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Transmission-blocking activity of a chitinase inhibitor and activation of malarial parasite chitinase by mosquito protease

(*Plasmodium*/peritrophic matrix/zymogen/vaccine/allosamidin)

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ABSTRACT **During development in the mosquito midgut, malarial parasites must traverse a chitin-containing peritrophic matrix (PM) that forms around the food bolus. Previously Huber *et al.* [Huber, M., Cabib, E. & Miller, L. H. (1991) *Proc. Natl. Acad. Sci. USA* 88, 2807–2810] reported that the parasite secretes a protein with chitinase activity, and they suggested that parasite chitinase (EC 3.2.1.14) plays an important role in the parasite's egress from the blood meal. We found that allosamidin, a specific inhibitor of chitinase, completely blocked oocyst development *in vivo* and thus blocked malaria parasite transmission. Addition of exogenous chitinase to the blood meal prevented the PM from forming and reversed the transmission-blocking activity of allosamidin. Using exogenous chitinase, we also found that the PM does not limit the number of parasites that develop into oocysts, suggesting that the parasite produces sufficient quantities of chitinase to penetrate this potential barrier. In addition, we found that treatment of parasite chitinase with a diisopropyl fluorophosphate-sensitive trypsinlike protease from the mosquito midgut or endoproteinase Lys-C increased its enzymatic activity. These results suggest that malaria parasite has evolved an intricate mechanism to adapt to the PM and the protease-rich environment of the mosquito midgut.**

A peritrophic membrane or matrix (PM) forms as a membranous sac around the ingested bloodmeal in most blood-sucking arthropods (1). Because arthropods are vectors for many human pathogens, including most filarial and many blood and tissue protozoan parasites, the PM may also act incidentally as a barrier in the development of these parasites (2). Clearly, for the successful transmission through an arthropod vector, human protozoan and filarial parasites have developed mechanisms to avoid or penetrate this potential barrier. Some parasites (e.g., *Wuchereria*) evade the PM by leaving the blood meal before the PM forms (3). In contrast, malaria parasites migrate out of the blood meal and cross the mosquito midgut epithelium while the PM is still intact around the food bolus; thus, these parasites must have evolved a means of penetrating this chitinous matrix. Early studies of the movement of *Plasmodium* sp. ookinetes as they penetrated the PM led Freyvogel and Jaquet to postulate that ookinetes "secrete enzyme-like substances from their 'mouth slit' " (4). Indeed, during the egress of *Plasmodium gallinaceum* ookinetes, the laminated structure of the PM appears to be disrupted focally and an electron-dense material appears to precede the anterior (apical) end of the parasite (5). Recent *in vitro* studies have demonstrated that about the time *P. gallinaceum* ookinetes would be binding to and penetrating the PM *in vivo*, a protein with chitinase activity is synthesized and secreted (6).

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In the life cycle of many organisms, particularly fungi, but also parasites, chitinases (EC 3.2.1.14) play an essential role in their interface with chitinous matrices: some organisms require chitinase to degrade chitin-containing structures produced by the parasite itself [*Onchocerca gibsoni* (7)], while others require this enzyme to degrade structures produced by the host [e.g., *Leishmania major* (8)]. Numerous studies of chitinase have been aided by the streptomycete antibiotic allosamidin (7, 8). Allosamidin, a potent inhibitor of various chitinases, consists of a dimer of β-*N*-acetylallosamine linked to dimethylaminocyclitol (9). β-*N*-Acetylallosamine is an epimer of the basic subunit of chitin, *N*-acetyl-β-D-glucosamine. Here we report that inhibition of chitinase activity by allosamidin interferes with normal malarial parasite development in the mosquito midgut by blocking the parasite's ability to cross the PM, and also that parasite chitinase is activated when treated with mosquito midgut protease and, therefore, apparently is a zymogen.

MATERIALS AND METHODS

Parasites, Mosquitoes, Media, Enzymes, and Chemicals. White Leghorn chickens were infected with the 8A strain of *P. gallinaceum* and the parasite was maintained by subpassage. Ookinetes were prepared by allowing zygotes, isolated as described earlier (10), to incubate for 20–24 hr at 26°C in complete M-199 medium (GIBCO) supplemented with 1 mM L-glutamine, streptomycin at 100 μg/ml, penicillin at 100 units/ml, and gentamicin at 125 μg/ml. Gametocytes of the 3D7 strain of *Plasmodium falciparum* were obtained by continuous culture as described (11). The Liverpool/blackeye strain of *Aedes aegypti* and the Maryville CA (1964) strain of *Anopheles freeborni* were raised at 26°C and 80% relative humidity and fed on sugar ad lib. *Streptomyces griseus* chitinase was purchased from Sigma (catalog no. 1525). Allosamidin was obtained from S. Sakuda (9) through Enrico Cabib (National Institutes of Health). Endoproteinases Lys-C, Glu-C, Asp-N, and trypsin were purchased from Boehringer Mannheim. The serine protease inhibitor diisopropyl fluorophosphate (DFP) was from Calbiochem.

