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## Characterization of proteolytic enzymes expressed in the midgut of *Haemaphysalis longicornis*

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### Abstract

The proteolytic activities present in midguts of both fed and unfed *Haemaphysalis longicornis* were assessed by using the gelatin-substrate gel electrophoresis and inhibitor sensitivity analyses. Three predominant (116, 48 and 40 kDa) and two weak (55 and 60 kDa) proteinase bands were commonly expressed in both unfed and fed ticks, while a weak 80 kDa band was only present in fed ticks. Consistent with observations on other tick species, proteolytic activity against the gelatin substrate was observed only under acidic conditions. Inhibition studies against the gelatin substrate using a panel of inhibitors showed that the predominant proteolytic enzymes of 40 and 48 kDa molecular mass are cysteine proteinases. These results are discussed in the context of host vaccination as an alternative tick control method to the current use of chemical acaricides.

Key words : Cysteine proteinases, *Haemaphysalis longicornis*, midgut proteolytic enzymes.

### Introduction

Ticks represent one of the most important ectoparasites affecting the livestock industry worldwide. They affect livestock production, directly by their feeding activity and indirectly through transmission of pathogens. Host vaccination against tick infestation has been shown to be the most sustainable alternative tick control method to the current use of acaricides which has serious limitations such as contamination of livestock food products and quick development of tick resistance against acaricides<sup>11,21</sup>. However, for this strategy to succeed, identification of key tick molecules for use as tick vaccine antigens is essential<sup>5,13</sup>. The factors used to define a key tick vaccine target antigen are highly subjective.

However based on the criteria to select candidate tick vaccine antigens, first proposed by Elvin and Kemp<sup>5</sup>, an ideal antigen is one to which host antibodies gain access and that the antibodies can inhibit the physiological function of the antigen. Tick midgut proteolytic enzymes associated with the tick blood meal digestion may fit this criteria, in that, when the tick takes in the blood meal it also imbibes the host antibodies which will ultimately come into contact with these enzymes in the midgut. Despite the potential for immunological targeting of digestive proteolytic enzymes, molecules participating in tick blood digestion have not been identified<sup>1,6,9,22</sup>. At present, digestion of the blood meal by the tick is believed to occur in 2 phases. The first phase which occurs extracellularly in the lumen of the

midgut involves lysis of host red blood cells by hemolytic enzymes<sup>14)</sup>, while the second phase occurs intracellularly in the tick midgut cells following uptake of first phase products<sup>1,4)</sup>.

A limited number of studies have described the properties of tick midgut digestive enzymes *in vitro*<sup>2,3,15,19,20)</sup>. Based on evidence from these studies, Akov<sup>1)</sup> concluded that the major digestive proteolytic enzyme in the midgut of ticks, is similar to the mammalian cathepsin D, an aspartic proteinase mostly localized in lysosomes. In related studies, Vundla *et al.*<sup>22)</sup> purified two aspartic proteinases from midguts of *Rhipicephalus appendiculatus*, while Mendiola *et al.*<sup>9)</sup> purified an aspartic and cathepsin L-like cysteine proteinase from midguts of *Boophilus microphilus*.

As part of our ongoing project aimed at identifying potential molecules for use as target tick vaccine antigens, this study was undertaken to get some basic information on proteolytic enzymes expressed in the midguts of *Haemaphysalis longicornis*. This species is a three-host tick commonly infesting cattle, dogs and man and a vector of *Theileria* spp. as well as *Coxiella burnetii*<sup>17)</sup>. In Japan and other east Asian countries, this tick is a major vector of *T. sergenti/buffeli* which is one of economically important diseases in cattle<sup>10)</sup>. We have used the gelatin-substrate gel electrophoresis and inhibitor sensitivity analyses to characterize proteolytic enzymes present in midguts of both fed and unfed adult *H. longicornis*.

#### Materials and Methods

##### Tick dissection :

The ticks used in this study were obtained from a colony of *H. longicornis* maintained on Japanese white rabbits in our laboratory. Midguts were dissected from female ticks both unfed and those that fed for 48 and 96 h. Tick dissection was carried out as described elsewhere<sup>14)</sup>. Briefly, under a light microscope,

ticks were submerged in sterile phosphate-buffered saline (PBS, 10mM sodium phosphate, 0.15M NaCl, pH 7.4) and held down with a soft tissue forceps. The dorsal cuticle was cut out and midguts were separated from other organs with the help of an 18 gauge needle. The dissected midguts were washed once in PBS and stored at -80°C until use.

