Different Effects of Modulation of Mosquito (Diptera: Culicidae) Trypsin Activity on the Infectivity of Two Human Malaria (Hemosporidia: Plasmodidae) Parasites

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ABSTRACT Trypsin production in the malaria vector *Anopheles tessellatus* Theobald peaks between 12 and 21 h after a blood meal. The presence of leupeptin or soybean trypsin inhibitor in a blood meal delayed the onset of maximal trypsin activity. Trypsin inhibitors in an infective blood meal increased the infectivity of *Plasmodium vivax* Grassi and decreased infectivity of *P. falciparum* Welch to *An. tessellatus*. The opposite effects of trypsin inhibitors on infectivity of the 2 malaria parasites were attributed to differences in the biology of the parasites within the midgut of the vector, particularly the time of ookinete formation and the requirement for activation of a chitinase.

KEY WORDS *Anopheles tessellatus, Plasmodium falciparum, Plasmodium vivax,* trypsin, trypsin inhibitors

FEMALE MOSQUITOES OBTAIN the protein requirements for egg development from a blood meal. From a malaria epidemiology viewpoint, a blood meal facilitates the entry of *Plasmodium* gametocytes into the vector or the entry of sporozoites into a vertebrate host. The intake of a blood meal triggers a series of physiological events in the mosquito that include the synthesis and release of the proteolytic enzymes trypsin, chymotrypsin, and aminopeptidase (Briegel and Lea 1975, Billingsley 1990, Hörler and Briegel 1995), the synthesis of yolk proteins in the fat body, and the development of eggs (Hagedorn 1985). Trypsin production in yellow fever mosquito, *Aedes aegypti* (L.) (Graf and Briegel 1989), and *Anopheles* (Horler and Briegel 1995) occurs in 2 stages—an early phase where presynthesised trypsin is secreted and a late phase that involves induced transcription of trypsin messenger RNA. In *Anopheles albimanus* Wiedmann, maximal trypsin activity is observed 12–18 h after a blood meal (Hörler and Briegel 1995), whereas in *An. stephensi* Liston, trypsin activity was reported to increase continuously up to 30 h after a blood meal before falling to baseline levels (Billingsley and Hecker 1991).

During the process of digestion, a chitinaceous peritrophic membrane is formed between the blood meal and the epithelial cells of the midgut. Ingested *Plasmodium* gametocytes transform into gametes, fertilization takes place, and a motile zygote, the ookinete, is produced within the lumen of the midgut. Ookinetes migrate through the peritrophic membrane and the midgut, lodge themselves within the basal lamina on the hemocoel side of the midgut and develop into oocysts.

The kinetics of synthesis and release of digestive enzymes and the rate of production of the peritrophic membrane in relation to ookinete formation are 2 factors that may have a role in the susceptibility of an *Anopheles* vector to malaria parasites. Immature ookinetes of the avian malaria parasite *P. gallinaceum* are damaged by trypsin from the mosquito *Ae. aegypti* (Gass 1977, Gass and Yeates 1979). However, mosquito trypsin is essential for activating an ookinete chitinase that is required for the penetration of the peritrophic membrane by ookinetes of *P. falciparum* Welch and *P. gallinaceum* Brumpt in *An. freeborni* Aitken and *Ae. aegypti,* respectively (Huber et al. 1991; Shahabuddin et al. 1993, 1995). Therefore, a close evolutionary relationship may exist between malaria parasites and trypsin-like enzymes of vector mosquitoes.

Our article describes the effects of modulating the activities of trypsin and chymotrypsin-like enzymes in the mosquito with protease inhibitors on the establishment of infections of the human malaria parasites *P. vivax* Grassi and *P. falciparum* in *An. tessellatus* Theobald, a vector of malaria in Sri Lanka (Mendis et al. 1990).

Materials and Methods

Anopheles tessellatus was obtained from a laboratory colony maintained at 28°C and 80% RH as described previously (Ramasamy et al. 1992).

