Bogin and Hadani (18), the proteolytic enzymes in the gut of adult female ixodid ticks show very little protease activity when feeding is initiated, and this activity gradually increases as feeding progresses, reaching its peak 3 days after attachment and, in fully engorged females, the protease activity is again very low, only 10% of the peak activity (5). In *H. longicornis* nymphs, the growth phase lasts for 1.5 to 2.5 days when the blood digestion is rapid (88). Feeding behaviours of larvae are almost similar to those of nymphs. The evidences obtained in the present study suggest that *H. longicornis* legumains are subcellularly localized in the membrane and membrane organelles of midgut epithelial cells. Thus, the intense expression of *HlLgm*- and *HlLgm2*-specific mRNA in midgut cells of 48 h-fed larvae and nymphs and 72 h-fed adult ticks followed by their dramatic decrease in 72 h- and 96 h-fed larvae and nymphs, and adults, respectively, coincides with the presence of the active digestive cells in the midgut and the proteolytic events in ticks of different stages during the blood-feeding process. Additionally, intracellular localization of legumains conceivably suggests their involvement in Hb digestion cascade in midgut cells. Together, these findings support our speculation that tick legumains contribute to the process of digestion of the host's blood components. Moreover, HlLgm and HlLgm2 might be involved as essential mediators of many other physiological processes/functions associated with survival, development and reproduction of *H. longicornis*. Also, based on the time-course of their expression, it is reasonable to speculate that of the two legumains, only the HlLgm enzyme participates in the blood-meal digestion and other physiological processes in the engorged ticks.

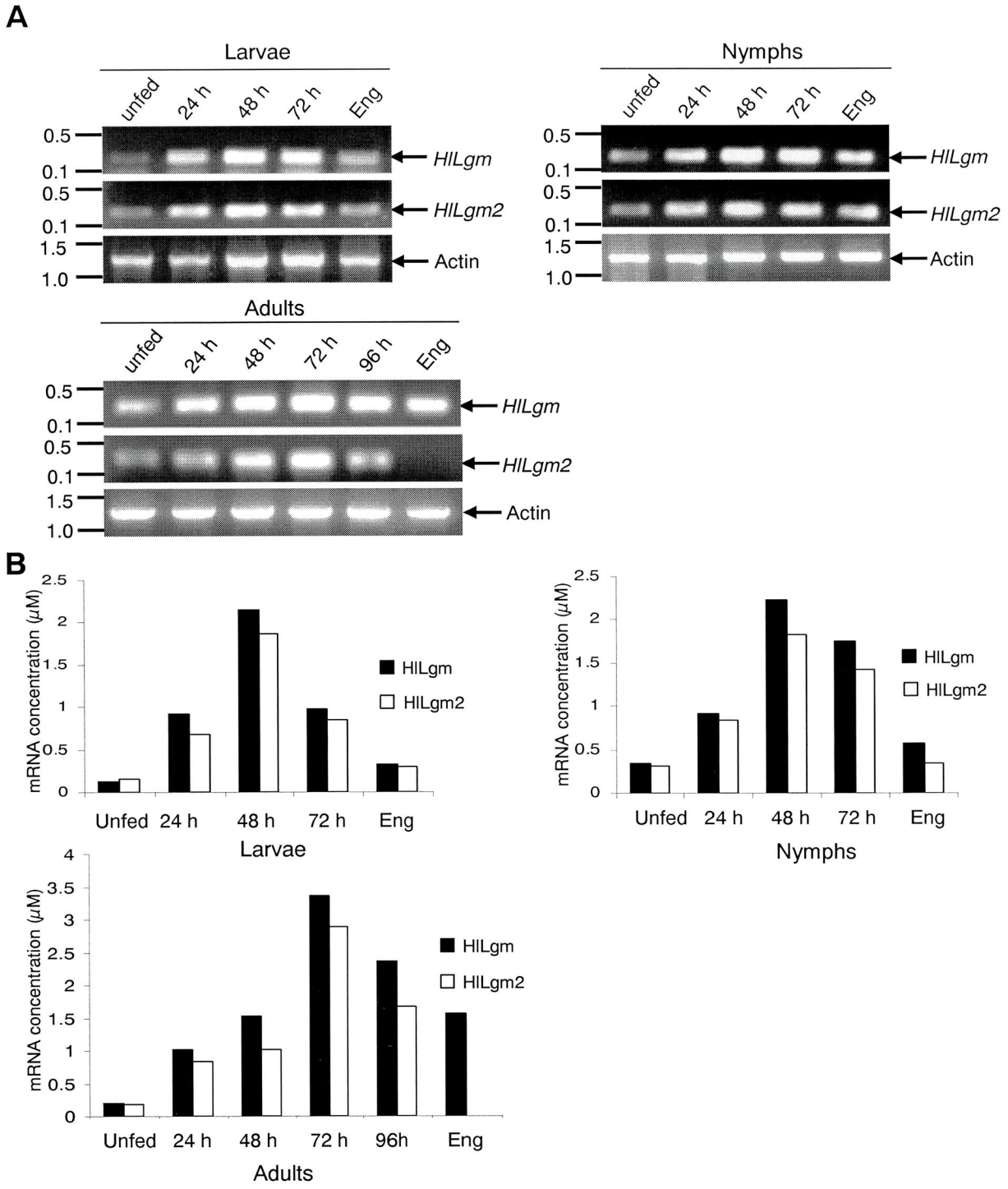


**Fig. 5. Expression profile of *HlLgm* and *HlLgm2* genes in different developmental and feeding stages of *H. longicornis***

(A) RT-PCR analysis. The whole body homogenates of larvae, nymphs, and midgut homogenates of adults were subjected to RT-PCR analysis using primers specific for *HlLgm* and *HlLgm2*. Arrows indicate the level of gene expression. Actin is shown as an internal control. Eng, engorged. (B) Quantitative RT-PCR was performed using the same total RNA and the same primers specific for *HlLgm* and *HlLgm2* as above.

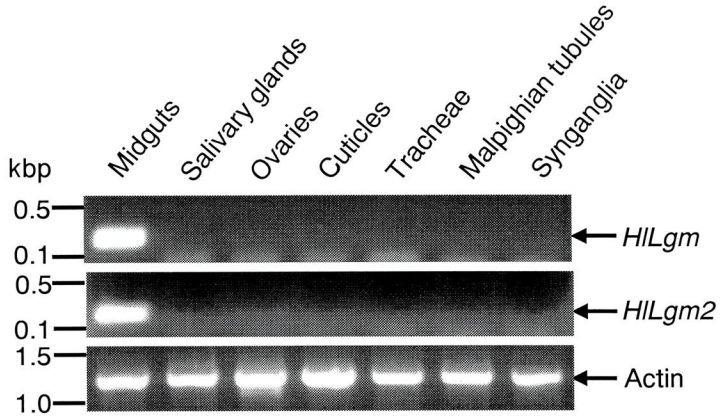


**Fig. 6. Organs-specific expression profile of *HlLgm* and *HlLgm2* in adult *H. longicornis***

The tissues were dissected from three partially fed (72 h) adult ticks and subjected to RT-PCR analysis. *HlLgm* and *HlLgm2* transcripts were expressed only in the midgut tissues (Arrows). *H. longicornis ß-actin* mRNA was used as an internal control.

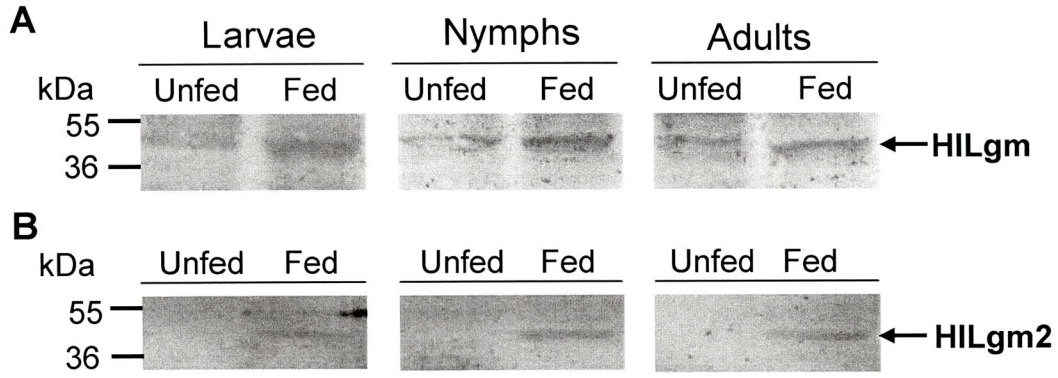


**Fig. 7. Up-regulation of endogenous legumains by the blood-feeding process**

Equal amount of tick antigens (40 μg) from unfed and partially fed (48 h) larvae, nymphs and adult *H. longicornis* were subjected to immunoblot analysis. Soluble antigens of ticks separated by 8% SDS–PAGE were transferred onto nitrocellulose membranes. The membranes were reacted separately with mouse anti-rHlLgm serum and mouse anti-rHlLgm2 serum at a dilution of 1:250.

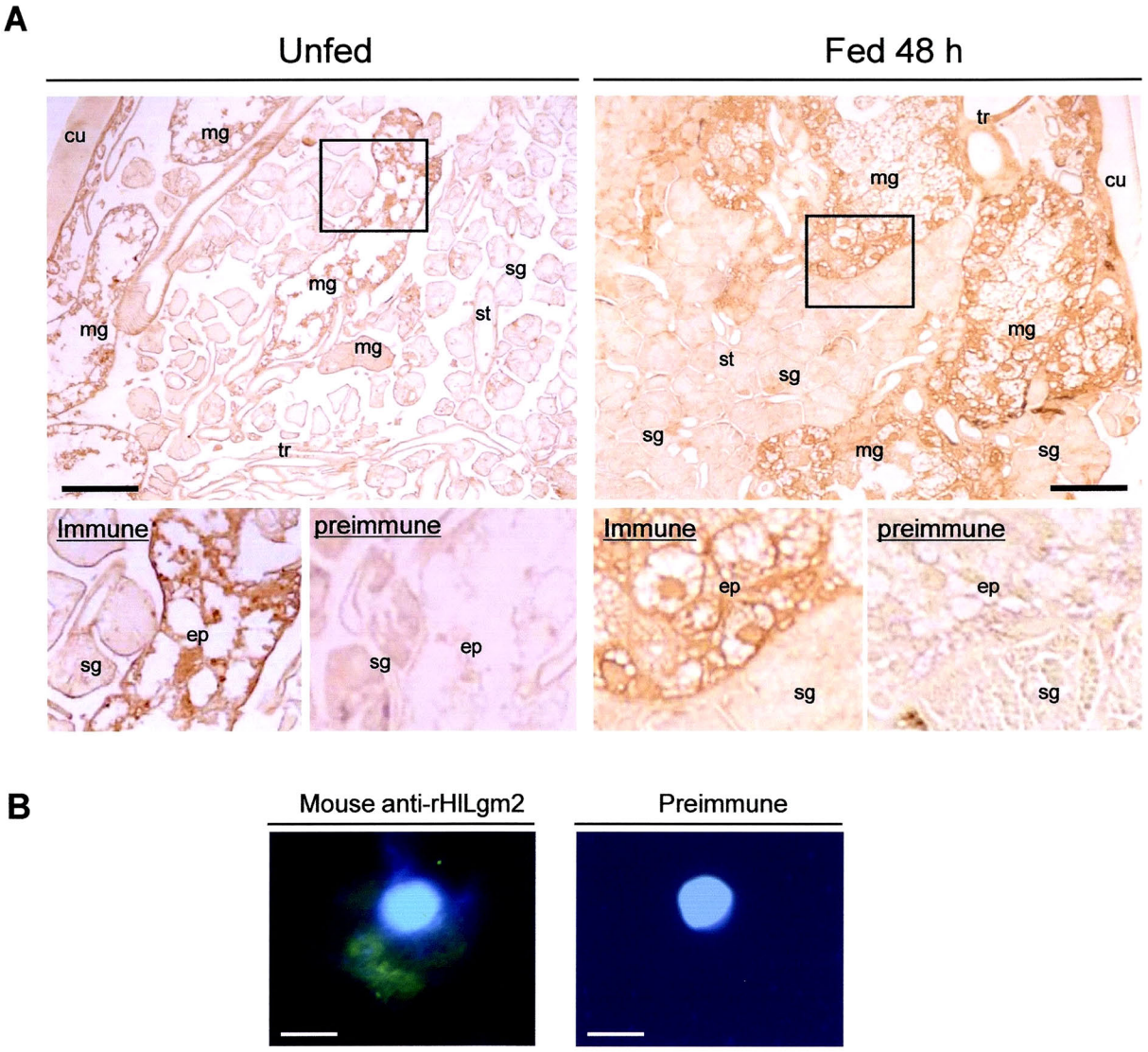


**Fig. 8. Localization of endogenous legumains in adult *H. longicornis***

(A). Immunohistochemical analysis. Unfed and partially fed (72 h) adult ticks were fixed in paraformaldehyde and embedded in paraffin as described in Materials and methods section. The flat sections of the ticks were exposed to either mouse anti-rHlLgm2 serum or preimmune serum. cu, cuticle; mg, midgut; ep, midgut epithelial cells; sg, salivary gland; st, salivary duct; tr, trachea. Areas marked by squares are shown at higher magnification. Scale bars indicate 50 μm. (B) Immunofluorescent staining of midgut epithelial cells to detect intracellular localization of endogenous legumains. The midgut epithelial cells were collected from 72 h fed adult *H. longicornis*. The cells were fixed in paraformaldehyde and permeabilized with Triton X-100. Then the cells were incubated with mouse anti-rHlLgm2 serum (1:250) and visualized with Alexa Fluor® 488 (green). The nuclei were stained with DAPI (blue). Scale bar indicates 20 μm.

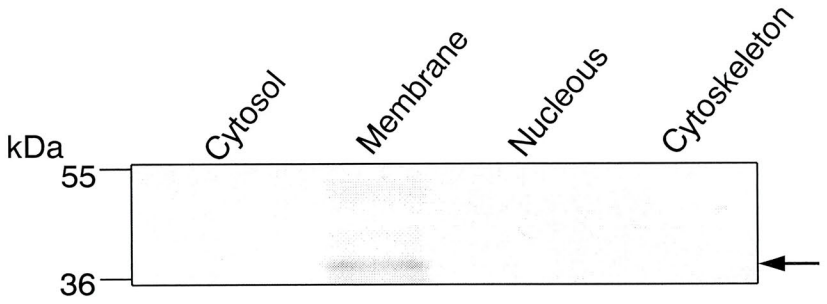


**Fig. 9. Subcellular localization of endogenous legumains in the midgut of adult *H. longicornis***

The midgut tissues from partially fed (72 h) adult *H. longicornis* ticks were dissected and the subcellular fractions were prepared using a ProteoExtract® Subcellular Proteome Extraction Kit. The proteins were separated by 8% SDS–PAGE, and blotted onto nitrocellulose membranes, and the membranes were treated with mouse anti-rHlLgm serum (1:250). The endogenous legumains were detected in the membrane and membrane organelle fraction (arrow) of the midgut tissues

Chapter 3:

# Enzymatic characterization of recombinant HlLgm and recombinant HlLgm2

## 1. Introduction

The endogenous HlLgm and HlLgm2 were expressed strictly in the midgut epithelial cells and their expression was up-regulated by blood-feeding process as it has been described in chapter 2. Blood digestion in ticks takes place in the midgut epithelium and digestion starts soon after attachment and reaches to the peak prior to the rapid feeding period for engorgement (5) . The midgut specificity of legumains and their expression profiles during blood-feeding strongly suggest their relationship with the blood-meal digestion. The biochemical properties, viz. the molecular mass of the mature active proteins, pH and temperature optima, metal cation dependency, substrate specificity, inhibitor sensitivity and blood proteins (Hb, BSA) digesting potentials of *E. coli* expressed rHlLgm and rHlLgm2 have been investigated and described in this chapter with a view to explore the speculated biological function of endogenous HlLgm and HlLgm2 in ticks in more detail.