Membrane Feeding Assay. Mosquitoes, starved for 6–18 hr, were fed blood through a Parafilm membrane as described by Sieber *et al.* (5). To assay for infectivity, mosquitoes were dissected 8 days after the blood meal and the midguts were stained with 1.0% mercurochrome in water for oocysts and scored by light microscopy. To assay for the effect of chitinase on the PM, *S. griseus* chitinase was diluted to a final concentration of 2 milliunits/ml in uninfected whole chicken

Abbreviations: DFP, diisopropyl fluorophosphate; PM, peritrophic matrix.

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blood and fed to mosquitoes through a membrane feeding apparatus as described above. Examination of the PM by light microscopy was performed by dissecting the midgut of the mosquito 15–20 hr after the blood meal and peeling back the anterior end of the midgut epithelium. For electron microscopy, midguts isolated at various times after a blood meal were fixed with 2.5% glutaraldehyde and 1% formaldehyde in phosphate-buffered saline (PBS) for 10 min and then stored at 4°C in PBS. Cross-sections of the fixed guts were examined by a JEOL CX100 electron microscope for PM as described previously (5).

Chitinase Activity Assay. Culture supernatants from *P. gallinaceum* ookinetes prepared as described above were clarified by centrifugation and stored at −20°C until used. The culture supernatants were filtered through a 0.45-μm low protein-binding membrane (Millipore), concentrated 50-fold in a centrifugal microconcentrator (Centricon-10; Amicon), and then assayed for chitinase activity as described (6); 1 unit catalyzes the production of 1 nmol of 4-methylumbelliferone per hr from 4-methylumbelliferyl *N,N',N''*-triacetyl-β-chitotrioside (Calbiochem). Kinetic studies of chitinase were performed in a Perkin–Elmer MPF 44B fluorescence spectrophotometer (excitation 350 nm, emission 440 nm) by continuously recording the change in fluorescence. Inhibition of chitinase activity was studied by using allosamidin at a final concentration of 5 μM in the reaction mixture. Reactions were started by the addition of enzyme.

Preparation of Mosquito Midgut Protease. Homogenates of 30 mosquito midguts isolated 24 hr after a blood meal were prepared in 1 ml of 0.1 M sodium phosphate buffer, pH 6.8, by using a Pyrex TenBroeck tissue grinder. The extract was centrifuged at 13,000 rpm for 30 min in an Eppendorf microcentrifuge. The resulting supernatant was freeze dried and resuspended in 100 μl of distilled water. Fifty microliters of this solution was mixed with Laemmli sample buffer without 2-mercaptoethanol, incubated at room temperature for 10 min, and subjected to electrophoresis in a 10% polyacrylamide gel for 90 min (125 constant volts). After electrophoresis, the gel was incubated for 15 min in renaturing buffer (Triton X-100, 2.5% vol/vol in water) with one change. The sample lane was then cut horizontally into 20 pieces. Each piece was crushed and put in 50 μl of Tris·HCl buffer (200 mM, pH 8.0) and left overnight on ice. The supernatants were then assayed for protease activity by using Chromozym-TRY as substrate (Boehringer Mannheim) and for chitinase activity as described above. Twenty microliters of the active fractions was then used to treat *P. gallinaceum* chitinase in an activation assay (see below).

Treatment of Chitinase with Proteases. Ten-microliter aliquots of concentrated culture supernatant of *P. gallinaceum* chitinase in 25 mM Tris·HCl/1 mM EDTA buffer, pH 8.5, were incubated with purified proteases (final concentration 10 ng/μl) or mosquito midgut protease (20 μl of supernatant, see above) for 2 hr at 37°C. Chitinase activity in each reaction mixture was then assayed as described above.