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Adults were fed ad libitum with 10% sucrose (wt: vol) supplemented with multivitamins and blood fed on rabbits. The care and use of rabbits were according to WHO guidelines (WHO 1988).

Assay for Trypsin and Chymotrypsin Activities. Groups of 3- to 4-d-old *An. tessellatus* were fed on a restrained rabbit and then held in the insectary for 48 h. Batches of 15 blood-fed females were frozen at intervals of 1–3 h and their midguts dissected intact in cold phosphate buffered saline pH 7.2 (PBS). Dissected tissues were homogenized at 4°C and homogenates stored at −20°C in eppendorf tubes as 3 replicates of 500 μl, each replicate consisting of 5 midgut equivalents.

Trypsin and chymotrypsin were assayed (Borovsky 1988) using the substrates N-benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPNA) and N benzoyl-L tyrosine p-nitroanilide hydrochloride (BTPNA) obtained from Sigma (St. Louis, MO). For the assay, 1 ml of 50 mM Tris buffer pH 8.5 containing 2 mM BAPNA or 1 mM BTPNA in DMSO was incubated with 10 μl of midgut homogenate for 30 min at 30°C. The reaction was stopped by the addition of 0.5 ml of 30% acetic acid. Absorbance at 410 nm was read on a CIBA-Corning 2800 Spectrascan spectrophotometer. One unit of enzyme activity (BAPNA or BTPNA units) was defined as a change in absorbance at 410 nm of 0.001/min at 30°C and pH 8.5.

Studies with Trypsin Inhibitors. A 20-mg/ml stock solution of soybean trypsin inhibitor (SBTI) from Sigma was prepared in serum from an unimmunized rabbit. Stock leupeptin (Sigma) was reconstituted in water at 5 mg/ml. SBTI is a potent irreversible inhibitor of chymotrypsin and trypsin, whereas leupeptin irreversibly inhibits trypsin (Laskowski 1955). SBTI and leupeptin were mixed with fresh rabbit blood at different concentrations and fed to 3- to 4-d-old *An. tessellatus* (same cohort for each experiment) in a water-jacketed membrane feeder fitted with parafilm and held at 40°C (Srikrishnaraj et al. 1995). Engorged mosquitoes were frozen at 6-h intervals and midgut homogenates prepared for the assay of trypsin as described above.

Parasite Infectivity Studies. Blood parasitized with either *P. vivax* or *P. falciparum* gametocytes were obtained voluntarily from patients reporting to the government hospitals in the vicinity of Kandy, according to procedures approved by the Institute of Fundamental Studies, committee on ethics in experiments involving human subjects. Parasitemia and gametocytemia were determined by examining Giemsa-stained blood smears. Venous blood (5–6 ml) was drawn aseptically and diluted immediately in 10 volumes of suspended animation solution (10 mM Tris, 170 mM NaCl, 10 mM glucose, pH 7.4), which reversibly suppresses gametogenesis (Carter and Nijhout 1977). Washed parasitised red blood cells were reconstituted 1:1 with normal rabbit serum containing SBTI or leupeptin and fed to 3- to 4-d-old *An. tessellatus* (same cohort for each experiment) through a membrane feeder (Srikrishnaraj et al. 1995). Blood-fed mosquitoes were maintained on 10% sucrose and on the 3rd d after infection provided with an oviposition substrate. A 2nd noninfective blood meal of unimmunized rabbit blood was given through a membrane feeder, 4 d after the infective meal. Mosquitoes were dissected 8–10 d after the infective blood meal, and the prevalence of *P. vivax* or *P. falciparum* infections in the vector determined by the presence of oocysts on the midgut. In addition, the number of oocysts (stained with methylene blue) on the midgut were counted. Chi-square analysis and the Mann–Whitney modification of Wilcoxon's sum of ranks test were used to analyze the significance of differences in proportions of infected mosquitoes and the numbers of oocysts respectively.

