RESEARCH NOTE

TRYPSIN AND AMINOPEPTIDASE ACTIVITIES IN BLOOD-FED FEMALES *ANOPHELES DIRUS* (DIPTERA: CULICIDAE) OF DIFFERING SUSCEPTIBILITY TO *PLASMODIUM YOELII NIGERIENSIS*

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Abstract. Midgut proteolytic enzymes contribute to the success or failure of *Plasmodium* infection of the mosquito. The present study investigated trypsin and aminopeptidase activities in the midgut of two strains of *Anopheles dirus* selected for susceptibility and refractoriness to *Plasmodium yoelii nigeriensis*. At intervals of 6 hours following a bloodmeal, the midguts of fully engorged female mosquitos were dissected, homogenized, and assayed for enzyme activity. No differences trypsin activity (nmole/min) were observed between the two strains throughout the course of blood digestion. By contrast, the aminopeptidase activity measured at 0 to 18 hours post-feeding was the same for the two strains, but at 24, 30 and 36 hours significantly less activity was observed in the refractory females. The results suggest neither trypsin nor aminopeptidase plays a role in the limitation of parasite development.

We recently established two lines of the oriental malaria vector mosquito *Anopheles dirus* Peyton and Harrison, one fully refractory and one fully susceptible to *Plasmodium yoelii nigeriensis* (an African rodent malaria parasite) (Somboon *et al*, 1999). The present study investigates activities of two major digestive enzymes, trypsin and aminopeptidase, in the female midgut of the two lines to elucidate their role in conferring susceptibility or refractoriness. Both enzymes have been shown to be involved in inhibition of the early stages of sporogonic development of malarial parasites *in vitro* and *in vivo* (in the midgut lumen of mosquitos) in some investigations (Gass and Yeates, 1979; Feldmann *et al*, 1990), but not in others (Chege *et al*, 1996).

Batches of 4- or 5-day-old females of the refractory and susceptible strains were allowed to feed on the arm of a human volunteer; fully engorged mosquitos were maintained at 25-26ºC and 70-80% RH with 2% sucrose. At each selected time point (0, 6, 12, 18, 24, 30, 36, 40, 46 and 52 hours post-feeding), four mosquitos of each strain were killed and their midguts dissected in cold phosphate-buffered saline (PBS), pH 7.2. Pairs of midguts were placed in 1.5 ml microcentrifuge tubes, each containing 1 ml of cold PBS, homogenized by hand on ice using a tight-fitting plastic grinder, and then centrifuged at 10,000*g* for 10 minutes at 4ºC. Supernatants were stored at -70ºC until they could be assayed for trypsin or aminopeptidase activity.

For the trypsin assay, 1.5 ml 0.05 M Tris-HCl buffer (pH 8.0 at 26ºC) and 40 µl 0.16 M benzoyl-arginine-*p*-nitroanilide (BA*p*NA) (Sigma) in dimethyl sulphoxide (DMSO) were first equilibrated at 26ºC for 15 minutes. Midgut homogenate (25 µl) was added, the reaction was run for 60 minutes at 26ºC and terminated by the addition of 0.5 ml 30% (v/v)

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acetic acid (Billingsley and Hecker, 1991). The absorbance of each reaction mixture was then read at 410 nm. Units of enzyme activity (nmol substrate hydrolysed/minute) were calculated using a molar extinction coefficient of 8,800 mM/cm (Erlanger *et al*, 1961). All assays were run in duplicate, with duplicate controls containing substrate and buffer to correct for spontaneous degradation of substrate and for absorbance intrinsic to the midgut sample.

The assays for aminopeptidase activity were as described for trypsin but with leucine-*p*-nitroanilide (L*p*NA) (Sigma) (40 mg/ml DMSO) as the substrate.

The results of this study revealed similar patterns of the trypsin activity between the two lines throughout the course of blood digestion (Fig 1A), suggesting that this enzyme plays no role in the limitation of parasite development. By contrast, the aminopeptidase activity measured at 0 to 18 hours post-feeding were similar in both lines, but at 24, 30 and 36 hours higher levels of enzyme activity (about 1.3 times) were observed in the susceptible females (Fig 1B). In order to confirm whether such increased aminopeptidase activity of the susceptible mosquitos was artefact, five fully engorged females of both lines, which has been kept for 28 hours, were individually assayed and the mean enzyme levels (unit/gut) were compared for significance using Student's *t* test. The result revealed a statistically significant difference ($t = 5.74$; df = 8; $p < 0.001$). Using a similar method, no statistically significant difference was observed in trypsin activity at 28 hours post-feeding in both lines ($t = 2.02$ df = 8; $p > 0.05$).