## 2. Materials and methods

### *2.1. Expression of recombinant HlLgm and HlLgm2 in* Escherichia coli

The open reading frame of the *HlLgm* gene excluding the signal sequence was amplified from pBS/*HlLgm* using a set of primers, 5′-CCATATGGTACCGCGGGAAAGTCGGCCG TCGCA-3′ and 5′-CGGAATTCTTACGTGCAAGCCAAGTCCATAGCATC-3′, which contained a *Kpn*I and an *Eco*RI enzyme restriction site (underlined letters), respectively. The purified PCR product was inserted into the *Kpn*I and *Eco*RI sites of plasmid pTrcHisB (Invitrogen). For *HlLgm2* amplification, one set of oligonucleotide primers derived from its open reading frame (excluding signal sequence) was used with the sense primer (5′-GCTTGGATCCGGCT GGGCAGGAAAGATCAGCG-3′) and the anti-sense primer (5′-CCGCTCGAGCGGTT ATACAATATCGACAACGGC-3′). The nucleotide sequence of the sense and anti-sense primers contained a *Bam*HI and an *Xho*I restriction site (underlined letters), respectively. The purified PCR product was inserted between the *Bam*HI and *Xho*I sites of plasmid pTrcHisB. The resultant plasmids (pTrcHisB/HlLgm and pTrcHisB/HlLgm2) were transformed separately into *E. coli* Top10F′ strain (Invitrogen) following the standard technique. The expression of the HlLgm and HlLgm2 cDNAs was performed as described previously (142). The transformed cells were grown in SOB containing 50 μg ampicillin/ml at 37 °C until an optical density of 1 at 600 nm ($OD_{600}$) was reached. Isopropyl-β-D thiogalactopyranoside (IPTG) was added to the culture media to 1 mM concentration to induce the expression of the fusion proteins. The cultures were grown for another 3 h at 37 °C, pelleted by centrifugation and resuspended in the lysis buffer (20 mM sodium phosphate, 500 mM sodium chloride, 8 M urea, pH 7.8). Lysozyme

was added to 100 μg/ml and the cell suspensions were incubated on ice for 15 min. The suspensions were sonicated with an ultrasonic processor (VP-15S, TAITEC) on ice for 2 min, frozen at –80 °C and thawed at 37 °C. After 3 cycles of sonication, freezing and thawing, the *E. coli* lysates were centrifuged at 25,000 × *g* for 30 min at 4 °C. The recombinant proteins in the supernatants were purified using Ni Sepharose™ 6 Fast Flow (Amersham Biosciences AB, Uppsala, Sweden) under native conditions according to the manufacturer's protocol and subsequently eluted with a stepwise gradient of imidazole (25–100 mM). The eluates were concentrated using Centrisart (MW cut off 20,000; Sartorius, Goettingen, Germany) and then dialysed extensively at 4 °C with several successive changes of 20 mM Tris–HCl (pH 7.5), and a decreasing concentration of NaCl (500 mM–250 mM) using a Slide-A-Lyser Dialysis Cassette (Pierce, Rockford, IL, USA). Protein concentration was determined using micro BCA reagent (Pierce). As a representative of HlLgm and HlLgm2, the His-tag from the rHlLgm2 was removed by digestion with enterokinase (EKMax™, Invitrogen) and purified using the enterokinase-eliminating column method (EK-Away™ Resin, Invitrogen) following the manufacturer's protocol. The His-tag removed rHlLgm2 was concentrated, dialysed, and its concentration was measured as above. The recombinant proteins were analysed by SDS–PAGE using equal amounts (5 μg) of rHlLgm2 with and without His-tag next to the purified His-tagged rHlLgm. The rHlLgm and rHlLgm2 were used for either antibody production or enzyme assays.

### *2.2. Enzyme assay*

The hydrolyzing efficiency of HlLgm and rHlLgm2 was assayed by using Z-Ala-Ala-Asn-MCA (Benzyloxycarbonyl-L-alanyl-L-alanyl-L-asparagine 4-methyl-coumaryl-7-amide)

(Code: 3209-v, Peptide Institute Inc, Osaka, Japan), a synthetic substrate specific for legumain as described previously (26) with a slight modifications. Briefly, a reaction mixture of 100 µl containing 50 mM buffer, 2 mM dithiothreitol (DTT), 1 mM EDTA, 0.05% CHAPS (3[(3-Cholamidopropyl)dimethylammonio]propanesulfonic acid) and 300 µM Z-Ala-Ala-Asn-MCA was prepared. The recombinant enzymes (rHlLgm and rHlLgm2) were assayed at different temperatures, from 10 °C–70 °C over a pH range of 3.0–11.0 using the following buffers, sodium citrate for pH 3–7, Tris–HCl for pH 7.5–9 and sodium carbonate-bicarbonate for pH 10–11. The reactions were started by adding 10 µg of either His-tagged rHlLgm or His-tagged rHlLgm2 to the reaction mixture. Simultaneously, to verify the specificity of HlLgm and HlLgm2, I also used the synthetic substrate Ac–Asp–Asn–Leu–Asp–MCA (Acetyl–L–aspartyl–L–asparaginyl–L-leucyl–L-aspartic acid α–(4–methyl–coumaryl–7–amide) (Code: 322-v, Peptide Institute Inc. Osaka, Japan) specific for caspase-3, under the same conditions. The enzyme activity was monitored using a Spectra Fluor fluorometer (TECAN, Männedorf, Switzerland) with excitation and emission wavelengths of 360 and 460 nm, respectively. All assays were performed in triplicate and one unit of activity was that releasing 1 µmol of AMC $min^{-1}$. To confirm the sensitivity of rHlLgm and rHlLgm2 to specific inhibitors of the C13 family, we performed enzyme inhibition assays with different peptidase inhibitors. Iodoacetamide, N-ethylmaleimide, and PMSF (phenylmethanesulfonyl fluoride) (Sigma) were used in the millimolar range and E-64 (*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane), leupeptin, pepstatin A, antipain (Peptide Institute Inc.) and egg white cystatin (Sigma) in the micromolar range.

### *2.3. Metal cation profile of recombinant legumains*

To determine the effects of the divalent cations on activities of *H. longicornis* legumains, rHlLgm2 was used as representative and the assay using Z-Ala-Ala-Asn-MCA as above was also performed in the presence of 5 mM $Mg^{2+}$, $Ca^{2+}$, $Cu^{2+}$, $Co^{2+}$, $Zn^{2+}$, $Mn^{2+}$, $Ni^{2+}$, or $Fe^{2+}$ at 37 °C and pH 7. To experimentally support the influence of His-tag on pH sensitivity and metal ion dependency of legumains, the pH profile and the effect of the above mentioned divalent cations were also verified by using His-tag removed rHlLgm2 against Z-Ala-Ala-Asn-MCA substrate in the same assay conditions as above.

### *2.4.* In vitro *proteolysis assay*

BSA (Pierce, Rockford, IL, USA) and bovine Hb (Sigma), as natural protein substrates, were used to test the activity of HlLgm and HlLgm2 against the host's blood proteins. To perform *in vitro* haemoglobinolysis assays with rHlLgm and rHlLgm2, both intact and cathepsin D (Sigma)-digested Hb were used as substrates. The enzyme assay to digesting intact Hb by cathepsin D was carried out according to the manufacturer's protocol. Briefly, a reaction cocktail was prepared with 40 $\mu$l of 2.5% Hb, 40 $\mu$l sterile deionized water and 10 $\mu$l of 400 mM sodium citrate buffer (pH 2.8). Ten micro liter of 5 units/ml cathepsin D was added to the reaction cocktail and incubated for 15 minutes at 37 °C. The digested Hb mixture was then dialysed in 20 mM Tris–HCl (pH 7) for 6 hours at room temperature using a Slide-A-Lyser Dialysis Cassette (Pierce). Twenty five micrograms of intact Hb or cathepsin D digested hemoglobin was reacted either with rHlLgm or rHlLgm2 in a 50 $\mu$l reaction mixture of 40 mM sodium citrate (pH 7.0), 1 mM EDTA, 1 mM DTT and 0.01% CHAPS at 30 °C overnight. The reaction was with or without (control) 15 $\mu$g of rHlLgm or rHlLgm2. Iodoacetamide at 5 mM concentration was added to the reaction mixture to verify its inhibitory effect on the degradation

of the natural substrate, Hb by HlLgm2. The products of degradation of hemoglobin by cathepsin D and recombinant legumains were analysed by SDS–PAGE using 10-20% Tricine-Gels (Invitrogen) under non-reducing conditions.

The proteolytic activity of rHlLgm and rHlLgm2 on BSA *in vitro* was determined as described previously (37). Briefly, BSA (20 μg in a 50 μl reaction mixture) was denatured by boiling in 1% SDS for 5 minutes. Twenty micrograms of denatured BSA was incubated at 30 °C for 6 h with or without (control) ~20 μg of either rHlLgm or rHlLgm2 in 40 mM sodium citrate (pH 7.0) containing 1 mM EDTA, 1 mM dithiothreitol and 0.01% CHAPS. The final SDS concentration in the reaction mixture was ≤0.02%. The reaction was stopped by boiling for 5 min with SDS sample buffer. The efficiency of BSA degradation by rHlLgm was tested over a pH range of 4–10. Considering the feeding behavior and host body physiology, and ecology of ticks, the proteolysis assay was also conducted over a wide range of temperatures, from 15 °C–55 °C. The thiol-blocking agent, iodoacetamide, was also used at 5 mM concentration to examine its inhibitory effect on the degradation of BSA by rHlLgm. BSA proteolysis assay was also performed in the absence of DTT while treated with rHlLgm2. The proteolytic products were analyzed by SDS–PAGE as described above and also by using 10-20% Tricine-Gel (Invitrogen) under reducing condition.

### *2.5. N-terminal micro-sequencing of degraded BSA*

For N-terminal micro-sequencing, the cleaved BSA was electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) at 90 mA for 1 h in CAPS buffer [3-(cyclohexylamino)-1-propane sulfonic acid] and stained with CBB.

The cleaved bands were excised and analyzed by N-terminal micro-sequencing using the Procise 494 cLC protein sequencing system (Applied Biosystems) (53, 97).

## 3. Results

### *3.1. Recombinant HlLgm and HlLgm2 and their autocatalytic cleavage sites*

The ORFs of HlLgm and HlLgm2 excluding the signal sequences were subcloned into the pTrcHisB (Invitrogen) vector. The rHlLgm and rHlLgm2 were expressed in *E. coli* and purified as described in Materials and methods section. SDS–PAGE showed a band of ~42.5 kDa for both His-tagged rHlLgm (Fig. 10A) and rHlLgm2 (Fig. 10B ) and a band of ~38 kDa His-tag removed rHlLgm2 proteins (Fig. 10C) which were further confirmed by immunoblot analysis. Either recombinant proteins were 99% pure as judged by SDS–PAGE analysis. The sequence analysis of both HlLgm and HlLgm2 showed that either preproteins have predicted molecular mass of ~47 kDa, and recombinant proteins with ~4 kDa His-tag would be ~51 kDa. However, both the recombinant and endogenous HlLgm and HlLgm2 on SDS–PAGE and immunoblot analysis showed lower MW (~42.5 kDa and ~38 kDa, respectively) than the values expected on the basis of their predicted MW (~51 kDa and ~47 kDa, respectively) indicating that post-translational processing of HlLgm and HlLgm2 occurred during biosynthesis to form a mature proteins through removal of ~9 kDa peptides from the precursor proteins. The alignment analysis of HlLgm and HlLgm2 with other legumain homologues from different sources (Fig. 3, Chapter 1) showed that there are C-termianl extensions in legumain sequences. Human

prolegumain is converted to mature active legumain through a C-terminal autocleavage (28). In the case of AEPs, the fully mature form is generated by living cells either from endogenous pro-AEP or following endocytosis and cellular processing of recombinant pro-AEP (93). Consistently, I isolated both rHlLgm and rHlLgm2 that had been converted to the mature active proteins (~42.5 kDa) either during expression in *E. coli*/or subsequent handling in *E. coli* expression medium, and it was not possible to experimentally determine the cleavage site of the pro-legumains. The plant legumain (vacuolar processing enzyme) recognizes asparagine residues in the hydrophilic regions of proproteins (62). The hydropathy profile (90) of the HlLgm and HlLgm2 sequence indicated that $Asn^{364-365}$ were present in the most hydrophilic regions (data not shown). Moreover, sequence analysis applying the server 'NetAEP: Predicting Asparaginyl Endopeptidase Specificity' (http://theory.bio.uu.nl/kesmir/AEP/) determined the theoretical cleavage sites predicted for HlLgm and HlLgm2 C-terminal extension at $Asn^{365}$ and $Asn^{364-365}$, respectively (Chapter 1) which further confirms the autocleavage sites of HlLgm and HlLgm2. Autocleavage at $Asn^{364-365}$ causes removal of a ~9 kDa peptide from both the HlLgm and HlLgm2 precursors, resulting in ~42.5 kDa recombinant and ~38 kDa endogenous proteins. Also, to confirm whether *E. coli* has endogenous legumain that activates recombinant legumains, I performed enzyme assays using *E. coli* lysate and the legumain-specific synthetic substrate Z–Ala–Ala–Asn–MCA in the same assay conditions for rHlLgm and rHlLgm2. Immunoblotting using *E. coli* lysate and mouse anti-rHlLgm serum was also performed. The results revealed that *E. coli* has no endogenous legumain (data not shown). Together, these results clearly indicate that the legumains have a unique feature of autocatalysed cleavage of the C-terminal extension resulting in conversion into the mature proteins. These results are in accordance with the previous reports on autocatalysis of the tick, *I ricinus* legumain (IrAE) (131) and castor bean

vacuolar processing enzyme (64), and the autocleavages described as post-translational modifications in the case of mammalian legumains (26, 27).

### *3.2. Functional characterization of the recombinant HlLgm and HlLgm2 towards synthetic substrates*

The legumain-specific fluorogenic substrate Z-Ala-Ala-Asn-MCA was used to characterize the activity of recombinant legumains as described in Materials and methods section. The assays revealed that rHlLgm and rHlLgm2 efficiently hydrolysed the substrate Z-Ala-Ala-Asn-MCA at the rate of $6.42 \times 10^{-4}$ and $13.30 \times 10^{-4}$ µmol/min/mg protein, respectively at 37 °C and at pH 7 while the recombinant enzymes showed very low efficiency for hydrolyzing the caspase-3 specific substrate, Ac–Asp–Asn–Leu–Asp–MCA, at the rate of $0.46 \times 10^{-4}$ and $0.41 \times 10^{-4}$ µmol/min/mg protein, respectively under the same assay conditions. Interestingly both rHlLgm and rHllgm2 hydrolyzed Z-Ala-Ala-Asn-MCA more efficiently at neutral to alkaline pH and over a wide range of temperatures, showing pH and temperature optima of pH 7 (Fig. 11A) and 37 °C (Fig. 11B), respectively. The assay using the His-tag removed rHlLgm2 also showed that the rHlLgm2 is more active at 37 °C in neutral to slightly alkaline condition, optimally at pH 7 (data not shown). The inhibition profile showed that thiol-blocking reagents, iodoacetamide and N-ethylmaleimide potently inhibited the activity of rHlLgm (Table 1) and rHlLgm2 (Table 2), but E-64, leupeptin and antipain, which are potent inhibitors of papain-like cysteine proteinases of family C1 had very minimal effects. Moreover, the serine peptidase inhibitor, PMSF and the aspartic peptidase inhibitor, pepstatin did not affect the enzymes activity significantly. Interestingly, egg white cystatin, an unusual potent inhibitor of mammalian

legumains (26), inhibited rHlLgm activity by only 45.13% at 2 μm concentration (Table 1) and rHlLgm2 activity by 62.30% at 3 μm concentration (Table 2). Surprisingly, the metal ion sensitivity showed that the activity of both the His-tagged rHlLgm2 and His-tag removed rHlLgm2 was inhibited strongly by $Fe^{2+}$, $Cu^{2+}$, $Co^{2+}$, and $Ni^{2+}$, and moderately by $Mg^{2+}$, $Ca^{2+}$, $Zn^{2+}$, and $Mn^{2+}$ (data not shown).

### *3.3. rHlLgm and rHlLgm2 proteolyse endogenous substrates*

The activities of rHlLgm and rHlLgm2 were tested *in vitro* against blood proteins, bovine Hb and BSA as natural substrates as described above. None of the recombinant enzymes had proteolytic effects on intact bovine Hb while cathepsin D degraded intact bovine hemoglobin into 14, 4.6, and 3.5 kDa fragments. Interestingly, when incubated with cathepsin D-digested dialysed (at pH 7) Hb overnight at 30 °C, both rHlLgm (Fig. 12A) and rHlLgm2 (Fig. 12B) digested the ~3.5-kDa band almost completely and also digested the ~4.6-kDa band partially, suggesting that legumains act in combination with the aspartic protease, cathepsin D to effect complete digestion of Hb in *H. longicornis*. No Hb digestion occurred when HlLgm2 was pre-incubated with 5 mM iodoacetamide inhibitor (Fig. 12B). Examination of the proteolytic activity of legumains against BSA *in vitro* showed that SDS-denatured BSA was readily cleaved by the recombinant enzymes. SDS–PAGE gel analysis revealed a major band of 58 kDa cleaved by both rHlLgm and rHlLgm2 while Tricine-Gel (10-20%) analysis showed two additional small fragments of ~4.5 kDa and ~3.5 kDa derived from 66 kDa BSA for each case (data not shown). No proteolysis of BSA, however, was seen in the control lane. rHlLgm was found to degrade BSA in a dose dependent manner at pH 7 and 30 °C during 6 h of incubation (Fig. 13A). As it

was found for the hydrolysis of the arylamide substrate, rHlLgm was more active at neutral to alkaline pH than acidic conditions, and cleaved BSA most efficiently at pH 7 (Fig. 13B). Remarkably, no cleavage of BSA was detected at or above 37 °C by rHlLgm (Fig. 13C) and also by rHlLgm2 (data not shown). Moreover, regardless of higher enzyme concentrations or prolonged incubation, the 58 kDa band remained stable, indicating that rHlLgm cleaves BSA in a limited proteolysis manner. N-terminal micro-sequencing of the bands, cleaved by both rHlLgm and rHlLgm2 showed that these resulted through cleavage from the carboxyl sides of the asparagine residues at $Asn^{506}$ (SLV*N*RRP) and $Asn^{573}$ (VME*N*FVA), confirming the asparaginyl specificity of HlLgm and HlLgm2. The proteolytic activity of rHlLgm towards BSA cleavage was inhibited by 5 mM iodoacetamide (Fig. 13D). Noteworthy, addition of 2 mM DTT to the reaction mixture did not show any significant effect on the rHlLgm2 activity for BSA cleavage (Fig. 13E).