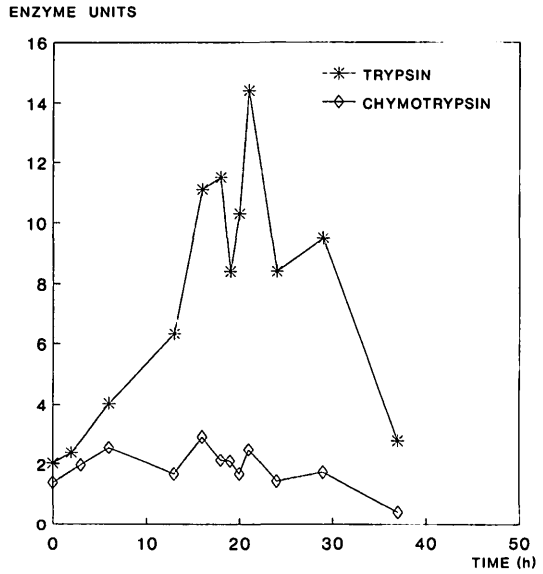

Fig. 1. Activity of trypsin and chymotrypsin in the midgut of *An. tessellatus* following a blood meal on a restrained rabbit.

Results

Trypsin and chymotrypsin activities in *An. tessellatus* were monitored for 37 h after a blood meal on a restrained rabbit; both enzymes were detected immediately after the intake of blood. Trypsin concentration before blood feeding was estimated at 0.2 BAPNA units, but immediately after a blood meal it was increased to 2.0 BAPNA units. Several peaks of trypsin production were observed in *An. tessellatus* (Fig. 1), notably between 18 and 21 h after blood feeding. Maximum trypsin activity and the time of its occurence varied between different cohorts of *An. tessellatus* that were used in different experiments (Figs. 1 and 2). Chymotrypsin activity in blood fed *An. tessellatus* was considerably lower than that of trypsin (Fig. 1).

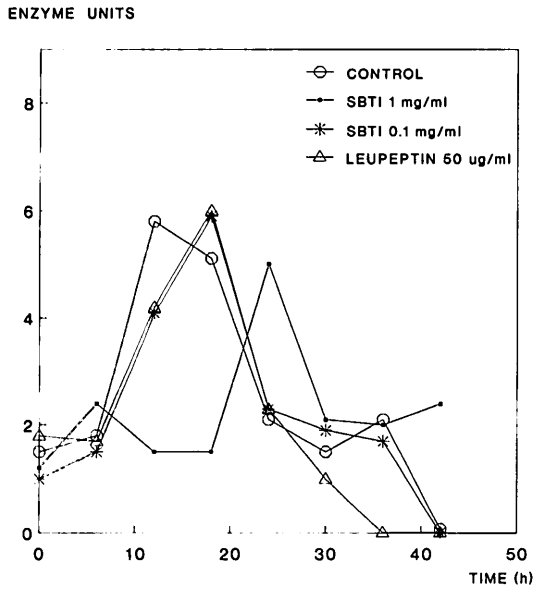

Fig. 2. Modulation of trypsin activity in the midgut of *An. tessellatus* with soybean trypsin inhibitor and leupeptin fed in vitro on rabbit blood.

The effects of SBTI and leupeptin ingested with an in vitro blood meal on trypsin activity of *An. tessellatus* is shown in Fig. 2. The presence of SBTI (0.1 mg/ml) and leupeptin (50 μg/ml) caused a delay of ≈6 h in maximum trypsin activity when compared with control mosquitoes. However, the peak activity of trypsin was not reduced significantly by 0.1 mg/ml SBTI and 50 μg/ml leupeptin (Fig. 2). Maximum trypsin activity of 5.0 BAPNA units was observed at 24 h when 1 mg/ml SBTI was present in blood meal, whereas in control mosquitoes the maximum activity of trypsin of 5.8 BAPNA units was observed at 12 h. The reduction in peak trypsin activity produced by 1 mg/ml SBTI was statistically significant (t = 4.3, df = 2, P < 0.05).