The relatively lower aminopeptidase activity within the midgut lumen of the refractory females compared with the susceptible ones suggests that this enzyme is unlikely to be involved in the limitation of parasite development. This is supported by a previous study (Somboon *et al*, 1999), in which both lines, which had been examined for 6-24 hours post-infection with *P. yoelii nigeriensis*, had plentiful ookinetes in the midgut lumen, and at 24 hours post-infection, oocysts (3-5 μm

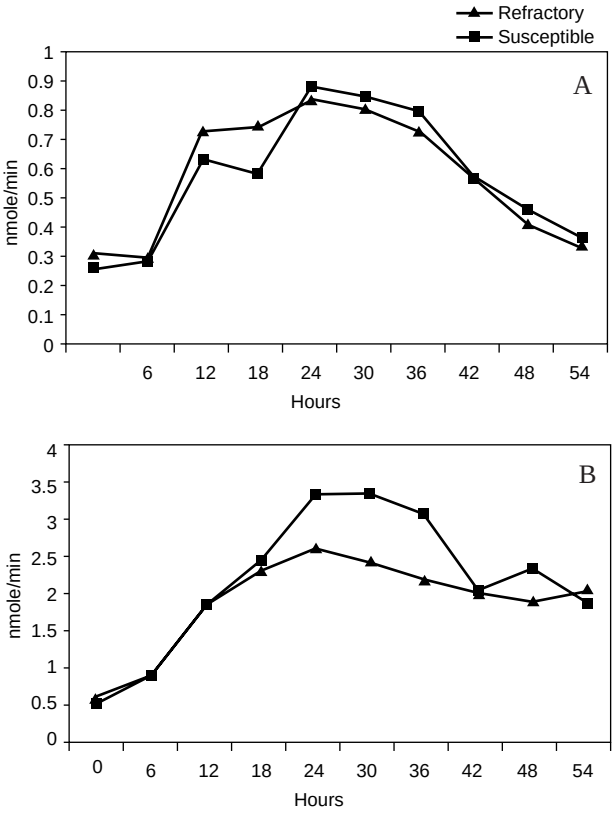

Fig 1A)–Trypsin and B) aminopeptidase enzyme activity for midgut homogenate supernatants from the *Plasmodium yoelii nigeriensis*-refractory and -susceptible females of *Anopheles dirus* at selected time intervals post-bloodmeal. Each time point represents the mean number of enzyme units (nmole/min) based on two samples (2 midguts per sample).

diameter) had already been observed between the epithelial cells and basal lamina; at 36 hours, the oocysts were 7-10 μm in midguts of the susceptible line, whereas those in females of the refractory line had stopped growing, thereby suggesting that disruption of parasite development had occured at the midgut wall, as observed in some other studies (Collins *et al*, 1986; Vernick *et al*, 1995). Increased activity of aminopeptidase in the susceptible females (Fig 1B) appeared to have no effect on the development of *P. falciparum* or *P. vivax* in the midgut, since both lines were equally susceptible to these two types of human malaria, based on oocyst count (Somboon *et*

al, 1999). However, in *An. stephensi*, high levels of aminopeptidase correlate with reduced susceptibility to *P. falciparum* (Feldmann *et al*, 1990). This study agrees with the findings of Chege *et al* (1996), who reported that *An. freeborni*, *An. gambiae* and *An. albimanus*, which had significantly different *P. falciparum* oocyst infection rates and different levels of trypsin or aminopeptidase activity, equally support the development of ookinetes. Proteolytic enzymes do not always limit the early stages of sporogonic development in *Anopheles* mosquito. Jahan *et al* (1999) reported that midgut peptidases of *An. stephensi* were unaffected by *P. y. nigeriensis* infection. In the present study, no evidence suggested that increased aminopeptidase activity in the susceptible mosquitos was due to the growing oocysts. It may be that the susceptible phenotype of the selected *An. dirus* is closely associated with the aminopeptidase allele.

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