RESULTS AND DISCUSSION

Allosamidin Inhibits *P. gallinaceum* Chitinase and Transmission. Allosamidin is a potent competitive inhibitor of insect, plant, and parasite chitinases (11). Isolated from fungus of the *Streptomyces* species, allosamidin weakly inhibits *S. griseus* chitinase (12). To determine if allosamidin inhibits the *P. gallinaceum* ookinete chitinase (6), concentrated culture supernatants of ookinetes were assayed for chitinase activity in the presence of 5 μM allosamidin. In contrast to *S. griseus* chitinase activity, which was inhibited less than 1% by 5 μM allosamidin (data not shown), parasite chitinase activity was reduced more than 90% (Fig. 1).

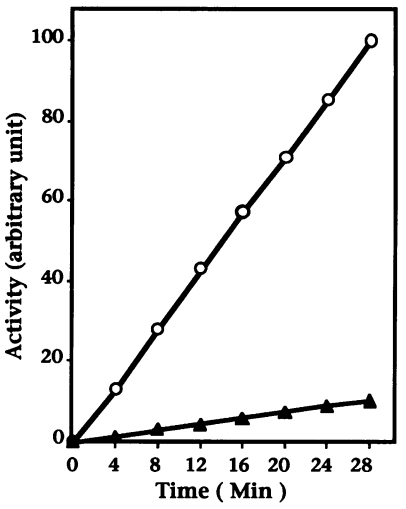

FIG. 1. Inhibition of *P. gallinaceum* chitinase activity by allosamidin. ○, No addition of allosamidin; ▲, with 5 μM allosamidin.

Mosquitoes were fed infected blood containing various concentrations of allosamidin to determine the effect that inhibition of chitinase has on normal sporogonic development of plasmodia *in vivo*. Allosamidin at concentrations of 0.1 mM or higher completely blocked the transmission of *P. gallinaceum* in *Ae. aegypti* and *P. falciparum* in *An. freeborni* (Table 1). Even at a concentration of 0.01 mM, the number of oocysts per gut that developed in allosamidin-fed mosquitoes was about 1% that of control mosquitoes. These data suggest that inhibition of chitinase has a profound effect on sporogonic development.

Although a likely target of allosamidin inhibition is parasite-produced chitinase, inhibition of some mosquito-derived factor(s) may also be responsible for blocking parasite transmission. In fact, allosamidin had at least two effects on the PM of mosquitoes fed uninfected blood: first, the PM appeared earlier, thicker, and, by electron microscopy, more disorganized than the PM in control mosquitoes (data not shown); second, at an allosamidin concentration of 1 mM, the

Table 1. Allosamidin completely blocks *in vivo* sporogonic development of plasmodia

Treatment*	Oocysts per gut†	Infectivity, % control	Infected/ total‡	P§
	Ae. aegypti/P. gallinaceum			
Allosamidin				
1.0 mM	0	0	0/19	<0.001
0.1 mM	0	0	0/25	<0.001
0.01 mM	0.5 (0–4)	1.1	4/11	<0.001
0.1 mM + chitinase	33.2 (10–117)	72.2	9/9	>0.1
Chitinase	11.8 (1–135)	25.6	9/9	>0.1
PBS	46 (6–193)	100	15/15	—
	An. freeborni/P. falciparum			
Allosamidin				
0.1 mM	0	0	0/21	<0.001
Chitinase	9.1 (0–114)	99	17/20	>0.1
PBS	9.2 (1–58)	100	23/23	—

*Parasite-infected blood was mixed with PBS or with allosamidin and/or chitinase (2 milliunits/μl in PBS) and fed to a natural definitive host.

†Data are presented as geometric mean with range in parentheses.

‡Number of mosquitoes infected/total number of mosquitoes dissected.

§*P* value determined by Wilcoxon rank sum analysis by comparing the number of oocysts in mosquitoes in each test group with that of the PBS-fed group.