Results of experiments where *An. tessellatus* was fed *P. vivax* or *P. falciparum* gametocytes in the presence of protease inhibitors are given in Tables 1 and 2. The presence of protease inhibitors in the infective blood meal increased the proportion of *An. tessellatus* that became infected with *P. vivax* (Table 1). In experiment 1, where the gametocytemia was high (0.16%), the prevalence of infections was similar in control mosquitoes and mosquitoes fed on blood that contained protease inhibitors. The high gametocytemia of the infective blood meal also produced high oocyst counts, which became elevated in the presence of SBTI and leupeptin. In experiments 2 and 3, where the gametocytemia was lower (0.02%), increases in the proportions of *An. tessellatus* infected with *P. vivax* in the presence of either leupeptin or SBTI were observed. SBTI at 0.1 mg/ml increased the prevalence of *P. vivax* infections by 50 and 110% in

Table 1. Effect of adding trypsin inhibitors to a blood meal on the infectivity of *P. vivax* to *An. tessellatus*

Treatment	% infection (*n*)	Mean no. oocysts (range)
Experiment 1: gametocytemia 0.16%		
Control	96.7 (30)	92.1 (1–346)
SBTI (0.1 mg/ml)	93.8 (49)	132.3** (15–350)
SBTI (1 mg/ml)	95.5 (66)	117.5 (2–450)
Leupeptin (50 μg/ml)	85.2 (54)	118.2 (1–474)
Experiment 2: gametocytemia 0.02%		
Control	48.3 (58)	9.4 (2–20)
SBTI (0.1 mg/ml)	70.5 (44)*	10 (1–24)
SBTI (1 mg/ml)	70.0 (30)	6.3 (2–16)
Leupeptin (50 μg/ml)	73.9 (23)*	10.3 (1–26)
Experiment 3: gametocytemia 0.02%		
Control	18.2 (66)	1.9 (1–4)
SBTI (0.1 mg/ml)	38.2 (34)*	2.7 (1–8)
SBTI (0.5 mg/ml)	27.5 (51)	3.1 (1–6)

n, number of mosquitoes; *, P < 0.05 by chi-square analysis; **, P < 0.05 by Mann–Whitney modification of the Wilcoxon sum of ranks test.

Table 2. Effect of adding trypsin inhibitors to a blood meal on the infectivity of *P. falciparum* to *An. tessellatus*

Treatment	% infection (*n*)	Mean no. oocysts (range)
Experiment 1: gametocytemia 0.07%		
Control	44.4 (27)	6.4 (2–17)
SBTI (0.1 mg/ml)	60.0 (15)	9 (2–20)
SBTI (1 mg/ml)	13.3 (15)*	12 (4–20)
Leupeptin (50 μg/ml)	83.3 (18)*	14 (1–69)
Experiment 2: gametocytemia 0.02%		
Control	73.7 (19)	19.9 (1–47)
SBTI (0.5 mg/ml)	15.0 (20)**	12.7 (3–20)
SBTI (2 mg/ml)	13.3 (15)**	1*** (1–1)
Experiment 3: gametocytemia 0.15%		
Control	77.8 (27)	7.6 (2–40)
SBTI (0.1 mg/ml)	71.4 (21)	4.3*** (1–29)
SBTI (1 mg/ml)	67.9 (28)	3.3*** (1–9)
Leupeptin (50 μg/ml)	80.0 (15)	12.9*** (3–46)

n, number of mosquitoes; *, P < 0.05; **, P < 0.001 by chi-square analysis; ***, P < 0.05 by Mann–Whitney modification of Wilcoxon sum of ranks test.

experiments 2 and 3, respectively. In experiment 2, an increase in prevalence of 53% was seen when leupeptin was present in the infective blood meal. Although oocyst counts were low in experiments 2 and 3, the presence of trypsin inhibitors in most instances increased the number of *P. vivax* oocysts.