##### Gelatin-substrate gel electrophoresis analysis :

Gelatin-substrate gel electrophoresis analysis was carried out according to methods described by others<sup>7,8,18)</sup>. The dissected midgut samples were solubilised in non-reducing sample buffer [0.5M Tris-HCl, pH 6.8, 2% (W/V) sodium dodecyl sulfate (SDS), 20% glycerol and 0.01% bromophenol blue] and left to stand at room temperature for at least 10 min before loading onto the gel. Proteins were fractionated in 10% polyacrylamide gels co-polymerized with 0.2% gelatin (W/V). Following the SDS polyacrylamide gel electrophoresis (SDS-PAGE), the separated proteinases were reactivated by washing the gel slices over 30 min in 3 changes of 2.5% Triton-X 100 to remove SDS. Subsequently gel slices were incubated overnight at 37°C, in one of the following buffers, 0.1M sodium acetate buffer, pH 4.0, 0.1M sodium phosphate buffer, pH 6.5, and 0.1M Tris-HCl buffer, pH 8.4. Proteolytic activity was detected by presence of a non-stained zone after staining with 0.1% (W/V) Coomassie brilliant blue R-250 in 30% methanol, 10% acetic acid and 60% H<sub>2</sub>O and destaining in 30% methanol, 10% acetic acid and 60% H<sub>2</sub>O.

##### Inhibitor sensitivity analysis :

The effect on midgut proteolytic activity of inhibitors with specificity for serine proteinases (aprotinin and Pefablock<sup>R</sup>), cysteine or serine proteinases (leupeptin), cysteine proteinases [(L-trans-epoxysuccinyl-leucylamido-(4-guanidino) butane (E64)], metalloproteinases [ethylenediamine tetraacetic acid (EDTA), 1,10 phenanthroline monohydrate (1,10 PM)] and aminopeptidases (bestatin) was assessed by in-

cubating gel slices in 0.1M sodium acetate buffer (pH 4.0) overnight at 37°C in the presence of an inhibitor. Inhibitors were dissolved in solvents recommended by the manufacturer (Boeringer-Mannheim, Germany) or distilled water and added to incubation buffer at concentrations indicated in Table 2. For the inhibitor sensitivity analysis, midguts dissected from ticks that had fed for 48 h were used.

### Results

The molecular mass (Mr) of proteinases active in the midguts of both unfed and fed adult *H. longicornis* were estimated by gelatin-substrate SDS-PAGE analysis (Fig. 1). Proteolytic activity was detected only when gel slices were incubated in acetate buffer, pH 4.0, while no activity was detected in buffers at higher pHs. Three predominant proteolytic zones at 116, 48, 40 kDa (Fig. 1, a, e and f, lanes 1 to 3) and 2 weak proteolytic zones at 55 and 60 kDa (Fig. 1, c and d, lanes 1 to 3) were commonly detected in midguts from both unfed and fed ticks. In addition, a weak band around 80 kDa was detected in midguts from fed ticks only (Fig. 1, b lanes 2 and 3). As shown in Fig. 1, lanes 2 and 3 the proteolytic zones for the 40 and 48 kDa proteinases were diffuse extending from 40 to about 60 kDa and hence the 55 and 60 kDa

proteinases could not be clearly detected in lanes 2 and 3.

Table 1 summarises the sensitivity of *H. longicornis* midgut proteolytic activity to various proteinase inhibitors. The 40 kDa proteolytic zone was significantly reduced by treatment with either E64 or leupeptin, while the 48 kDa proteinase was completely inhibited by these inhibitors and partially by EDTA (Fig. 2). Apro-

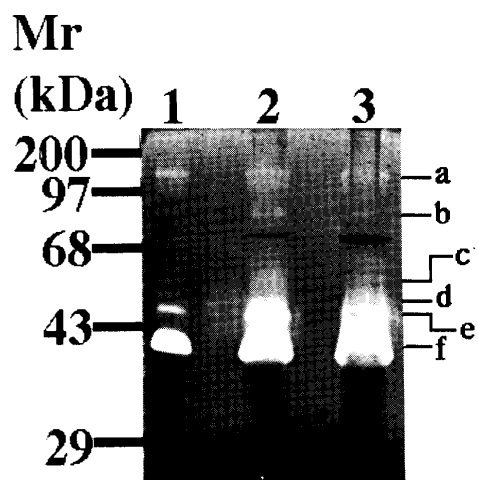


Fig. 1. Detection of *H. longicornis* midgut proteinases by gelatin-substrate SDS-PAGE analysis.

Gelatin was co-polymerised with a 10 % polyacrylamide gel at a final concentration of 0.2%. About 100 µg of the tick midgut homogenate was loaded to each lane. Lane 1 = midguts from unfed ticks, lanes 2 and 3 = midguts from ticks that fed for 48 and 96 h respectively. a to f = positions of proteolytic enzymes

Table 1. Inhibitor sensitivity analysis of *H. longicornis* midgut proteolytic enzyme activity against the gelatin substrate.