## 4. Discussion

The enzyme assays demonstrated that recombinant legumains hydrolysed the synthetic peptides and natural substrates efficiently at neutral to alkaline medium, and optimally at pH 7. The pH optima of the legumains of blood-feeding helminth parasites *S. mansoni* and *H. contortus* are pH 6.8 and pH 7, respectively (36, 116). In contrast, the pH optima of IrAE for hydrolyzing the synthetic substrate and Hb degradation are 5.5 and 4.5, respectively. IrAE activity declined sharply at pH 6.0 and was unstable at neutral pH (131). Also, the mammalian and plant legumains are active in an acidic environment and very labile at neutral pH (26, 27, 62,

75). Thus, although HlLgm and HlLgm2 phylogenetically belong to the mammalian-arthropod subfamily, biochemically they share similar properties with those of haematophagous helminth parasites rather than with those of mammals and plants. Remarkably, the broad range of temperature dependency of both rHlLgm and rHlLgm2 (from 10–70 °C, optima 37 °C) for hydrolyzing peptide substrates indicates that legumains in *H. longicornis* are active during blood-feeding on hosts as well as off the hosts periods, even under extreme environmental conditions. It is surprising that all divalent cations tested ($Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Mg^{2+}$, $Fe^{2+}$, $Cu^{2+}$, $Zn^{2+}$, and $Ca^{2+}$) inhibited the rHlLgm2 activity. The activity of a leucine aminopeptidase from *H. longicornis* (HlLAP) was highly activated by $Mn^{2+}$. HlLAP activity was also enhanced by other cations ($Co^{2+}$, $Ni^{2+}$, $Mg^{2+}$, $Fe^{2+}$, $Cu^{2+}$, and $Ca^{2+}$) (65). Yamane et al. (154) reported that $Hg^{2+}$ and $Cu^{2+}$ cations strongly, and $Cd^{2+}$ moderately inhibited the bovine legumain enzyme activity. The mechanisms by which metal ions inactivate legumains from various sources are yet to be clarified. rHlLgm and rHlLgm2 activities were potently inhibited by the thiol-locking reagents, iodoacetamide and N ethylmaleimide, but was unaffected by E-64 and leupeptin, which potently inhibit the papain-like cysteine proteinases of the C1 family. Moreover, no significant inhibitory effects of the serine peptidase inhibitor, PMSF or the aspartic peptidase inhibitor, pepstatin on legumains were detected. These results accord with the earlier reports on helminth (36, 116), plant (1) and mammalian legumains (26). This inhibition profile corresponds to the specific biochemical features of enzymes belonging to peptidases of the legumain family (C13 family) and indicates that HlLgm and HlLgm2 reported here are authentic members of the C13 family. The egg white cystatin was found to weakly inhibit rHlLgm (45.13% at 2 μm concentration) and rHlLgm2 (62.30% at 3 μm concentration) activities. Cystatin is known to be a potent inhibitor of the peptidases of the papain family, and egg white cystatin was reported to potently inhibit

(100%) the structurally unrelated pig kidney legumain at 50 nM concentration (26). The weak inhibition of recombinant legumains by egg white cystatin might be due to the absence of some reactive site(s) on HlLgm and HlLgm2, a possibility which awaits verification in future studies.

Both rHlLgm and rHlLgm2 effectively digested the ~4.6 and ~3.5 kDa fragments of cathepsin D-degraded bovine Hb, suggesting that *H. longicornis* legumains act in cooperation with a cascade of aspartic proteases to effect complete degradation of Hb to absorbable peptides and amino acids. Multi-enzyme, synergistic networks of Hb digestion by proteases have also been reported in the cases of the protozoan, *Plasmodium falciparum* (44); haematophagous helminths (34, 152) and *H. longicornis* (20, 65, 101). Hb digestion takes place entirely in the acidic environment within the digestive vesicles of tick gut cells (33) which is not in accord with the pH sensitivity of HlLgm and HlLgm2. However, intracellular localization of legumains (Fig. 8B, Chapter 2) conceivably suggests their role in intracellular Hb digestion cascade. Either recombinant proteins cleaved BSA selectively at $Asn^{506}$ and $Asn^{573}$, yielding a major band of 58 kDa and two other small peptides of ~4.5 and ~3.5 kDa. Pig and bovine legumains cleaved BSA and human vitamin D binding protein, respectively, in a limited proteolytic manner (37, 154). The fact that DTT had almost no activating effect on rHlLgm2 is consistent with the result of *S. mansoni* legumain activity assay (36). However, the moth bean AEP was inactive in the absence of thiol activator (83) and jack bean AEP was activated and remained more stable in the presence of DTT (1, 83). These findings suggest that haematophagous tick (*H. longicornis*) and helminth (*S. mansoni*) legumains are self-activated without thiol activators. The similarities in the enzyme assay profile and natural substrate hydrolyzing specificities between HlLgm and HlLgm2 indicate that two legumains in *H. longicornis* share very similar biochemical properties. This

may be due to the use of the same catalytic site residues by these two enzymes, despite some differences (75% identity) in the overall sequences of their genes.

Two novel genes from the midgut of the *H. longicornis* encoding the asparaginyl endopeptidases/legumains HlLgm and HlLgm2 are strictly localized in the midgut of the ticks. The results also suggested that HlLgm and HlLgm2 are critically associated with the host blood-meal digestion cascade and they take part in the terminal phase of Hb digestion process and help liberating absorbable amino acids and small peptides.

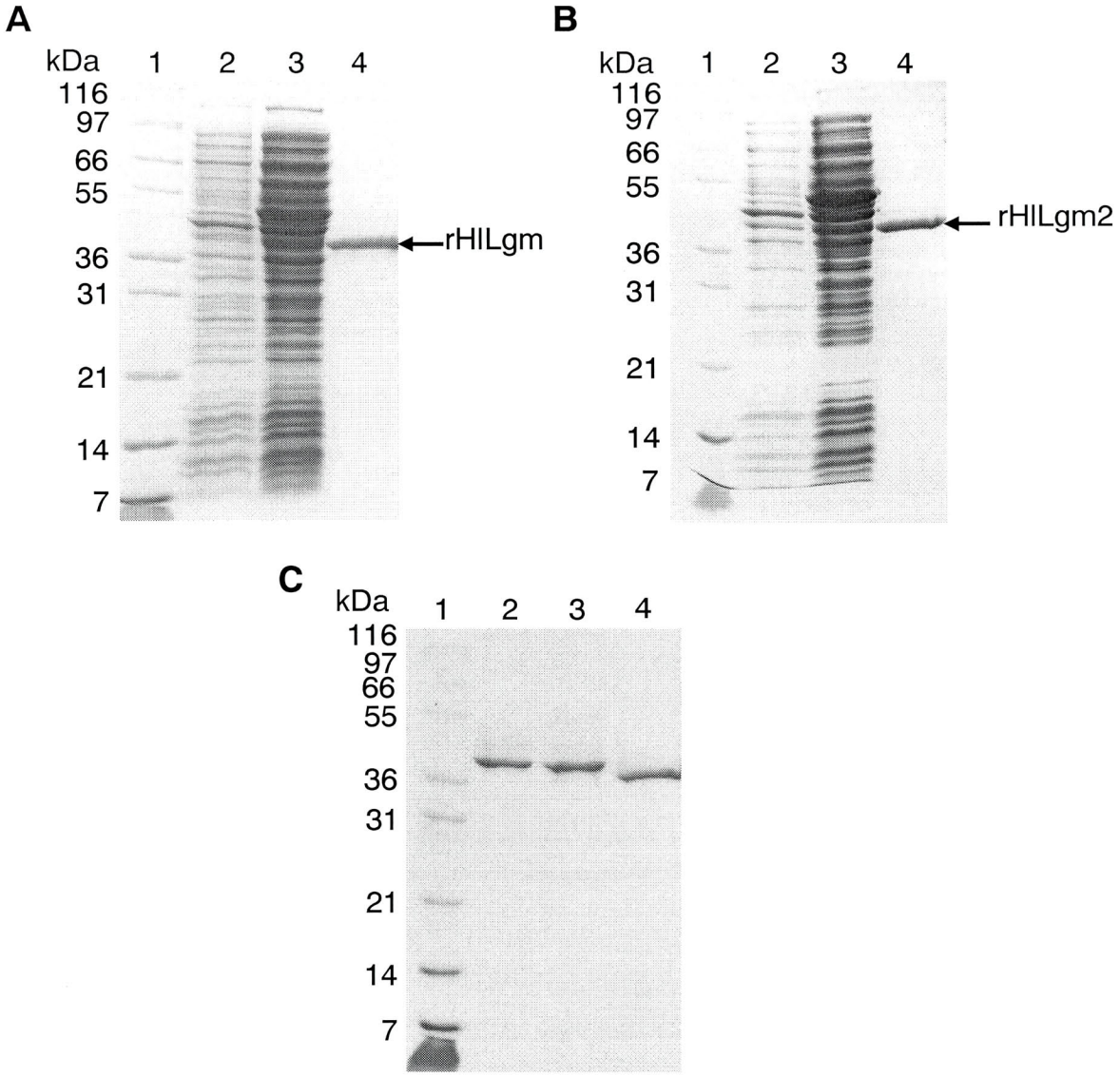


**Fig. 10. Expression of recombinant HlLgm and recombinant HlLgm2 in *E. coli***

The proteins expressed from pTrcHisB/HlLgm and pTrcHisB/HlLgm2 vectors were separated by 12.5% SDS–PAGE and were detected by CBB staining. (A) and (B) *E. coli* expressed rHlLgm and rHlLgm2, respectively. Lane 1, marker; Lane 2, crude lysate of *E. coli* before induction; Lane 3, crude lysate of *E. coli* 3 h after induction with 1.0 mM IPTG and Lane 4, purified rHlLgm/rHlLgm2 (arrow). (C) Purified His-tagged and His-tag removed legumains. Lane 1, Marker; Lane 2, purified His-tagged rHlLgm; Lane3, purified His-tagged rHlLgm2; lane 4, purified His-tag removed rHlLgm2.

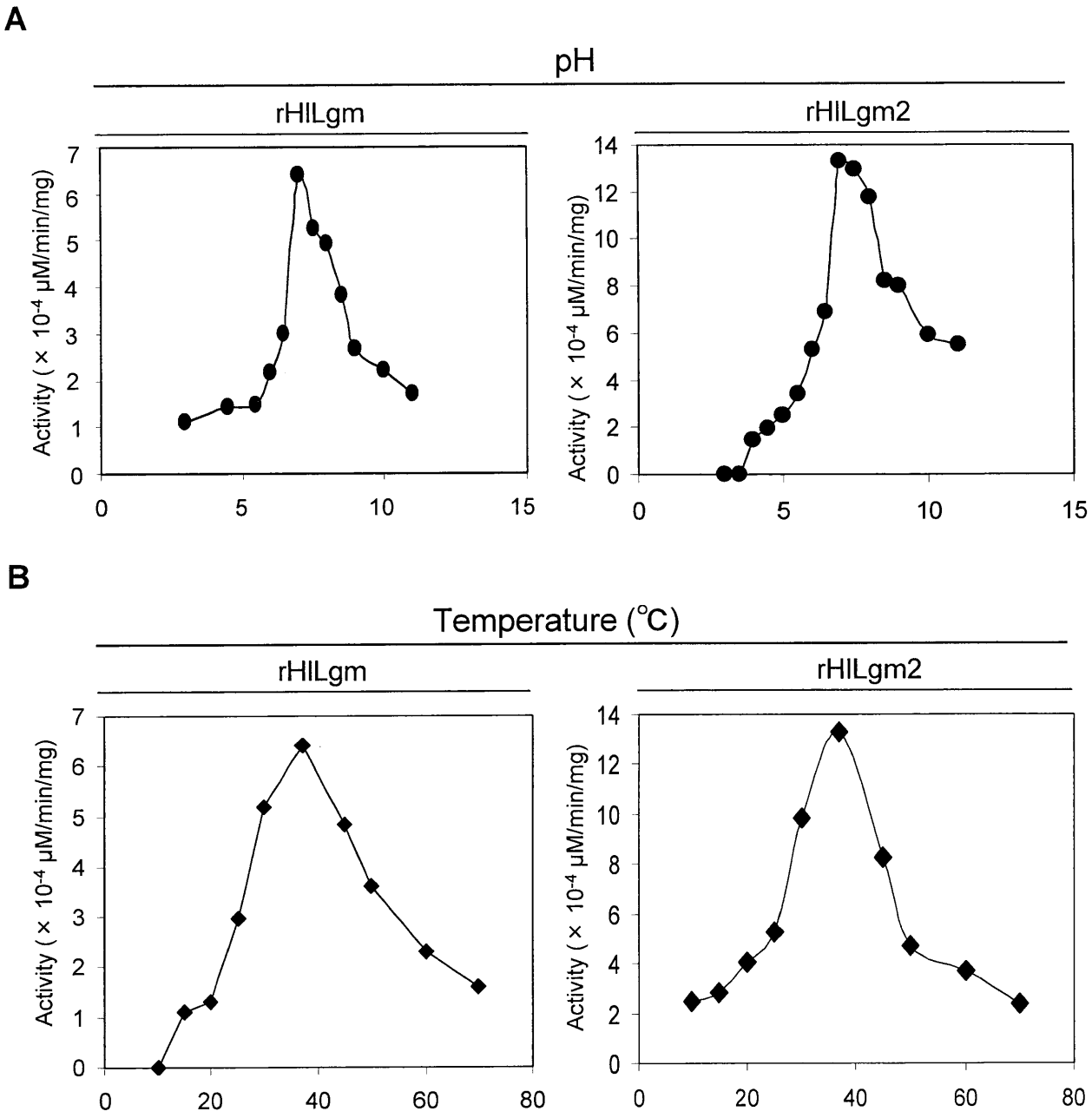


**Fig. 11. Hydrolysis efficiency of rHlLgm and rHlLgm2**

(A) rHlLgm and rHlLgm2 were assayed in a reaction mixture with the indicated pH at 37 ° C and their activities toward the fluorogenic substrate Z–Ala–Ala–Asn–MCA were determined. (B) The activities of rHlLgm and rHlLgm2 toward the same substrate were measured at various temperatures (pH 7), as indicated.

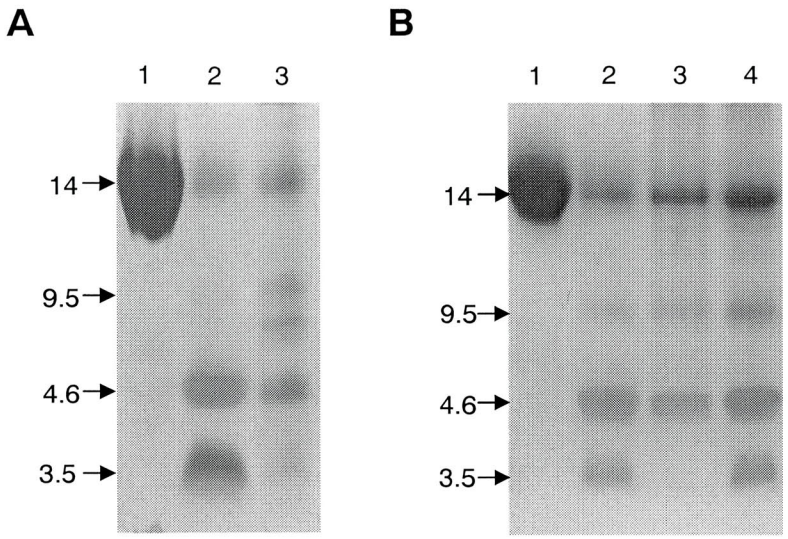


**Fig. 12.** ***In vitro*** **proteolysis assay by using bovine Hb and BSA as natural substrates**

(A) Intact Hb or cathepsin D-digested Hb was reacted with 15 $\mu$g of rHlLgm or incubated in its absence (cont: control) at 30 ° C overnight at pH 7 as described in Materials and methods section. The products of degradation of Hb by cathepsin D and rHlLgm were analysed by SDS–PAGE using a 10-20% Tricine-Gel under non-reducing condition. Lane 1, intact Hb; lane 2, Hb digested with cathepsin D; Lane 3, cathepsin D-digested Hb reacted with rHlLgm. (B) Hb digestion by rHlLgm2 as in (A). The reaction was also performed in presence of 5 mM iodoacetamide. Lanes 1–3, as in A; Lane 4, cathepsin D-digested Hb reacted with rHlLgm2 in presence of 5 mM iodoacetamide.