In contrast, inhibiting trypsin activity with 0.5–2.0 mg/ml SBTI reduced the prevalence of *P. falciparum* infections in *An. tessellatus* (Table 2). In experiments 1 and 2 (gametocytemia 0.07 and 0.02%, respectively), the prevalence of infections was significantly reduced by 70 and 82%, respectively, when SBTI was present in the blood meal at 1–2 mg/ml. In experiment 3 where the gametocytemia was 0.15%, the observed 13% reduction in the prevalence of infection with 1 mg/ml SBTI was not statistically significant. The numbers of oocysts were significantly reduced in experiments 2 and 3 in the presence of 1–2 mg/ml SBTI, although a reduction in oocyst numbers was not seen in experiment 1. The effect of 0.1 mg/ml SBTI and 50 μg/ml leupeptin on *P. falciparum* infectivity was variable. In experiment 1, the prevalence of *P. falciparum* infections increased significantly by 46.7% in the presence of leupeptin, but this was not seen in experiment 3.

The presence of SBTI at concentrations up to 2 mg/ml and leupeptin at 50 μg/ml in a *P. falciparum* or *P. vivax* infected blood meal did not increase mosquito mortality for up to 8 d after blood feeding in the above experiments.

Discussion

Anopheles tessellatus midguts showed at least 2 peaks of trypsin activity after blood feeding, consistent with the observations made in other anophelines (Hörler and Briegel 1995) and *Ae. aegypti* (Graf and Briegel 1989). Variation in the time course of trypsin activity observed in different experiments probably is caused by a variability among mosquito cohorts and by the use of blood from different rabbits. Some of the SBTI and leupeptin ingested in the blood meal is likely to be excreted within minutes during diuresis. The concentrations of the inhibitors in the blood bolus in the midgut therefore may be different from that in the blood meal. However, sufficient inhibitors appear to remain in the midgut to influence trypsin activity. SBTI at 0.1 mg/ml and leupeptin at 50 μg/ml delay the appearance of peak trypsin activity, but do not significantly affect the magnitude of the peak, indicating that the retained inhibitors rapidly are depleted in irreversibly bound and neutralized trypsin. However, 1 mg/ml SBTI reduced and more markedly retarded peak trypsin activity, in *An. tessellatus* compared with 0.1mg/ml SBTI probably as a result of the greater quantity of inhibitor retained in the blood bolus.

The infectivity of the parasite to the vector is not solely dependent on host gametocytemia. The maturity of the gametocytes and other host factors may influence infectivity. This probably explains the variations in infectivity seen with blood from patients with similar gametocytaemias in Tables 1 and 2. Our results indicate that inhibiting trypsin activity can reduce infectivity of *P. falciparum* to *An. tessellatus*. This is in agreement with the results of Shahabuddin et al. (1993, 1995) who demonstrated transmission blocking activity of *P. falciparum* in *An. freeborni* and *P. gallinaceum* in *Ae. aegypti*. The reduction in infectivity is manifest in the numbers of mosquitoes infected (prevalence of infection) and the intensity of infection (the numbers of oocysts per infected mosquito). Two mechanisms have been proposed to explain the action of the trypsin inhibitors. Trypsin may destroy components of the alternative pathway of complement activation that is deleterious to zygotes (Grotendorst and Carter 1987) or trypsin may be required to activate an ookinete chitinase that is required for penetration of the chitinaceous peritrophic membrane (Shahabuddin et al. 1993, 1995). According to these hypotheses, the presence of trypsin inhibitors in the blood meal will lead to greater complement mediated lysis of zygotes or retard the passage of ookinetes across the peritrophic membrane.