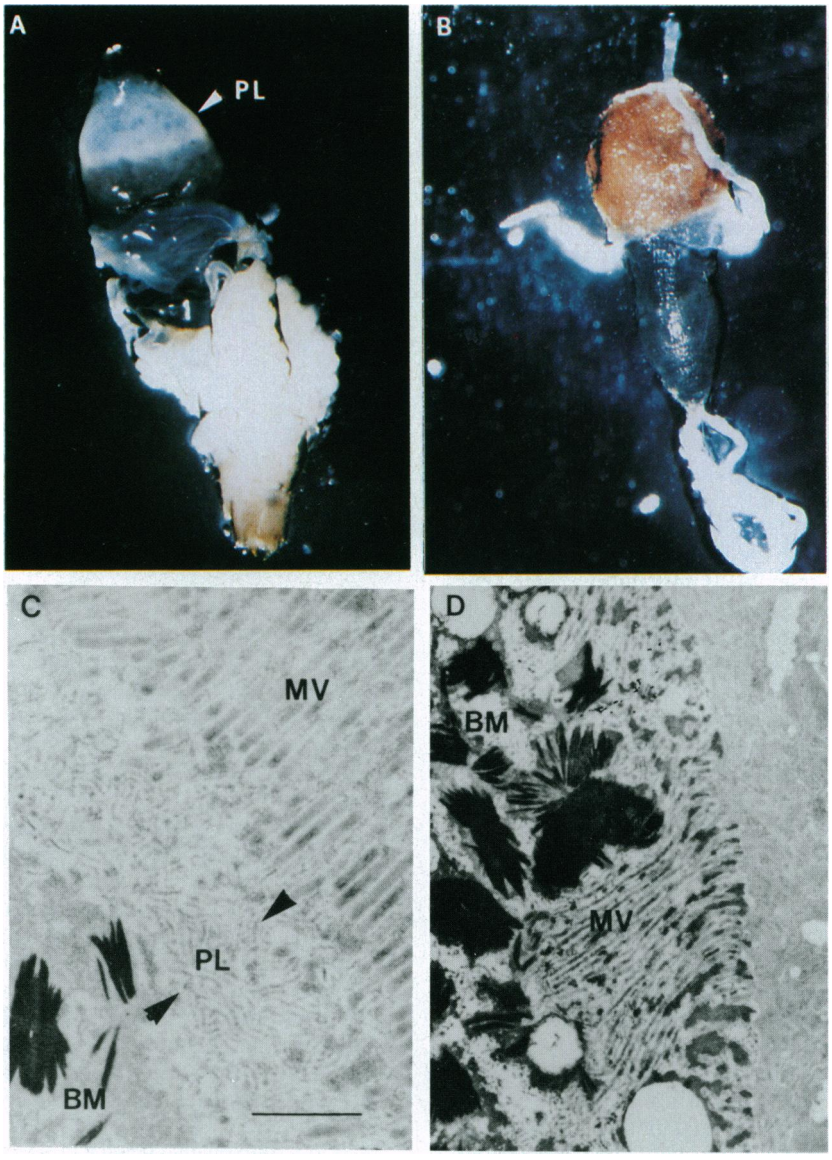

FIG. 2. *In vivo* disruption of the PM in *An. freeborni* mosquito by *S. griseus* chitinase. Six- to 8-day-old mosquitoes were fed human blood at a 50% hematocrit in heat-inactivated human serum either alone (*A* and *C*) or with chitinase at 2 milliunits/μl (*B* and *D*). Fifteen to 17 hr after a blood meal mosquitoes were dissected and the gut wall was partially peeled back. The resulting midguts were fixed and examined by light microscopy (*A* and *B*). (×25.) For electron microscopy, the midguts were dissected 35 hr after the blood meal and immediately fixed (*C* and *D*). (Bar = 0.6 μm.) PL, peritrophic matrix; MV, microvilli; BM, blood meal.

PM was still present in 90% of mosquitoes 8 days after the blood meal, whereas in control mosquitoes, the PM was detectable only for 4 days after the blood meal (data not shown). That the PM persists for at least 8 days after a blood meal in allosamidin-fed mosquitoes is at least suggestive evidence for the existence of a mosquito chitinase and inhibition of that chitinase by allosamidin.

We suspect, however, that the transmission-blocking effect of allosamidin is probably due to the inhibition of parasite chitinase rather than mosquito chitinase, for the following reasons. During the egress of malaria parasites from the blood meal, chitin is still forming and "maturing" in the thickening PM (20–30 hr after a blood meal, depending on the species). At a similar time period the parasite is producing large quantities of chitinase (6). Furthermore, because the PM does not begin degrading before 48 hr after a blood meal and does not completely disappear until 72–96 hr after a blood meal (depending on the species), the maximal activity of mosquito chitinase is likely to occur after most of the parasites have already penetrated the PM and formed oocysts. Nevertheless, presently we cannot exclude the possibility that whatever causes allosamidin to thicken the PM may also be responsible, at least in part, for its transmission-blocking activity.