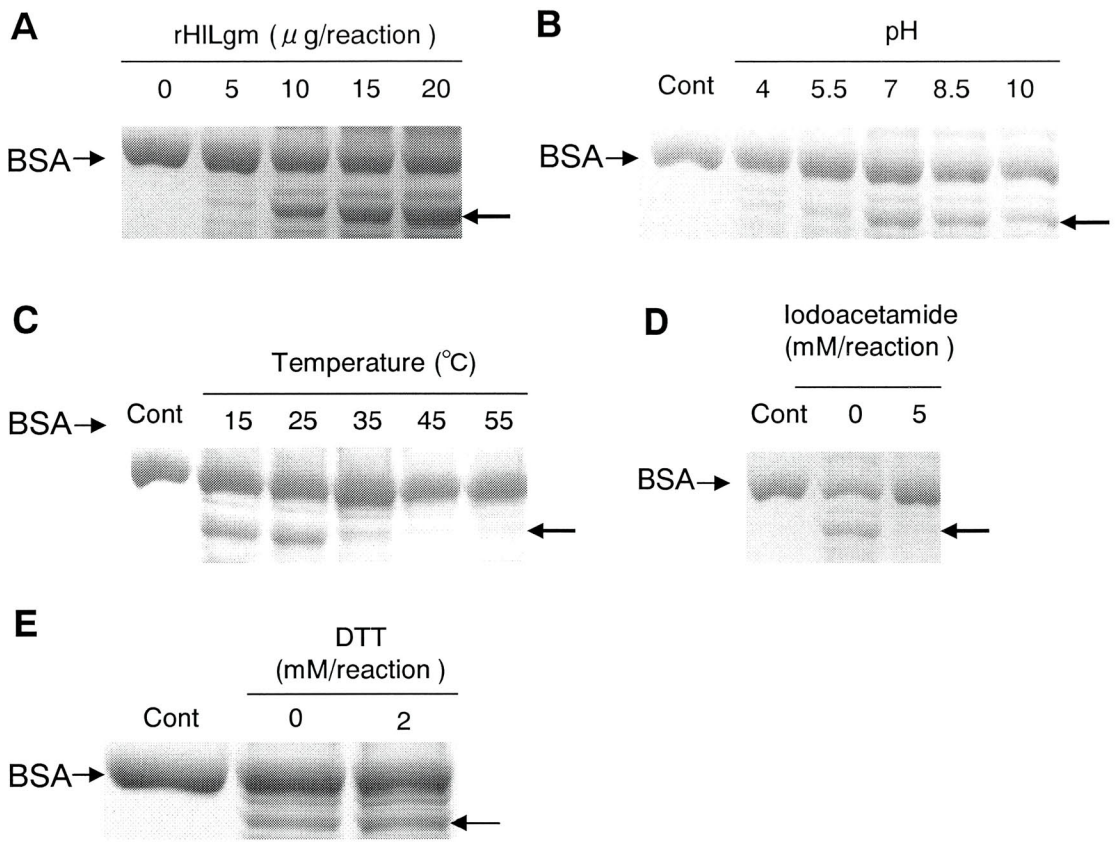


**Fig. 13.** ***In vitro*** **proteolytic effects of recombinant legumains on the native protein substrate, BSA**

(A-D), BSA cleavage by rHlLgm. SDS-denatured purified BSA (20 μg in 50 μl reaction mixture) was incubated with ~20 μg of rHlLgm or in its absence (cont: control) at various pHs and temperatures for 6 hours in 40 mM sodium citrate, 1 mM EDTA, 1 mM dithiothreitol and 0.01% CHAPS. The resultant proteolytic products (arrows) were analysed by 8% SDS–PAGE. (A) Dose-dependency of rHlLgm for BSA degradation at pH 7 and temperature 30 °C. (B) pH dependency of the enzyme for BSA cleavage at a concentration of 20 μg of rHlLgm and at 30 °C. (C) Temperature-dependency of enzyme for BSA degradation at a concentration of 20 μg of rHlLgm at pH 7. (D) Inhibition of rHlLgm activity for BSA cleavage by iodoacetamide. Twenty micrograms of BSA were reacted with 20 μg of rHlLgm at 30 °C and pH 7. (E) BSA degradation by rHlLgm2 and effects of DTT on enzyme activation. Twenty micrograms of SDS-denatured purified BSA was treated with 15 μg of rHlLgm2 as above in presence of 2 mM DTT or its absence at pH 7, 30 °C.

**Table 1 Influence of different protease inhibitors on rHlLgm activity**

| Inhibitor | Concentration | Inhibition (%) |
|---|---|---|
| Cysteine proteases | | |
| Iodoacetamide | 3 mM | 100 |
| N-etheylmaleimide | 3 mM | 95.74 |
| E-64 | 500 μM | 8.88 |
| Egg white cystatin | 2 μM | 45.13 |
| Serine proteases | | |
| PMSF | 5 mM | 19 |
| Leupeptin | 300 μM | 16.07 |
| Aspartic proteases | | |
| Pepstatin A | 500 μM | 7.8 |
| Control | – | 0 |

The enzyme was pre-incubated with the inhibitor in the usual assay buffer at 37 °C for 20 min before the reaction was initiated by addition of substrate.

**Table 2 Inhibition of rHlLgm2 activity by different protease inhibitors**

| Inhibitor | Concentration | Inhibition (%) |
|---|---|---|
| Cysteine proteases | | |
| Iodoacetamide | 3 mM | 100 |
| N-etheylmaleimide | 3 mM | 100 |
| E-64 | 300 μM | 7.51 |
| Antipain | 100 μM | 28.77 |
| Egg white cystatin | 3 μM | 62.30 |
| Serine proteases | | |
| PMSF | 5 mM | 13.48 |
| Leupeptin | 300 μM | 3.9 |
| Aspartic proteases | | |
| Pepstatin A | 300 μM | 12.22 |
| Control | – | 0 |

The enzyme was pre-incubated with the inhibitor in the usual assay buffer at 37 °C for 20 min before the reaction was initiated by adding the substrate.

Chapter 4:

# Impacts of *HlLgm* and *HlLgm2* gene knock-down on tick biology by RNA interference

## 1. Introduction

Ticks depend entirely on host blood proteins for nutrition and energy. Also, in ticks, the vectored microbial pathogens generally do not reproduce and typically do not cause obvious disease until the ticks begin feeding on a host (86, 117). The fecundity of ticks is directly related to the volume of blood ingested and more than 50–60% of the engorged female body weight converts into eggs (66, 132). The high fecundity and the unique capacity of ticks for transmitting various virus, bacteria, rickettsiae and protozoa to their progeny by way of transovarial transmission (86, 115) make the ticks highly successful as disease vectors. Knock-down of the genes that modulate blood-feeding and digestion seems to be the most promising strategy in reducing the abundance of ticks and thereby reducing the tick infestations and burden of tick-borne disease.

In the recent past years, the nucleic acid based reverse genetic approach, RNA interference (RNAi) has successfully been employed to investigate and identify the role of proteins hypothesised to be involved in blood-feeding by different ticks (6, 81, 107, 118, 120) and also for screening the tick protective antigens (39, 40, 109). Attempts have also been made to unmask the biological role of some bioactive molecules in the hard tick *H. longicornis* through RNAi (20, 66, 74, 103). However, the regulatory genes involved in blood digestion and reproductions in ticks await elucidation. The expression profiles of legumain transcripts and endogenous legumains, their localization, and blood proteins (Hb and BSA) digesting potentials of rHlLgm and rHlLgm2 *in vitro*, as described in above chapters, suggest the important roles of legumains in tick blood-feeding and digestion. This chapter describes the biological roles of *H. longicornis* legumain genes (*HlLgm* and *HlLgm2*) *in vivo* in tick blood-feeding and digestion, midgut cellular remodelling and reproduction as determined by RNAi. Abrogation of *HlLgm* and *HlLgm2* genes by RNAi treatment resulted in reduced tick survival, blood-feeding, disruption of midgut cellular development and differentiation, reduced oviposition and strikingly, impaired embryogenesis suggesting their critical roles in development and reproduction of ixodid ticks.

## 2. Materials and methods

### *2.1.* In vitro *transcription of* HlLgm *and* HlLgm2 *gene double-stranded RNA*

The ORFs of *HlLgm* and *HlLgm2* genes inserted into *E. coli* expressed pTrcHisB/*HlLgm* and pTrcHisB/*HlLgm2* plasmids (Chapter-3) were used as templates and cloned into pBluescript II SK+ plasmid (Toyobo, Osaka, Japan). In parallel, PBS and double-stranded RNA (dsRNA) complementary to the non-functional portion of the *E. coli* malE gene (*malE*) for ticks that encodes the maltose-binding protein was used as a negative control (30). mRNA from *E. coli* (BL21 strain, Invitrogen) was isolated using an mRNA isolation kit (QIAGEN) and following the manufacturer's protocol. The mRNA template was employed to prepare single-stranded cDNA by reverse transcription (RT)-PCR using a Takara RNA PCR Kit (AMV) Ver.3.0 (Takara, Shiga, Japan) and following the protocol provided by the manufacturer. The cDNA of *malE* was cloned into pBlueskript II SK+ plasmid using the oligonucleotides 5′-CCGCTCGAGCGGTTATGAAAATAAAAACAGGTGCA-3′and 5′-GAATTCGCTTG TCCTGGAACGCTTTGTC-3′ as forward and reverse primer, respectively. The inserted sequences for *HlLgm*, *HlLgm2* and *malE* were amplified by PCR using the oligonucleotide T7 (5′-GTAATACGACTCACTATAGGGC-3′) and CMo422 (5′-GCGTAATACGACTCACTATAGGGAACAAAAGCTGGAGCT-3′) as primers to attach T7 promoter recognition sites on both the 5′ and 3′ends (T7-HlLgm-T7, T7-HlLgm2-T7 and T7-malE-T7). The PCR products were purified by agarose gel electrophoresis using the QIAquick® Gel Extraction kit (QIAGEN Sciences, Germantown, MA, USA) and following the manufacturer's protocol. Using approximately 2 µg T7-HlLgm-T7, T7-HlLgm2-T7 and T7-malE-T7 as templates, 50–100 µg dsRNA complementary to the sequences encoding ORFs of *HlLgm*, *HlLgm2* and *malE*, respectively, was synthesised by an *in vitro* transcription method using the T7 RNA polymerase (Ribomax™ Express Large Scale RNA Production System, Promega) in accordance with the manufacturer's instructions. The purity of the synthesised

dsRNAs was checked by 1% agarose gel electrophoresis and their concentration was determined by spectrophotometer (GE Healthcare Biosciences KK, NJ, USA).

### 2.2. *Injection of ticks with dsRNA and tick feeding*

The ticks were microinjected with dsRNA as described previously (140). Briefly, 1 µg of *HlLgm* dsRNA and 1 µg of *HlLgm2* dsRNA either separately or in combination in 0.5 µl of PBS was injected into the haemocoel through the fourth coxa in 10–14 day post-moult adult unfed female ticks fixed on a glass slide with adhesive tape. The control ticks were injected with 0.5 µl of PBS alone or 0.5 µl of PBS containing 1 µg dsRNA of *malE*. A total of 75, 110, 73, 65 and 115 ticks were injected with PBS alone, *malE* dsRNA, *HlLgm* dsRNA, *HlLgm2* dsRNA and with a combination of *HlLgm* and *HlLgm2* dsRNA, respectively. Following treatment, the ticks were allowed to rest for 18–24 h at 25 °C and then placed on the ears of rabbits for attachment. The ticks that dropped on repletion were picked up and those that did not engorge on day 7 post-infestation were removed forcibly using forceps.

### 2.3. *Analysis to confirm gene silencing by reverse transcription (RT)-PCR and quantitative RT-PCR*

The midguts from three randomly collected ticks from control and RNAi groups during feeding (24 h, 48 h, 72 h and 96 h), repletion, pre-oviposition (4 days post engorgement (PE)) and oviposition period (10 days PE) were dissected and used for mRNA detection by RT-PCR

and quantitative RT-PCR using the primers specific for *HlLgm, HlLgm2,* and for the positive control cDNA encoding *β-actin* as described in Chapter 2.

### *2.4. Protein expression analysis by Western blotting*

The impact of RNAi on protein translation was determined by Western blotting. Midguts from six partially fed (72 h) ticks of all RNAi and control groups were dissected separately in PBS and antigens were prepared as described in Chapter 2. Equal amounts of protein (40 μg) from control and RNAi groups were separated by SDS–PAGE under reducing condition and processed for immunoblotting. For detection of endogenous legumains, the immunoblots were incubated with mouse anti-rHlLgm serum and mouse anti-rHlLgm2 serum separately at a dilution of 1:250 and subsequently processed for protein bands development as described in Chapter 2.

### *2.5. Immunofluorescent staining of the midgut tissues*

The protein translational disruption by RNAi was also investigated by immunofluorescent staining of *H. longicornis* midgut tissues. The midgut tissues from 72 h fed ticks of the *HlLgm* and *HlLgm2* dsRNA dually injected RNAi group and the *malE* dsRNA treated control group were dissected separately in PBS and thin sections were prepared as described previously (140). The midgut tissue sections were incubated with mouse anti-rHlLgm serum (1:250) overnight at 4 °C. Following treatment with green fluorescence labelled secondary antibody (Alexa Fluor® 488 goat anti-mouse IgG (H + L) (Invitrogen)), the slides were mounted with VECTASHIELD® (Vector) with DAPI (Vector Laboratories, Burlingame,

CA, USA) and photographed with a fluorescence microscope (Leica Microsystems) as described in Chapter 2.

### 2.6. *Analysis of impact of RNAi on midgut tissues*

The tick midgut cells undergo changes during different phases of blood-feeding and post-feeding periods for effective digestion of blood-meal (3). The impacts of RNAi on midgut tissues were investigated on the basis of gross and histological changes of the midgut tissues compared with the control. The midgut tissues were obtained from ticks of the combined *HlLgm* dsRNA and *HlLgm2* dsRNA injected group and the *malE* dsRNA injected control group during feeding (24 h, 48 h, 72 h and 96 h) and immediately after repletion. Three ticks from each group were collected randomly during each phase. Dissection of midgut tissues and preparation of fixed tissue sections were performed as above. The midgut sections of each phase of feeding from each group were stained with H&E and examined under a microscope (DM 4000B, Leica) to analyse the impact of legumain gene silencing on midgut epithelium.

### 2.7. *Determination of effect of* HlLgm *and* HlLgm2 *gene silencing on tick blood-feeding and reproduction*

The effects of RNAi on tick blood-feeding and reproductive parameters were investigated by measuring the attachment rate, death of the ticks after attachment, blood-feeding periods, number of ticks engorged, engorged tick body weight, pre-oviposition periods, weight of egg mass, number of eggs per tick and egg conversion ratio (total egg mass/engorged body weight).

The Reproductive Efficiency Index (REI) (number of eggs/weight of engorged female at the time of host detachment) (42) and Reproductive Fitness Index (RFI) (number of eggs that hatch into larvae/weight of the engorged female at the time of host detachment) (31) were measured for each tick. To count the number of eggs laid by each tick, arbitrarily, we weighed 200 eggs taken from each of three randomly selected ticks from each group. The total number of eggs/tick was calculated as (weight of total egg mass (mg)/mean weight of 200 eggs (mg)) × 200. The weight of individual tick and egg mass/tick was taken by using a digital balance (Model: 321-3357, Shimadzu, Kyoto, Japan). Photographs of ticks were taken with a digital camera (Cannon, Tokyo, Japan). For convenience of investigating the effects of RNAi on reproduction we measured only the engorged ticks from all groups.

### *2.8. Statistical analysis*

Statistical analyses of data obtained on feeding and reproductive parameters for RNAi treated and control groups of ticks were performed by using Student's *t*-test with unequal variance. Tick mortality was compared between the RNAi treated and control ticks by the $\chi^2$-test. *P* values of ≤0.05 were considered statistically significant.