The peritrophic membrane of *An. tessellatus* is formed by 24 h after a blood meal and undergoes further thickening up to 48 h (Ramasamy et al. 1996). The ingestion of a blood meal containing rabbit antibodies against mosquito midgut tissue prevents the formation of a peritrophic membrane in the posterior midgut (Ramasamy et al. 1996), and the infectivity of *P. vivax* to *An. tessellatus* is reduced when Immunoglobulin G antibodies to mosquito midgut tissue are present in an infective blood meal (Srikrishnaraj et al. 1995). *P. falciparum* and *P. vivax* oocysts are found predominantly in the posterior region of the *An. tessellatus* midgut. Because the absence of a peritrophic membrane does not increase infectivity of *P. vivax* to the vector, the peritrophic membrane in *An. tessellatus* does not appear to be a significant barrier to the establishment of a *P. vivax* infection. Mosquito trypsin damages immature ookinetes of *Ae. aegypti* (Gass 1977, Gass and Yeates 1979, Yeates and Steiger 1981). An estimated 1–2% of macrogametocytes of *Plasmodium* are fertilized and form ookinetes (Vanderberg et al. 1977, Sluiters et al. 1986) and 1% of ookinetes develop into oocysts (Sluiters et al. 1986). Proteolytic activity in the mosquito midgut, particularly that of trypsin, may be partly responsible for the high attrition rate during the development of *Plasmodium* sexual stages in the mosquito midgut. Because in *An. tessellatus* the activity of chymotrypsin is negligible compared with that of trypsin and because leupeptin which is a specific inhibitor of trypsin also produces effects similar to SBTI, the observed effects on *P. vivax* and *P. falciparum* transmission are most likely to be the result of an inhibition of trypsin.

The opposite effects of inhibiting trypsin on infection with *P. vivax* and *P. falciparum* clearly are due to specific differences in the biology of the sexual stages of these species within the midgut. One difference may lie in the kinetics of zygote formation and ookinete migration through the midgut. Maximal ookinete penetration of the midgut epithelium is reported to occur between 30 and 35 h after a blood meal in *Ae. aegypti* infected with *P. gallinaceum* (Torii et al. 1992) and 32–36 h in *An. stephensi* infected with *P. falciparum* (Meis and Ponnadurai 1987). The corresponding maximal ookinete penetration activity occurred at 22 h for *An. atroparvus* infected with *P. berghei* (Sluiters et al. 1986) and at 15–18 h in *An. omori* infected with *P. yoelli* (Syafruddin et al. 1991). In *P. yoelli* and *P. berghei,* ookinete formation, maturation, and midgut penetration therefore occurs more rapidly than in *P. falciparum* and *P. gallinaceum.* There are little or no published data for *P. vivax.* We have observed *P. vivax* ookinetes in the midgut of *An. tessellatus* as early as 12 h after a blood meal (unpublished data). *P. falciparum* is phylogenetically closer to the avian malaria parasite *P. gallinaceum* than to *P. vivax* (Waters et al. 1993), and hence differences between *P. falciparum* and *P. gallinaceum* on one hand and *P. vivax* on the other, with regard to the mosquito stages are possible. Because trypsin levels in *An. tessellatus* peak between 12 and 21 h, delaying the action of trypsin in the blood meal (particularly with 1–2 mg/ml SBTI) may reduce damage to zygotes and immature ookinetes and thereby enhance *P. vivax* infection. However, a delay in peak trypsin formation in the midgut may be deleterious to *P. falciparum* where ookinete formation and maturation may occur more slowly. The passage of *P. vivax* ookinetes through the peritrophic membrane may take place when the membrane is not fully formed and therefore be relatively independent of a putative parasite chitinase. There is no evidence, yet, for the production of chitinase by *P. vivax* ookinetes. In contrast, because of the delayed ookinete formation in *P. falciparum,* the thicker peritrophic membrane probably constitutes a significant barrier to penetration (Meis and Ponnadurai 1987) and the parasite therefore is dependent on a chitinase to facilitate penetration (Shahabuddin et al. 1993, 1995). The obligatory dependence on trypsin for activation of a prochitinase may be another factor that causes *P. falciparum* transmission to be inhibited in the presence of trypsin inhibitors.

The geographical distributions of *P. vivax* and *P. falciparum* overlap in many parts of the world such as Sri Lanka and often involve identical vectors (Ramasamy et al. 1992a, b). Our results indicate that mosquito trypsin is not a suitable target for blocking the transmission of *falciparum* malaria as tentatively proposed by others (Shahabuddin et al. 1993).

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