INHIBITORS (final concentration)	INHIBITION SPECIFICITY	PROTEOLYTIC ENZYME INHIBITION AGAINST		
		116	48	40(kDa)
Bestatin (130 µM)	Aminopeptidases	— <sup>a</sup>	—	—
1,10PM (10mM)	Metalloproteinase	—	—	—
EDTA (5mM)	Metalloproteinase	—	p	—
E64 (2.8 µM)	Cysteine proteinase	—	+	p
Leupeptin (1 µM)	Cysteine/Serine proteinase	—	+	p
Aprotinin (0.3 µM)	Serine proteinase	p	—	—
Pephablock (100 µg/ml)	Serine proteinase	—	—	—

a(—) = no proteolytic activity inhibition

+ = Proteolytic activity inhibited

p = proteolytic activity partially inhibited

Note: the weak proteinase bands at 80, 60 and 55 kDa (shown in Fig. 1) were not considered in this analysis.

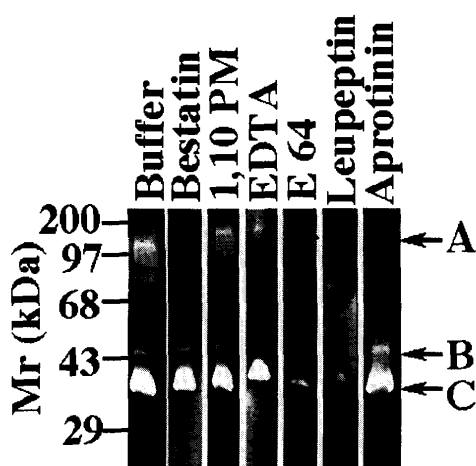


Fig. 2. Inhibition of tick midgut proteolytic activity against gelatin substrate.

After separation of the proteinases by electrophoresis, gel slices were incubated at 37°C overnight in the presence of buffer only (negative control) or indicated inhibitors. A, B, C = positions of the 116, 48, and 40 kDa proteinases

tinin partially reduced the 116 kDa proteolytic zone (Fig. 2), but Pefhablock did not show any significant inhibition on any of the proteinases (result not shown). The classification of the 3 weak proteinase bands at 80, 60 and 55 kDa was not conclusive by inhibitor sensitivity analysis because they were undetectable in the test shown in Fig. 2.

#### Discussion

This study demonstrated presence of at least 5 proteinases in the midguts of both unfed and fed adult *H. longicornis*. Based on inhibitor sensitivity analysis findings, it is most likely that both the 40 and 48 kDa proteinases are members of the cysteine proteinase family, while the 116 kDa may be related to a class of serine proteinases. Consistent with previous studies, where an acidic optimum pH was demonstrated for crude midgut proteolytic enzymes in *B. microplus*<sup>15)</sup>, *Hyalomma excavatum*<sup>3)</sup>, as well as purified midgut proteinases from *B. microplus*<sup>9)</sup> and *R. appendiculatus*<sup>22)</sup>, proteolytic activity was detected at pH 4.0 in the present study.

The fact that proteinases detected in the present study were commonly expressed in midguts from both unfed and fed ticks, rules out the possibility that the observed proteolytic activity was derived from the host. The proteolytic zones around 40 and 48 kDa proteinases appeared clearer in fed than unfed ticks suggesting that these proteinases increased with feeding. The increase in quantity of some proteins, like the 40 and 48 kDa proteinases in this study, between the unfed and fed ticks is an established phenomenon.<sup>2,3,12,16)</sup> While the underlying mechanism is not established, proteins upregulated in fed ticks are thought to be involved in the ability of the tick to feed and transmit disease pathogens<sup>16)</sup> and hence can be targeted as antigens for an anti-tick vaccine. For example, a recombinant *H. longicornis* tick 29 kDa salivary gland protein, which showed increased expression in fed ticks, conferred significant anti-tick immunity resulting in 40 and 56% mortality of larval and nymphal ticks, respectively, as well as a significant reduction in engorgement weights of adult ticks that had fed on immunized rabbits (Mulenga *et al.* submitted).

We recently cloned and characterized two cathepsin L-like cysteine proteinase genes (HLCG-A and HLCG-B) with 33 kDa and 37 kDa predicted Mr from *H. longicornis* (Mulenga *et al.* submitted). Because eukaryotes express a wide variety of cysteine proteinases, the relationship of proteinases in the present study to HLCG-A and HLCG-B is not clear based on current data. Purification of proteinases in this study, analysis of their primary structures, determination of their localization and functions are needed to clarify their relationship to HLCG-A and HLCG-B gene products. If these proteinases are key enzymes for the digestion of the blood meal, it is logical to assume that there is a possibility of blocking their activity by drugs or antibodies specifically targeted against these molecules and thus compromising the capacity of

the tick to feed and transmit pathogens.

In conclusion, while this study may not be exhaustive, we have obtained baseline information on the *H. longicornis* midgut proteinases.

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