## 3. Results

### *3.1. Demonstration of gene silencing by RT-PCR and quantitative RT-PCR*

The total RNA isolated from the midgut of ticks during different phases of feeding, engorgement, pre-oviposition (4 days PE), oviposition period (10 days PE), and eggs of control groups (PBS and *malE* dsRNA injected), and RNAi treated groups (*HlLgm* and *HlLgm2* dsRNA injected separately or in combination) were analysed by RT-PCR and quantitative RT-PCR to evaluate the effect of RNAi on *HlLgm* and *HlLgm2* mRNA expression as described above. The results demonstrated that there was significant reduction or absence of detectable mRNA expression corresponding to the *HlLgm* and *HlLgm2* genes in ticks injected with *HlLgm* dsRNA and *HlLgm2* dsRNA either individually or in combination while equal levels of *HlLgm* and *HlLgm2* mRNA expression were detected in PBS and *malE* dsRNA injected ticks (Fig. 14A and B) indicating that disruption of *HlLgm* and *HlLgm2* mRNA transcription was achieved by RNAi. There were no detectable *HlLgm2* amplicons in engorged ticks of either control groups which is consistent with our observations on the expression profile of the *HlLgm2* gene during different phases of blood-feeding until engorgement in adult *H. longicornis* (Fig. 5A and B, Chapter 2). Interestingly, it was revealed that the *HlLgm2* mRNA expression profile was bimodal where the transcript was further expressed during pre-oviposition and oviposition periods and also in eggs. However, the reasons for the missing of *HlLgm2* signal in engorged ticks are obscure and await elucidation. There is no obvious sequence homology between *E. coli malE* and the *H. longicornis* legumain family (*HlLgm* and *HlLgm2*), and therefore, we did not expect any direct interaction between the *malE* dsRNA and legumain genes. Targeting *HlLgm* and *HlLgm2* mRNA did not affect the transcription of the *H. longicornis ß-actin* control gene indicating that dsRNA treatment was gene-specific. Although not consistent in different RNAi groups of ticks, there was detectable expression of *HlLgm* and *HlLgm2* mRNAs during 96 h of blood-feeding and beyond (Fig. 14A and B). This may be due variation in the tick population. For mRNA

detection during each phase pools of midgut extracts from three randomly collected ticks were used and it is very likely that RNAi targeting was not uniform in each microinjected tick. Also, dilution of injected dsRNA *in vivo* during prolonged period of tick blood-feeding (81) may be a cause of detectable expression of legumains at a later phase of blood-feeding and onward. However, the results suggest that RNAi targeting effectively silenced the expression of legumains mRNAs in *H. longicornis*. RNAi is a robust and target-specific phenomenon that abrogates only the mRNAs complementary to the introduced dsRNA leading to the silencing of the specific genes (46, 119). However, off-target silencing can be achieved as RNAi can trigger the destruction of mRNAs containing significant stretches of nucleotide sequence identity (77, 107). *HlLgm* and *HlLgm2* mRNA nucleotide sequences (GenBank Accession Nos. AB279705 and AB353127, respectively) share 78% identity to each other and individual injection of dsRNA corresponding to the ORFs of *HlLgm* and *HlLgm2* genes caused a 39% and 45.56% reduction of *HlLgm2* and *HlLgm* mRNA expression levels, respectively, compared with PBS and *malE* dsRNA injected control ticks as revealed by quantitative RT-PCR (Fig. 15A and B) suggesting some off-target silencing of the homologues.

### *3.2. Demonstration of gene silencing by immunoblot and immunofluorescence analyses*

Protein extracts from the midguts of 72 h fed ticks injected with *HlLgm* and *HlLgm2* dsRNA individually or in combination and *malE* dsRNA and PBS alone were analysed by immunoblotting. On immunoblots of the antigens from RNAi and control groups probed with mouse anti-rHlLgm serum and mouse anti-rHlLgm2 serum, reactive bands of ~38 kDa protein corresponding to both the endogenous HlLgm and HlLgm2 (Fig. 7A and B, Chapter-2 ) were

observed in control groups whereas no visible reactive band in the combined *HlLgm* and *HlLgm2* dsRNA introduced group was seen. However, positive reactive bands with a lower intensity were detected in either individual *HlLgm* and *HlLgm2* knock-down tick extracts (Fig. 16A) that might be due to the cross-reactivity of the polyclonal antibodies against the legumain isomers as it was observed that polyclonal antibodies raised against rHlLgm and rHlLgm2 cross-reacted with rHlLgm2 and rHlLgm, respectively (Chapter 2). Immunofluorescence analysis using fixed midgut sections of 72 h fed ticks from control (*malE* dsRNA injected) and RNAi groups (combined *HlLgm* and *HlLgm2* dsRNA injected) incubated with mouse anti-rHlLgm serum (1:250) and fluorescence labelled goat anti-mouse IgG (H+L) revealed strong fluorescence reaction in the sections from the control group while there was very little or no reaction in the sections prepared from the RNAi group (Fig. 16B). These results indicate the blocking of translation of endogenous proteins by RNAi.

### *3.3. Impact of gene silencing on blood-feeding of* H. longicornis

The impact of legumain gene silencing on tick feeding was evaluated by comparing attachment rate, feeding period, engorged tick body weight and mortality of ticks among the control and RNAi groups. None of the ticks from any group died during the post-injection incubation period prior to infesting rabbits for feeding. All ticks from each group were found to attach successfully on rabbit ears 24 h post-placement. None of the ticks from the control groups, either PBS injected or *malE* dsRNA injected, died after attachment. By contrast, death of the ticks in RNAi groups was observed 48 h after successful attachment and blood-feeding (Fig. 17A). Among the RNAi groups, four (5.48%) *HlLgm* dsRNA injected and two (3.1%) *HlLgm2*

dsRNA injected feeding ticks died and the mortality rate was relatively higher (11 ticks, 9.6%) when the ticks were injected with *HlLgm* and *HlLgm2* dsRNA together (data not shown). All ticks from control groups fed to repletion, however, two (2.73%) *HlLgm* dsRNA injected, one (1.54%) *HlLgm2* dsRNA injected and 11 (9.6%) combined *HlLgm* and *HlLgm2* dsRNA injected ticks failed to engorge (data not shown). Phenotypically, the RNAi treated engorged ticks were smaller than those in control groups. Additionally, the cuticle of these ticks appeared to be tense and there was no cuticular wrinkling on the dorsum of ticks (Fig. 17B). There was no notable variation in the blood-feeding period toward repletion among the PBS injected (5.11 ± 0.35 days), *malE* dsRNA injected (5.20 ± 0.40 days), *HlLgm* dsRNA injected (5.35 ± 0.48 days) and *HlLgm2* dsRNA injected (5.34 ± 0.37 days) groups of ticks. However, introduction of combined *HlLgm* dsRNA and *HlLgm2* dsRNA resulted in slower tick blood-feeding (Fig. 17A) and the blood-feeding period of this group (6.28 ± 0.73 days) of ticks was significantly longer ($P < 0.05$ than all other groups of ticks (Fig. 17C). The mean body weight of engorged ticks in PBS and *malE* dsRNA injected control groups was identical, 285.3 ± 30.05 mg and 281.9 ± 27.90 mg, respectively. A significant ($P < 0.01$) decrease in mean engorged body weight compared with control groups was observed in *HlLgm* dsRNA (196.82 ± 34.50 mg) and *HlLgm2* dsRNA (195.99 ± 30.57 mg) groups separately injected. Interestingly, when the ticks were injected with *HlLgm* and *HlLgm2* dsRNA together, the impact on body weight gain was more pronounced with a highly significant ($P < 0.001$) reduction of the mean engorged body weight (111.8 ± 23.30 mg) compared with the control groups (Fig. 17D). Taken together, the results indicate that HlLgm and HlLgm2 play a vital role in blood-feeding and survival of *H. longicornis*.

*3.4. Impact of gene silencing on remodelling of midgut epithelium during different phases of blood-feeding*

The effects of *HlLgm* and *HlLgm2* gene silencing on *H. longicornis* midgut tissues were analysed macroscopically and microscopically using sections prepared from partially fed (24 h, 48 h, 72 h and 96 h) and engorged ticks of control (*malE* dsRNA) and RNAi (*HlLgm* dsRNA and *HlLgm2* dsRNA) treated groups. Macroscopically, the midguts of RNAi treated ticks were atrophied with narrower lumina compared with the controls (data not shown). The midgut epithelia in control ticks showed cellular changes during different phases of blood-feeding. Following attachment and feeding, the midgut epithelial cells underwent dramatic changes including massive proliferation and differentiation of the stem cells (3, 146). The stem cells are dome shaped cells with rounded nuclei and are located on the basement membrane. These cells were found to maintain their original morphology throughout the feeding period. The prodigest cells which are morphologically slightly elongated cells with large rounded nuclei appeared soon after attachment and blood-feeding. With the progression of feeding and enlargement of the lumen, the prodigest cells rapidly transformed into sessile digest cells and residual sessile digest cells during 48 h and 72 h of feeding, respectively. The sessile digest cells are comparatively large cells, columnar in shape, having a broad base in contact with the basement membrane. The residual sessile digest cells are larger club-shaped cells which are thinly attached to the basement membrane and appear to move into the lumen. Sometimes there is apical movement of nucleus. Beyond 96 h of feeding, the residual sessile digest cells started sloughing off into the lumen resulting in reduction of sessile and residual sessile digest cells on the basement membrane of the midgut. In the engorged ticks, the midgut was distended with blood and its epithelial lining

consisted mainly of stem cells. Strikingly, no such cellular events were observed in RNAi group of ticks; rather there was damage to midgut tissues and loss of the epithelium with retention only of the stem cells and sometimes prodigest cells which were sparsely distributed on the basement membrane of the midgut tissues throughout the feeding periods (Fig. 18A). These findings suggest that legumains play an important role in midgut epithelial cell development and differentiation and in maintaining the integrity of the midgut tissues in ticks.

### 3.5. *Role of blood-induced proliferating and differentiated mature midgut epithelial cells in legumains expression*

Striking developmental variations in the midgut epithelium during feeding indicative of major functional differences were seen between the control and RNAi groups of ticks. The role of the proliferating and differentiated mature midgut cells with legumain expression was therefore investigated. Immunofluorescent staining using fixed midgut sections of 24 h and 96 h fed control (*malE* dsRNA injected) and RNAi treated (*HlLgm* and *HlLgm2* dsRNA injected) ticks revealed strong expression of endogenous legumains in the sessile digest cells and residual sessile digest cells of 96 h fed control ticks. Interestingly, the stem cells and the prodigest cells in both 24 h fed and 96 h fed control tick sections showed little fluorescence. Furthermore, there was very little or no reaction in the counterparts of these sections of RNAi treated ticks (Fig. 18B). These results show that endogenous legumains are mostly expressed in the proliferating and differentiated midgut cells.

### 3.6. *Effects of gene silencing on* H. longicornis *reproduction*

The impact of RNAi on tick reproduction was investigated by determining the pre-oviposition period, egg mass weight and number of eggs laid per tick, egg conversion ratio, REI, number (%) of eggs hatched and RFI. All these phenotypic parameters of reproduction were significantly affected by RNAi treatment, especially in *HlLgm* and *HlLgm2* gene knock-down ticks. Some ticks from control groups started ovipositing 4 days after repletion and ticks injected with *HlLgm* dsRNA and *HlLgm2* dsRNA separately, started laying eggs on day 5 post-repletion showing no significant variations in mean pre-oviposition periods among these groups. Interestingly, in ticks injected with a combination of *HlLgm* dsRNA and *HlLgm2* dsRNA, the onset of oviposition was on day 6 post-repletion and their mean preoviposition period was significantly ($P < 0.01$) longer than that of ticks of all other groups (Fig. 19A). Gene knock-down resulted in death of the engorged ticks where three (7.70%) *HlLgm* dsRNA injected and two (4.65%) *HlLgm2* dsRNA injected engorged ticks died without laying any eggs. Notably, the mortality of engorged ticks was significantly higher in *HlLgm* and *HlLgm2* dsRNA dually injected ticks (13, 16.88%) compared with the controls ($P < 0.05$). However, due to unknown reasons, one (2.70%) PBS injected, and one (1.30%) *malE* dsRNA injected engorged ticks died during incubation for oviposition. It was observed that eggs laid by all groups of ticks were normal in shape with a glistening appearance indicating that they were successfully coated with the secretion from Gene's organ. The mean egg mass weight and total number of eggs laid by the ticks injected with *HlLgm* dsRNA (108.15 ± 24.12 mg and 1,615.82 ± 360.61, respectively) and *HlLgm2* dsRNA (113.80 ± 23.20 mg and 1,700.95 ± 351.96, respectively) were significantly ($P < 0.01$) lower than those of the PBS injected (162.41 ± 23.31 mg and 2,450.84 ± 347.94, respectively) and *malE* dsRNA injected (159.91 ± 22.83 mg and 2,391.81 ± 340.77, respectively) groups. In the case of the combined *HlLgm* dsRNA and *HlLgm2* dsRNA injected group, the

mean egg mass weight (48.93 ± 17.14 mg) and the total number of eggs (730.11 ± 255.75) were significantly ($P < 0.001$) lower than those of control groups (Fig. 19B and C). Interestingly, the egg conversion ratio and REI of ticks injected with *HlLgm* dsRNA (54.78 ± 5.86% and 8.15 ± 0.88, respectively) and *HlLgm2* dsRNA (57.04 ± 6.97% and 8.60 ± 1.07, respectively) were almost similar to PBS (56.73 ± 5.17% and 8.50 ± 0.81, respectively) and *malE* dsRNA (58.13 ± 5.04% and 8.60 ± 0.76, respectively) injected control groups. These two reproductive parameters were significantly ($P < 0.01$) lower in the *HlLgm* and *HlLgm2* knock-down group of ticks (41.27 ± 10.90% and 6.16 ± 1.64, respectively) than those of all other groups (Fig. 19D and E). The most significant impact of *HlLgm* and *Hllgm2* gene silencing was on the embryogenesis and hatching of eggs. Successful embryogenesis and hatching of eggs, showing a uniformly constant rate of hatchability and RFI, was detected in eggs laid by the PBS treated (98.80 ± 1.25% and 8.4 ± 0.78, respectively) and *malE* dsRNA treated (98.83 ± 1.47% and 8.5 ±0.77, respectively) control ticks. The egg hatchability and RFI were significantly ($P < 0.05$) affected in individually *HlLgm* dsRNA (85.60 ± 3.56% and 7.6 ± 1.08, respectively) and *HlLgm2* dsRNA (88.15 ± 4.1% and 7.6 ± 1.08, respectively) introduced ticks compared with controls. Strikingly, the majority of the eggs oviposited by engorged females injected with combined *HlLgm* dsRNA and *HlLgm2* dsRNA failed to hatch and the mean hatchability (42.35 ± 30.92%) and RFI (2.88 ± 1.25) were significantly lower compared with controls ($P < 0.001$) and other RNAi groups (Fig. 19F and G). These eggs showed an aberrant phenotype with an undifferentiated mass inside and the embryos did not develop within these eggs (Fig. 20). The eggs that failed to hatch eventually dried up and shriveled after incubation at 25 °C /95% relative humidity for 4 weeks. These results clearly show that *HlLgm* and *HlLgm2* play important roles in oviposition and RFI in *H. longicornis*.

## 4. Discussion

Blood-meal digestion in ticks occurs as a slow intracellular process in the midgut epithelium (33), although the molecular basis of *in vivo* blood digestion in ticks is yet to be elucidated. It is reasonable to speculate that specific gene-products are involved in blood-meal digestion in ticks, identification of which is important not only to understand the biology of ticks but also to evaluate them as the suitable targets for the development of chemotherapy/vaccines.

The knock-down of legumain genes in adult *H. longicornis* (*HlLgm* and *HlLgm2*) impacted markedly on its midgut tissues and was characterised by atrophy, disruption of normal cellular development and differentiation and damage of the gut tissues. In ixodid ticks, the midgut epithelium follows a specific cellular cycle while feeding. During the preparatory phase or slow feeding period (1–3 days post-attachment) when continuous digestion of the blood-meal takes place, there are rapid cellular changes in the midgut epithelium. The prodigest cells transform into sessile digest cells and later into residual sessile digest cells. In the rapid feeding period, for a further 2 days, when digestion is limited, most of the active sessile and residual sessile digest cells slough off into the lumen (3, 136). A recent report shows that legumain controls the extracellular matrix remodelling in mouse renal proximal tubular cells through degradation of fibronectin, a component of the extracellular matrix (104). Overexpression of legumain under stress conditions such as tumour hypoxia leads to increased tumour progression, angiogenesis and metastasis (95). Angiogenesis is the formation of new blood vessels which is essentially dependent on proliferation of endothelial cells and is a normal process in growth and development, as well as in wound healing (48). Modulation of angiogenesis and tumour growth by mammalian legumains indicates its intensive involvement in cellular growth and

differentiation. It is plausible that ticks have molecules which specifically modulate orderly proliferation and differentiation of midgut cells during feeding. The present study revealed that there was disruption of midgut cellular proliferation during feeding in legumains knock-down ticks, whereas the midgut cells underwent normal proliferation and differentiation (3) in the control ticks. Furthermore, legumains were mostly expressed by the proliferating, differentiated, mature midgut cells in the control ticks. From these observations, it is reasonable to speculate that legumains may play a vital role in maintaining midgut epithelial cell integrity and remodelling in ticks. However, the precise molecular pathway is yet to be defined. Disruption of midgut cellular developmental events and damage of midgut tissues by RNAi suggests that legumains may play roles in combating host defense systems and in gut-associated disease transmission since the tick midgut and its epithelial lining is a major physical barrier between the tick and the host defense mechanisms (3) and tick midguts and salivary glands are major sites of pathogen infection, development and transmission (87).

Silencing of legumain genes resulted in perturbation of feeding leading to significant changes in feeding period, repletion, body weight gain and survival of the feeding and engorged ticks. Failure of ticks to feed to repletion and significant reduction in engorged body weight of legumain-silenced ticks support the speculation that HlLgm and HlLgm2 are involved in blood-feeding and the Hb digestion cascade in *H. longicornis*. These findings are also in accord with the host Hb-degrading function of the orthologus legumain, IrAE in the midgut of the haematophagous tick, *I. ricinus* (131). The causes of death of the feeding and engorged ticks are not clear but they might be due to extensive damage of the midgut tissues or blocking/inactivation of legumain-induced biological functions of other protease(s) essential for tick survival.

The most pronounced impacts of legumain RNAi was on tick reproduction. RNAi treatment impacted on *H. longicornis* oviposition, fecundity, embryogenesis and hatchability of eggs. The development of egg masses was directly related to the amount of blood imbibed by individual ticks. It was noticed that the egg conversion ratio and REI of individually *HlLgm* dsRNA and *HlLgm2* dsRNA injected groups were not affected by RNAi when compared with those of control ticks. Assuming one larva from one egg, a significant impact of RNAi was on the number of eggs laid by individual ticks. However, the ultimate reproductive success of each tick depends on the number of progeny it can produce and it serves as the driving force behind tick infestations and transmission of diseases. Decreased hatchability and RFI in the RNAi treated groups compared with those of the control ticks clearly indicate the critical roles of legumains in tick embryonic development. Simultaneous knock-down of two legumains impacted more on tick feeding, reproduction and survival than when the genes were silenced individually. Death and failure of more individuals to reach repletion and the dramatic falls in engorged body weight, egg conversion ratio, number of eggs laid, hatchability and RFI in the group with two legumains silenced suggest the synergistic effects of RNAi. These results also further reinforce the role of legumains in the modulation of tick feeding and reproduction. The disruption of oviposition and hatching in RNAi groups of ticks may be due to disturbances in oocyte development (vitellogenesis) and embryogenesis. Recently, it has been reported that, vitellogenesis, the central event in reproduction, is regulated by the ingested blood-meal in anautogenous mosquitoes (10). As with mosquitoes, vitellogenesis is also induced in response to a blood-meal in adult female ticks (137). Pertinently, the absorbable amino acids of Hb digestion in ticks serve as raw materials and a trigger for vitellogenesis as it has been described for mosquitoes (10, 61). Reduced egg mass and impaired embryogenesis in RNAi treated

groups of ticks are obviously due to less blood intake and interference in Hb digestion to absorbable amino acids resulting from legumain deletion. However, ticks have several proteases that act in a cascade to effect complete digestion of blood-meal (130). It is likely that expression of multiple midgut-specific proteases would have to be ablated simultaneously to abolish tick feeding and reproduction. Recombinant HlLgm and HlLgm2 proteins share almost similar biochemical properties and digest BSA and Hb *in vitro* in the same manner (Chapter 3). It was expected that there would be functional compensation by the homologue in case of individual silencing of either *HlLgm* or *HlLgm2*. It is clear that this expected functional compensation was hampered due to off-target silencing of the homologue.

The data above suggested highly specialised and conserved biological functions for legumains. RNAi experiments provided evidences that legumains play key roles in mediation of blood-feeding, digestion, midgut cellular remodelling and, in reproduction including vitellogenesis and embryo development in *H. longicornis*. Deletion of *HlLgm* and *HlLgm2* genes in *H. longicornis* would likely impact on both feeding and reproductive ability of ticks, thereby reducing the field population size and hence disease transmission.

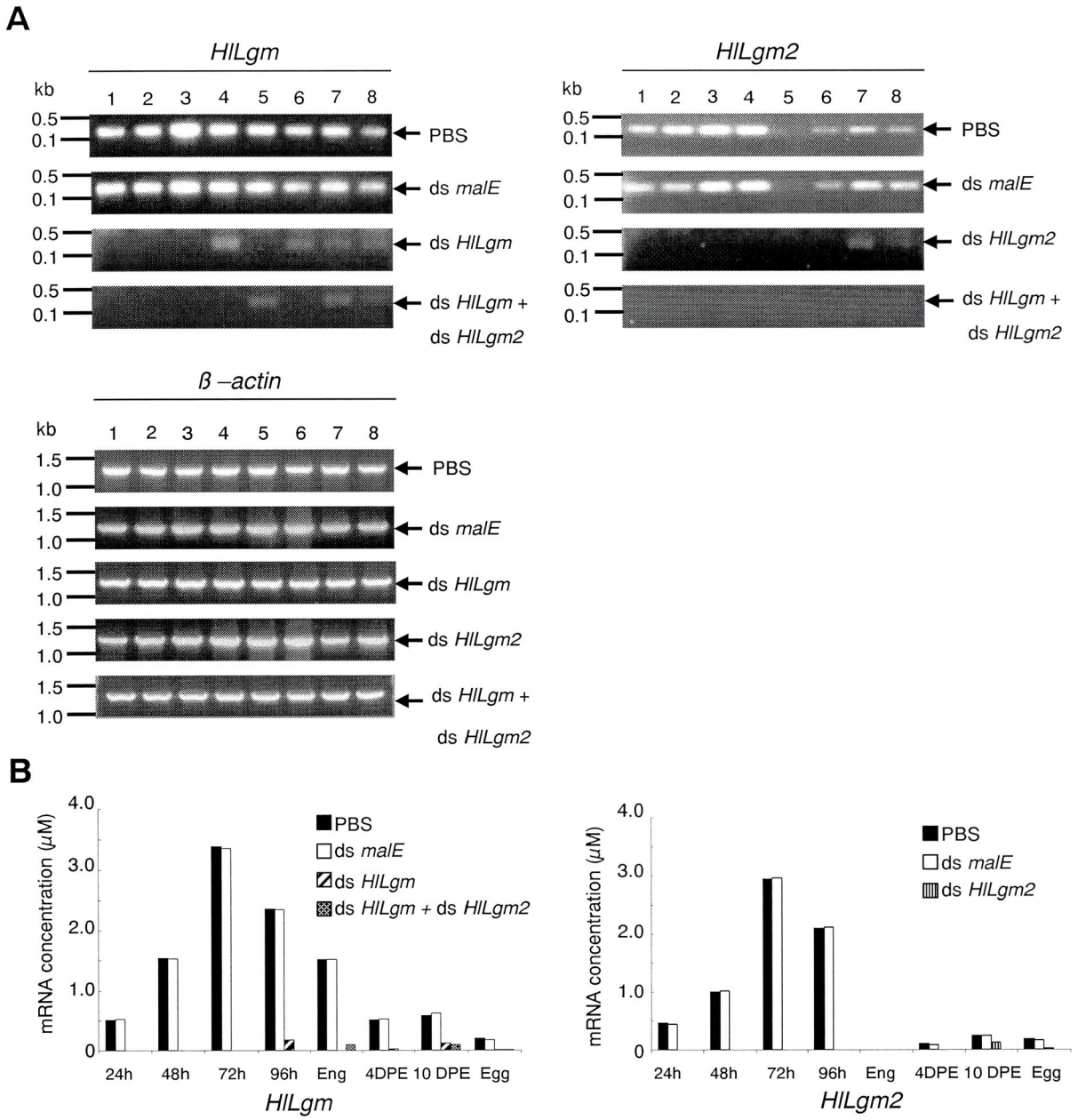


**Fig. 14. Silencing of *HlLgm* and *HlLgm2* genes from adult *Haemaphysalis longicornis* by treating with double-stranded RNA (dsRNA)**

(A) Reverse transcription (RT)-PCR analysis. The control groups of ticks were injected with either PBS alone or PBS containing 1 μg of *Escherichia coli malE* dsRNA. The RNA interference groups of ticks were treated with 1 μg *HlLgm* dsRNA and 1 μg *HlLgm2* dsRNA either individually or in combination. The level of expression of *HlLgm*- and *HlLgm2*- specific mRNAs are indicated by arrows. Actin is shown as an internal control. Lane 1, 24 h post-attachment; lane 2, 48 h post-attachment; lane 3, 72 h post-attachment; lane 4, 96 h post-attachment; lane 5, engorged; lane 6, 4 days post-engorgement; lane 7, 10 days post-engorgement; lane 8, eggs. (B) Quantitative RT-PCR was performed using the same total RNA and the same primers specific for *HlLgm* and *HlLgm2* as in (A).

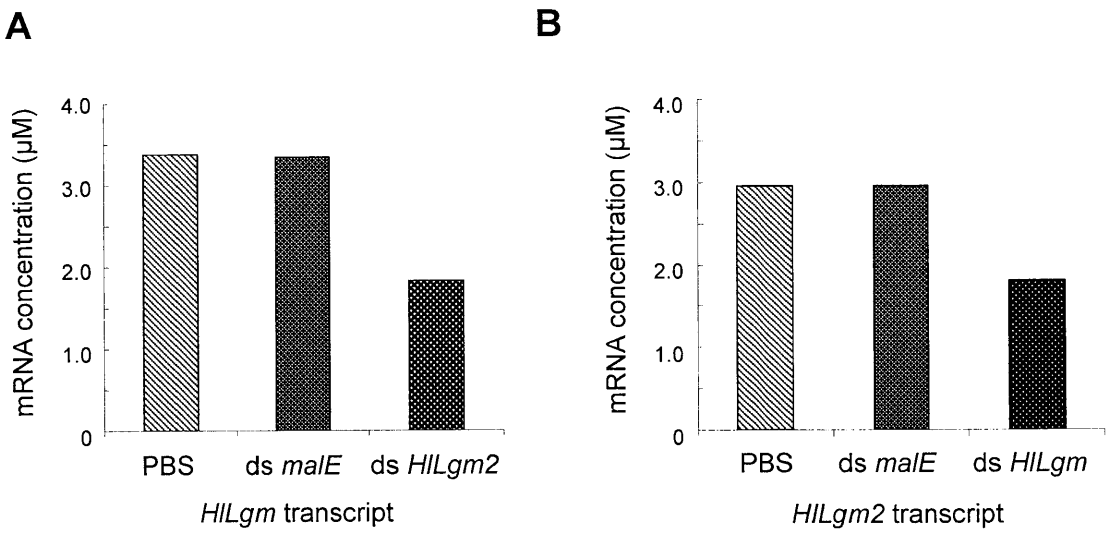


**Fig. 15. Off-target silencing of *HlLgm* and *HlLgm2* transcripts in RNA interference groups compared with control ticks**

(A) Off-target silencing of *HlLgm* demonstrated by quantitative RT-PCR analysis using the total RNA isolated from 72 h fed *HlLgm2* double-stranded RNA (dsRNA) treated ticks and the primers specific for *HlLgm*. (B) Off-target silencing of *HlLgm2* transcript demonstrated by the same way using the total RNA from *HlLgm* dsRNA treated ticks and primers specific for *HlLgm2*.

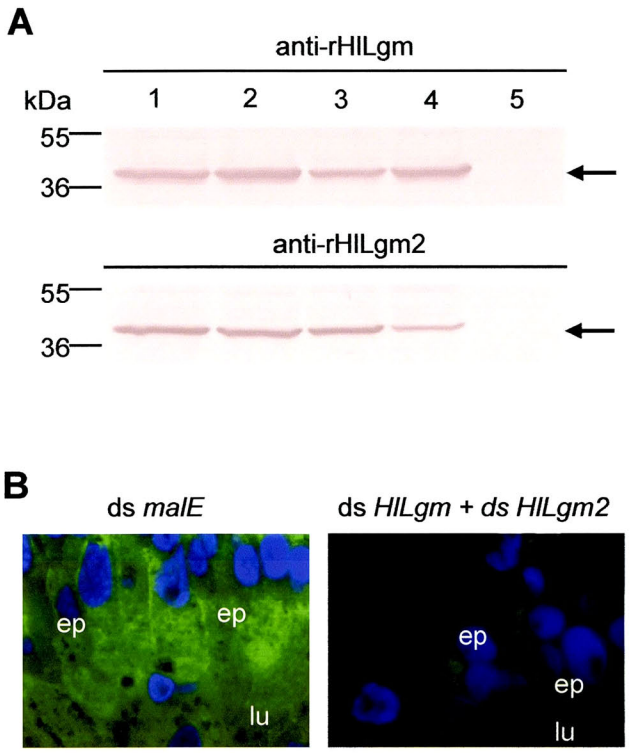


**Fig. 16. Translational effects of *HlLgm* and *HlLgm2* gene knock-down by introduction of dsRNA**

Ticks were injected with PBS alone, *malE* dsRNA or *HlLgm* and *HlLgm2* dsRNA either individually or in combination. (A) Comparison of endogenous *HlLgm* and *HlLgm2* expression in between the control and RNAi treated groups by Western blot analysis. Lane 1, ticks injected with PBS alone; lane 2, ticks injected with *malE* dsRNA; lane 3, ticks injected with *HlLgm* dsRNA; lane 4, ticks injected with *HlLgm2* dsRNA; lane 5, ticks injected with a combination of *HlLgm* and *HlLgm2* dsRNA. Arrows indicate endogenous legumain. (B) Immunofluorescence analysis of the midgut tissues. Midgut tissues from partially fed (72 h) ticks of the *malE* dsRNA treated group and combined *HlLgm* and *HlLgm2* dsRNA treated group. ep, midgut epithelial cells; lu, midgut lumen. Data reported here is from one of the three replicates.

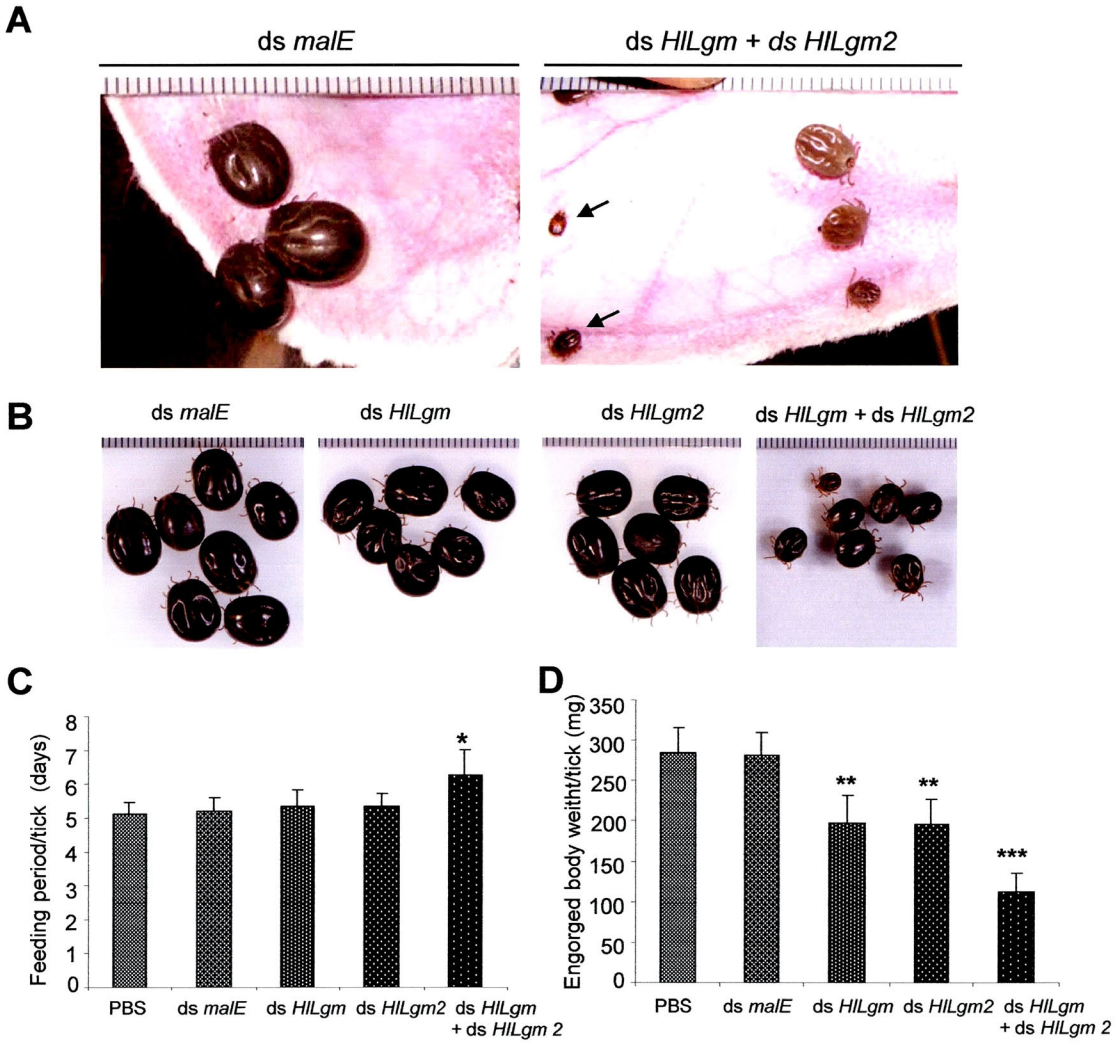


**Fig. 17. Effects of *HlLgm* and *HlLgm2* gene silencing on blood feeding and survival of adult *H. longicornis***

Ticks were injected with PBS (control) or 1 µg of *malE* dsRNA (control) or 1 µg each of *HlLgm* and *HlLgm2* dsRNA either individually or in combination and allowed to feed on rabbit for 7 days. (A) Disruption of blood-feeding and death of feeding ticks in the *HlLgm* and *HlLgm2* knock-down group compared with control ticks (*malE* dsRNA). Photographs were taken on day 5 post-infestation. Arrows indicate the dead ticks. (B) Comparison of the phenotype of the engorged ticks from control and RNAi treated groups. The replete and spontaneously dropped ticks (from all groups) and those that were attached at 7 days post-infestation (combined *HlLgm* and *HlLgm2* dsRNA injected group) were forcibly collected. Scale: 1 unit = 1 mm. (C) Blood feeding period (days) toward repletion. Blood feeding period was calculated only for the engorged ticks from the time of attachment to spontaneous dropping-off the host on repletion. (D) Engorged body weight. Bars represent mean values and the error bars indicate S.D. Asterisks (*) denote the difference compared with the control group is significant as determined by Student's *t*-test with unequal variance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

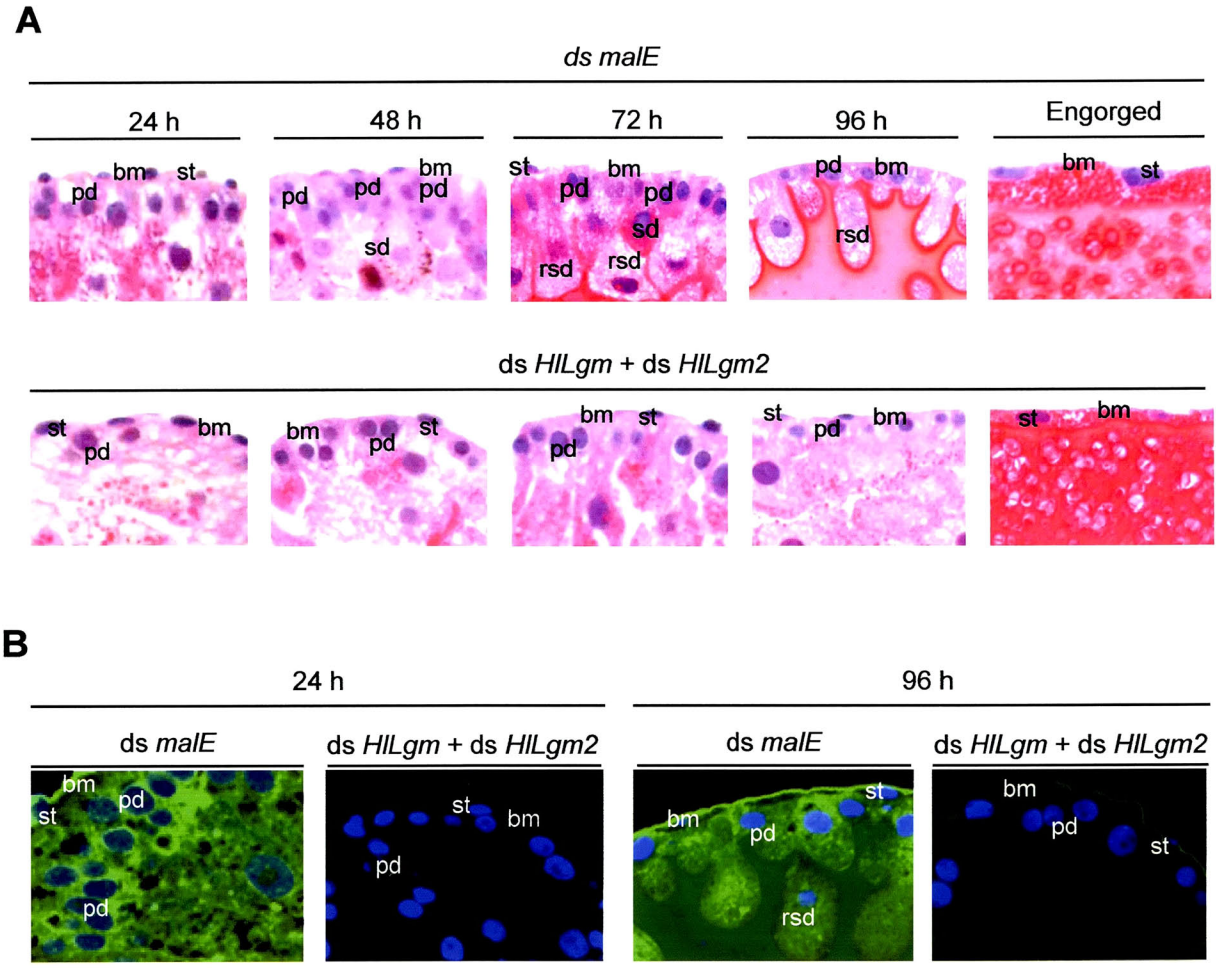


**Fig. 18. Endogenous legumain expression by midgut cells during blood feeding and impact of legumain genes silencing on midgut cellular remodelling in adult *H. longicornis*.**

The midgut tissues were collected from the *malE* dsRNA injected control group and combined *HlLgm* and *HlLgm2* dsRNA group during different phases of feeding (24 h, 48 h, 72 h, 96 h) and engorgement. (A) Impacts of *HlLgm* and *HlLgm2* gene knock-down on midgut cellular remodelling. On attachment and progression of feeding, the midgut epithelial cells in the control group followed the normal cellular cycle characterized by massive proliferation and differentiation of cells. Compared with controls, no cellular remodelling in the midgut epithelium, but rather damage of midgut tissues, was observed in RNAi treated ticks. (B) Immunofluorescent staining showing endogenous legumain expression by midgut epithelial cells during blood-feeding. bm, basal membrane; st, stem cells; pd, predigest cells; sd, sessile digest cells; rsd, residual sessile digest cells.

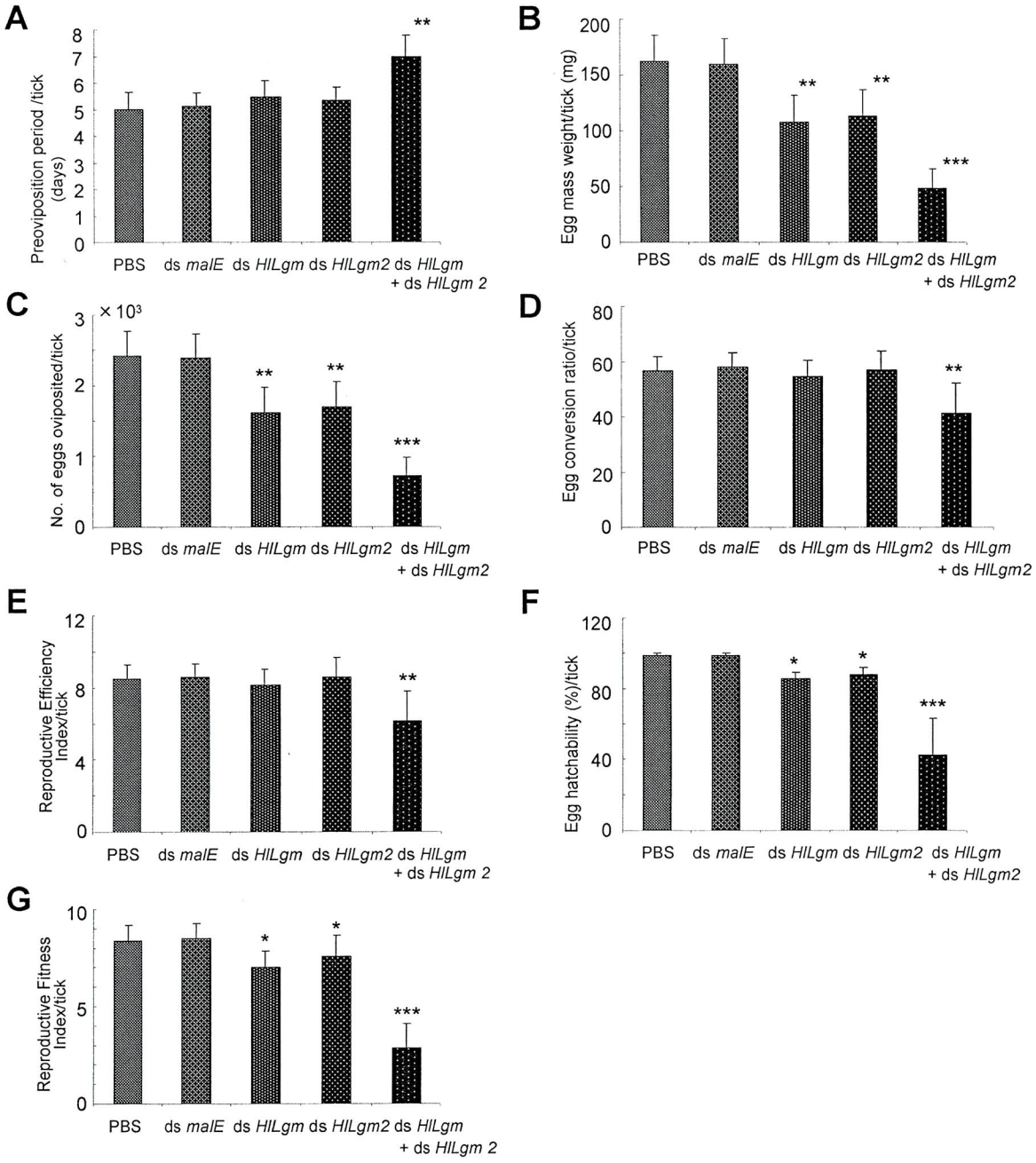


**Fig. 19. Impact of legumain gene knock-down on reproduction of adult female *H. longicornis***

The ticks that spontaneously dropped-off the host after engorgement were collected and incubated at 25 ° C and 95% relative humidity. (A) Pre-oviposition period (days). (B) Egg mass weight (mg)/tick. (C) Number of eggs oviposited/tick. (D) Egg conversion ratio/tick. (E) Reproductive Efficiency Index (REI)/tick. (F) Egg hatchability (%)/tick. (G) Reproductive Fitness Index (RFI)/tick. The bars represent mean values and the error bars indicate S.D. Asterisks (*) denote the difference compared with the control group is significant as determined by Student's *t*-test with unequal varianceand by $\chi$ 2-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

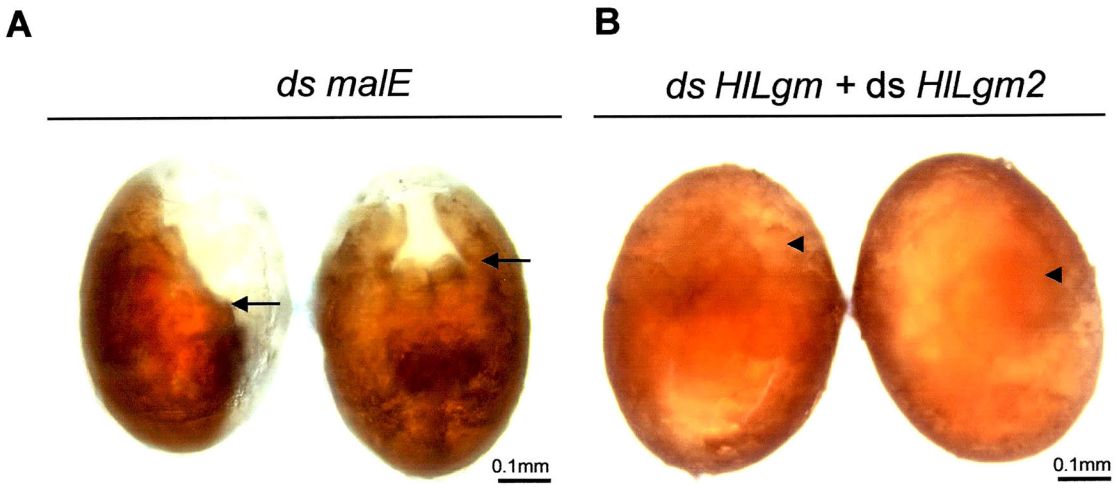


**Fig. 20. Effects of RNA interference treatment on embryogenesis**

(A) Representative eggs from female *H. longicornis* injected with *malE* dsRNA (A) or a combination of *HlLgm* and *HlLgm2* dsRNA (B) 18 days after oviposition. Arrows indicate normal development of embryo, arrowheads indicate an undifferentiated mass.

# General Discussion and Conclusions

Ticks are non-permanent but obligate parasites of most of the terrestrial vertebrates requiring a blood-meal for all active instars (larvae, nymphs and adults). The blood-meal ingested from the hosts largely consists of red blood cells. Following haemolysis, Hb is released in the tick midgut lumen (2, 5) which is the most important source of nutrients for ticks. In ticks, Hb is metabolized via catabolism and anabolism in the midgut cells by the proteases (58). Proteases (proteinases) refer to a group of naturally occurring enzymes in all organisms, involved in a multitude of physiological functions including digestion of food proteins. Recently varieties of blood-meal digesting proteases such as, cubilin-related serine proteases (101), aspartic protease (20), leucine aminopeptidase (65), serine carboxypeptidase (105), and cysteine proteases (142, 143) have been identified from *H. longicornis*. The potential of the proteases in Hb digestion suggests that there exists a multi-enzyme cascade to complete digestion of Hb into absorbable amino acids in tick. More recently, an autophagy related gene hypothesized to be involved in digestion of Hb or cytoplasmic proteins during off-host period facilitating survival of ticks for long period of starvation (145) and a Vg receptor essential for oogenesis and transovarial transmission of diseases (19) have been reported from *H. longicornis*. In the present study, I have selected and characterized two legumain genes (*HlLgm* and *HlLgm2*) from the EST

data base constructed from midgut cDNA libraries of *H. longicornis* with the aim of exploring their roles in Hb digestion necessary for survival and reproduction of tick.

Legumain is an AEP the biochemical properties and functional significance of which have been described from a wide variety of organisms (25, 26, 32, 37, 62, 64, 83, 92, 98, 116, 124). The distinctive life-cycle pattern and feeding behaviour of ticks have led to the evolution of unique metabolic pathways which differ from those in other animals. It is speculated that exploration of biochemical properties of tick legumains will contribute to our knowledge about tick biology including blood digestion and reproduction and would help in evaluating the legumains as drug targets or vaccine candidates for the purpose of better control of ticks and tick-borne diseases.

Blood-meal digestion occurs in the midgut in ticks. Endogenous HlLgm and HlLgm2 were expressed in all developmental stages and the expressions were up-regulated by the host blood-feeding process which obviously suggests the critical relationship of legumains in blood proteins digestion. Blood digestion is rapid during the growth phase of feeding which lasts for 1.5 to 2.5 days of feeding in *H. longicornis* nymphs (88) and the proteolytic activities reaches to the peak at 72 h of feeding in adult ticks (18). Blood digestion during the initial stage of feeding (preparatory phase) and in extension phase when ticks feed rapidly to engorge is very low (5). The expression profiles of legumains during feeding (Chapter 2 and 4) and the strict localization of legumain transcripts in the midgut tissues (Chapter 2) substantiate the postulation that the legumains act as blood protein digesting enzymes in *H. longicornis*.

HlLgm and HlLgm2 exhibited typical AEP/legumain features for hydrolyzing the specific synthetic substrates, for their sensitivity to the specific inhibitors (1, 26, 36, 116), and metal ions (154). The temperature profiles of the enzymes suggest their adaptation with the host

body physiology (~ 37 °C) for digesting blood proteins while feeding as well as their ability to proteolyse in cold or in xeric environmental conditions in habitats. Other proteases from *H. longicornis* have also been reported to show wide temperature dependency (65, 102). HlLgm and HlLgm2 were shown to be more active at neutral to alkaline conditions (Chapter 3) and their pH optima (pH 7) and DTT sensitivity suggest that, biochemically HlLgm and HlLgm2 are more closely related with the haematophagous *S. mansoni* (36) and *H. contortus* (116) legumains. Both HlLgm and HlLgm2 failed to cleave intact Hb but effectively digested cathepsin D degraded Hb (Chapter 3). Proteolytic enzymes frequently function not only as individual enzymes but also in cascades or networks, and in invertebrates the aspartic protease and cysteine proteases play the roles (12, 41). Identical semi-ordered catalytic pathway of Hb digestion has been described in case of Hb proteolysis in haematophagous helminths (34, 99, 152) and in human malaria parasite (44). In *P. falciparum*, Hb digestion is initiated in the food vacuoles by the aspartic protease, termed plasmepsins (49, 55). The vacuolar cysteine proteases, termed falcipains, digests native proteins and denatured globin (125, 127, 129). After initial digestion with plasmepsins and falcipains, the metalloprotease, falcilysin, digests globin fragments even further (44). In haematophagous helminths, the AEPs (legumains) synergize with cathepsin B and L to effect Hb digestion (41). Hb digestion takes place within the digestive vesicles of tick gut cells (33). The membrane-bound natures and the localization of the endogenous proteins in some intra-cellular compartments in the midgut epithelial cells (Chapter 2) convincingly recognize legumains as haemoglobinases. The haemoglobinolysis assay also suggests that legumains take part in the final stage of Hb digestion into absorbable small peptides and amino acids in a Hb digestion cascade in arthropods. The selectivity of HlLgm and HlLgm2 in choosing the asparaginyl bonds of BSA for hydrolysis (at $Asn^{506}$ and Asn573 only, Chapter 3)

and the autocatalyzed cleavage for removal of their C-terminal extensions (Chapter 2) to convert into mature active proteins as the unique features of the peptidases of legumain family (26–28, 62, 93, 131) furthermore conforms HlLgm and HlLgm2 as the asparaginyl endopeptidases.

Death of the ticks, inability of ticks to replete, significant reduction in engorged tick body weight, and marked impact on reproductive events in legumains knock-down ticks (Chapter 4) strongly support the speculation that HlLgm and HlLgm2 serve as tick blood-feeding and digestion modulating enzymes, and play critical roles in vitellogenesis and tick embryonic development. In mosquitoes, blood-meal ingestion induces the translation of Hb digesting enzymes in the midgut (111) and the level of hemolymph amino acids and the stimuli by them are crucial for vitellogenic genes expression (10, 61, 144). Oogenesis in ticks is induced by blood-meal. The amino acids level in the hemolymph along with the hormone, 20-hydroxyecdysone (20E) stimulates the synthesis of vitellogenin (Vg) (126, 137). Vg protein secreted in the tick hemolymph is absorbed by the ovary up-taking by a Vg receptor (19, 122). Reasonably, the amino acids of Hb digestion play vital roles in tick body metabolism at multiple levels including reproduction. It is very likely that several other proteases in Hb digestion cascade (130) would play roles for functional compensations of legumains. However, lower fecundity and disrupted embryogenesis in RNAi treated groups of ticks reflects decreased free amino acids levels in the haemolymph resulted from reduced blood ingestion and impaired Hb digestion due to legumains deletion. Impact of RNAi on tick midgut tissues revealed an interesting cycle of interdependence between legumain expression and cell growth and maturation (Chapter 4) that take place during feeding (3, 136). Cysteine proteases from *H. longicornis* midgut have been reported to play critical roles in tick innate immunity against *Babesia* parasite development, and bacterial and fungal growth resulting in killing of the

pathogens (140, 141, 143). Peptides of Hb digestion exert antimicrobial activity in the tick midgut and hemolymph and suggest their possible use in tick defense mechanism against microorganisms (47). Legumains might have vital roles directly or indirectly in evading host defense systems as well as in microbial disease transmission by ticks through modulation of gut cell proliferation and differentiation and Hb digestion.

In conclusion, the present study suggested that two novel genes, *HlLgm* and *HlLgm2* encoding the functional asparaginyl endopeptidases/legumains from the ixodid tick *H. longicornis* belong to the legumain family C13. The enzymes are strictly localized in the midgut epithelia of the ticks and might be critically involved in mediation of host blood-feeding and in the final stage of Hb digestion cascade to release free amino acids from blood-derived proteins, particularly Hb. Furthermore, legumains may play crucial roles in midgut cellular remodelling and reproduction, most critically, in vitellogenesis and embryo development in *H. longicornis*. The structural and biochemical features of HlLgm and HlLgm2 may help in designing site-directed mutagenesis with a view to probing the functionally important residues. Deleterious effects of legumain knock-down on gut architecture also suggest that legumains may be used as therapeutic targets. Understanding the mechanisms underlying legumain-mediated blood-feeding, midgut cellular remodelling and amino acid signalling relating to vitellogenesis are of paramount importance to develop legumain-based novel strategies for controlling ticks and tick-mediated disease transmission.

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# 学 位 論 文 要 旨

氏　　名　　ALIM, Md. Abdul

題　　目　　Novel asparaginyl endopeptidases/legumains from the hard tick *Haemaphysalis longicornis* and their roles in tick blood-feeding and embryogenesis
（フタトゲチマダニ新規アスパラギンエンドペプチダーゼ/レグマインの吸血および胚発生における役割）

論文要旨

Ticks are ectoparasites that live entirely on haematophagy and it is believed that ticks acquire nutrition through digestion of blood protein, haemoglobin (Hb) by evolutionarily gained proteolytic mechanisms. However, the molecular basis of Hb digestion in ticks is still unclear. This study was performed to characterize two novel cDNAs encoding the asparaginyl endopeptidases/legumains (HlLgm and HlLgm2) from the midgut of the ixodid tick *Haemaphysalis longicornis* with a view to explore their possible roles in Hb digestion. The biochemical profiles and the functional analyses revealed that the enzymes are involved in blood-meal digestion and play modulatory roles in midgut cellular remodelling and embryogenesis in *H. longicornis*.

In Chapter 1, *HlLgm* and *HlLgm2* genes were cloned from the midgut cDNA library of adult female *H. longicornis*. Cloning and partial characterization of *HlLgm* and *HlLgm2* genes showed that *HlLgm* cDNA was 2,335 base pairs (bp) long and the proprotein consists of 442 amino acid residues with a predicted molecular weight (MW) of 48.84 kDa and an isoelectric point (pI) of 6.25. The full-length *HlLgm2* cDNA sequence is 1387 bp long and the proprotein consists of 442 amino acids with a predicted MW of 49.63 kDa and a pI of 7.05. Both the deduced HlLgm and HlLgm2 sequences possess a single peptidase C13 family domain with evolutionarily conserved regions and active catalytic residues ($His^{155}$ and $Cys^{196}$). Sequence analysis revealed that HlLgm and HlLgm2 shared high sequence similarities with arthropods, mammals, helminths and plant legumains. Phylogenetically HlLgm and HlLgm2 are most closely related to the hard tick, *I. ricinus* legumain and belong to the arthropod and mammalian subfamily representing a separate group from haematophagous helminth legumains.

In Chapter 2, the transcriptional and translational profiles of legumains in relation to different developmental stages and blood-feeding phases in *H. longicornis*, and localization of endogenous legumains in adult ticks were determined. *HlLgm*- and *HlLgm2*-specific transcripts were well expressed in all developmental stages of *H. longicornis* as revealed by reverse transcription (RT)-PCR and quantitative RT-PCR.

Noteworthy, HlLgm2 mRNA was expressed at a lower level during all phases of blood-feeding in larvae, nymphs and adults, and also in eggs. More strikingly, there was no expression of *HlLgm2* mRNA in engorged adult ticks. Endogenous legumains (HlLgm and HlLgm2) were detected in soluble extracts of larvae, nymphs and adults. Western blotting using tick extracts probed with polyclonal antibodies raised against recombinant HlLgm (rHlLgm) and recombinant HlLgm2 (rHlLgm2) revealed ~38 kDa bands for both endogenous HlLgm and HlLgm2. Immunohistochemistry and RT-PCR analyses revealed that legumains were strictly localized in the midgut of adult ticks. Immunofluorescent staining showed that endogenous legumains were expressed intracellularly in some digestive vacuoles of the midgut cells. The legumains expressions were up-regulated by blood-feeding process, indicating their involvement in blood protein digestion.

In Chapter 3, the expression of recombinant legumains in *Escherichia coli* and their biochemical properties have been determined. The rHlLgm and rHlLgm2 efficiently hydrolyzed the legumain-specific synthetic substrate in neutral to alkaline conditions and in a wide range of temperature, optimally at pH 7 and at 37 °C. The activities of the recombinant proteins were potently inhibited by the thiol blocking reagents, iodoacetamide and N-ethylmaleimide and also by $Fe^{+2}$, $Cu^{+2}$, $Co^{+2}$ and $Ni^{+2}$. However, the inhibitors for other proteinases had very minimal effects on rHlLgm and rHlLgm2 activity. HlLgm and HlLgm2 were shown to possess a unique feature of having an autocatalysed cleavage at $Asn^{365}$ and $Asn^{364-365}$, respectively, at the C-terminus of both endogenous and recombinant enzymes. Both the recombinant enzymes digested bovine serum albumin (BSA) showing their strict specificity for hydrolysis of the peptide on the carboxyl side of the asparagines, as demonstrated by internal amino acid sequence analysis of proteolytic products. rHlLgm and rHlLgm2 could not degrade intact bovine Hb but readily digested cathepsin D degraded small Hb peptides indicating that legumains act in a cascade to effect a complete digestion of Hb in ticks. These results suggested that legumains play pivotal roles in host blood-meal digestion and possibly in the final process of Hb digestion in ticks.

In Chapter 4, the biological roles of HlLgm and HlLgm2 *in vivo* have been investigated by employing RNA interference (RNAi) technique. Silencing of legumain genes through injection of *HlLgm*- and *HlLgm2* gene-specific dsRNAs into unfed adult female *H. longicornis* caused death of the feeding ticks, failure of ticks to reach repletion and significant reductions in engorged tick body weight. Deletion of legumains resulted in damage of the midgut tissues and disruption of normal cellular remodelling during feeding. RNAi also caused significantly delayed onset of oviposition, reduced number of eggs and most strikingly, structurally deformed eggs that failed to hatch suggesting imperfect embryogenesis. Synergistic impacts of RNAi were reflected on all evaluated parameters when *HlLgm* and *HlLgm2* were silenced together. These findings suggest that legumains play modulatory roles in blood-feeding and digestion, midgut cellular remodelling and embryogenesis in *H. longicornis*.

The results obtained in the present study indicate that HlLgm and HlLgm2 are functional asparaginyl endopeptidases in the midgut of *H. longicornis*. They are critically involved in host blood-feeding and digestion, and midgut cellular remodelling in ticks. HlLgm and HlLgm2 also play crucial roles in tick reproduction, most critically in vitellogenesis and in embryo development in *H. longicornis*. The results also suggest that HlLgm and HlLgm2 may be used as novel vaccine- or drug-targets for controlling ticks and tick-borne disease.

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# 学 位 論 文 要 旨

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論文要旨

マダニは一生を通じて宿主動物の血液のみを生存基盤とする寄生性節足動物であり，宿主血液中のヘモグロビンを独自に獲得した消化機構により分解し栄養源にすると考えられている。しかしながらマダニの血液消化の分子基盤に関して詳細はいまだ不明である。本研究では，フタトゲチマダニ中腸より分離された新規アスパラギンエンドペプチダーゼ/レグマイン（HlLgm, HlLgm2）遺伝子と遺伝子産物の分子性状について検討した。生化学的機能解析により，これら二つの分子は，マダニ中腸における血液消化に関与する機能を有することが示唆された。

第１章では，フタトゲチマダニ cDNA ライブラリより，レグマイン遺伝子 *HlLgm* および *HlLgm2* を分離した。*HlLgm* cDNA は全長 2,235bp で推定遺伝子産物は 442 アミノ酸残基から構成され，推定分子量は 48.84kDa，等電点は 6.25 であった。*HlLgm2* cDNA は全長 1,387bp からなり，推定産物は 442 アミノ酸残基，分子量 49.63kDa，等電点 7.05 の蛋白質であると考えられた。HlLgm, HlLgm2 は共にペプチダーゼ C13 ファミリーのドメインを含み，ペプチダーゼ活性に不可欠な $His^{155}$ および $Cys^{196}$ 残基が保存されていた。HlLgm, HlLgm2 の推定アミノ酸配列は，節足動物，哺乳類，蠕虫，植物由来レグマインと高い相同性を示したが，系統学的解析においては，マダニ *Ixodes. ricinus* のレグマインと最も近縁であり，節足動物および哺乳類由来レグマインのグループに属し，住血性蠕虫由来レグマインとは異なることが示唆された。

第 2 章では，マダニ各発育期および吸血期間における内在性レグマイン遺伝子の発現，さらに内在性タンパク質のマダニ組織内における発現について検討した。reverse transcription (RT) およびリアルタイム PCR によって，*HlLgm, HlLgm2* はフタトゲチマダニの全ての生育ステージでの発現が確認されたが，*HlLgm2* の発現は，幼・若・成ダニの吸血時と卵のステージにおいては低く，また，飽血成ダニでは全く発現がみられなかった。ウエスタンブロットによって，HlLgm, HlLgm2 内在性蛋白質は，幼・若・成ダニ抽出蛋白質の可溶性分画より，いずれも約 38kDa の分子として検出された。さらに，免疫組織化学と RT-PCR の結果から，内在性レグマインは成ダニ中腸に発現していることが示された。レグマインの発現は吸血時に増強されることから，血液成分の消化に関与することが示唆された。

第 3 章では，大腸菌発現による組換え体レグマインを用い，マダニレグマインの生化学的性状について検討を加えた。組換え HlLgm (rHlLgm)・組換え HlLgm2 (rHlLgm2) はレグマイン特異的基質に対し，中性-塩基性の pH 下，比較的広い温度領域において強い加水分解活性を示し，その至適 pH は 7，至適温度は 37℃であった。組換えレグマインのペプチダーゼ活性は，チオール結合阻害剤，ヨードアセトアミド，N-エチルマレイミドの他，$Fe^{+2}$，$Cu^{+2}$，$Co^{+2}$，$Ni^{+2}$によって顕著に阻害された。しかし，他のプロテアーゼ阻害剤に対しては感受性を示さなかった。HlLgm，HlLgm2 はそれぞれ $Asn^{365}$ と $Asn^{364-365}$ において自己触媒的にクリベージが起こるという特徴を持つことが示された。組換えレグマインは，ウシ血清アルブミンに対して加水分解を示した。また，通常状態のウシヘモグロビンを分解することは不可能であるが，カテプシン D によって処理されたヘモグロビン断片ペプチドに対しては加水分解活性を示した。以上の結果より，マダニレグマインは宿主血液成分の分解に不可欠な役割を持ち，中腸におけるヘモグロビン分解カスケードにおいて最終的な段階を担うものであることが示唆された。

第 4 章では，HlLgm，HlLgm2 の，マダニ個体内における機能について，RNAi の手法を導入することにより明らかにした。*HlLgm*，*HlLgm2* の dsRNA の血体腔への注入によって，死亡，飽血不能，飽血時体重の減少などが確認された。また，レグマイン遺伝子の発現抑制により，中腸組織の損傷，吸血時における中腸細胞の再構築の阻害などが惹起された他，産卵の遅延，産卵数の減少，形態異常卵（孵化不能）の産生などの現象も確認された。*HlLgm*，*HlLgm2* 両方の発現を阻害した個体では，上記の現象について相乗的な作用がみられた。これらの結果より，マダニレグマインはマダニの吸血及び消化について調節機能を持ち，中腸細胞の再構築や胚発生にも深く関与していることが示唆された。

本研究の成果より，HlLgm および HlLgm2 はフタトゲチマダニ中腸で機能するアスパラギンエンドペプチダーゼであり，吸血行動や血液消化，中腸細胞再構築に関与するのみならず，ビテロジェニン合成や胚発生など生殖過程にも不可欠な機能を持つことが考えられた。従って，これらのマダニレグマインは新規のマダニおよびマダニ媒介性感染症制圧法の創出における標的分子としても非常に有用な候補であると思われる。