Disruption of the PM Reverses the Transmission-Blocking Effect of Allosamidin. To exclude the possibility that allosamidin was affecting the parasite or mosquito at some point other than the penetration of the PM, we developed a means of disrupting the PM *in vivo*. Previously Huber *et al.* (6) had shown that fungal chitinase accelerates the degradation of the PM *in vitro*. We found that *S. griseus* chitinase, when added to a blood meal, resulted in disruption of normal PM formation. In the absence of exogenous chitinase, the food bolus containing the blood meal retained the oblong shape of the midgut and was encapsulated by a thin membranous structure (Fig. 2*A*). When exogenous chitinase was added to the blood meal, no such membranous structure was seen, and the food bolus immediately dispersed in the dissecting fluid (Fig. 2*B*). Without exception ($n > 100$), midguts from chitinase-fed mosquitoes lacked PMs when examined at 12, 20, 24, and 36 hr after the blood meal, whereas all of the midguts from

mosquitoes that were not fed chitinase developed PMs. To confirm the absence of the PM in chitinase-fed mosquitoes, midguts of *An. freeborni*, 35 hr after the blood meal, were fixed in 2.5% glutaraldehyde/1% formaldehyde in PBS and examined by transmission electron microscopy. PMs were seen only in midguts from mosquitoes that were not fed chitinase (Fig. 2*C*). Chitinase-fed mosquitoes had no detectable PMs (Fig. 2*D*).

Having established that chitinase completely disrupts PM formation *in vivo*, we sought to exclude the possibility that allosamidin nonspecifically killed the parasite or interfered with normal sporogonic development by some mechanism other than by inhibiting the parasite's penetration of the PM. *S. griseus* chitinase and allosamidin were mixed and fed to *Ae. aegypti* mosquitoes together with *P. gallinaceum* parasites. Twenty-four to 30 hr after the blood meal, the PM was undetectable by light microscopy in those mosquitoes fed the combination of allosamidin and chitinase; 8 days after the blood meal, the number of oocysts per gut was similar to that of the control group (Table 1). Thus, in the presence of a PM, allosamidin inhibition of parasite chitinase significantly impedes parasite development, but in the absence of a PM, inhibition of chitinase has little to no effect.

Interestingly, disruption of the PM by feeding exogenous chitinase, either with or without inhibitor, did not significantly increase or decrease the number of oocysts that formed per gut as compared with control mosquitoes. Therefore, contrary to the current notion that the PM is a barrier to the parasite (5), the data presented here indicate that the PM by itself does not limit the number of parasites that develop into oocysts because the parasite chitinase allows penetration of ookinetes. In addition, these *in vivo* data are consistent with the *in vitro* studies of Warburg and Miller (13), which indicate that the presence of a PM is not required for normal sporogonic development.

Activation of Parasite Chitinase by Mosquito Midgut Protease. The protease-rich environment of the PM and the periphery of the blood meal would seem to be a hostile place for most proteins. To examine the stability of parasite chitinase to proteolysis, purified mosquito midgut protease was isolated 24 hr after blood meal and added to concentrated ookinete culture supernatants. After 2 hr, the serine protease inhibitor DFP (final concentration 10 μM) was added. The resulting reaction mixture was then assayed for chitinase. To our surprise the mosquito midgut protease increased the chitinase activity at least 3-fold after 2 hr of treatment, instead of destroying the activity. In a control experiment midgut protease and DFP were mixed together before adding them to parasite chitinase. No activation of the parasite chitinase was observed with DFP-inactivated midgut protease (Fig. 3*A*). Heat-inactivated protease had no effect, and the protease preparation did not have any chitinolytic activity. These results clearly suggest that the parasite secretes an inactive or partially active chitinase that is activated by a mosquito-produced serine protease. Activation by trypsin has also been described for *Candida albicans* (14) and *Mucor mucedo* (15) chitinases. In these organisms microsomal but not cytosolic chitinases are activated when treated with this protease.

The major proteases produced by the mosquito midgut are trypsins, which cleave peptide bonds C-terminal to arginine and lysine residues. To examine the nature of the peptide cleavage responsible for activation of parasite chitinase, we treated the chitinase with endoproteinases Glu-C, Asp-N, Arg-C, and Lys-C and assayed for chitinase activity. Glu-C, Asp-N, and Arg-C did not activate the parasite chitinase (data not shown). Lys-C, which is a thermolabile trypsin-like enzyme that specifically cleaves peptide bonds C-terminal to lysine residues, increased chitinase activity 12- to 13-fold after incubation for 2 hr at 37°C (Fig. 3*B*). Lys-C did not have chitinase activity, nor did heat-treated Lys-C activate parasite chitinase (Fig. 3*B*). Thus, the activation of the parasite chitinase by Lys-C appears to be due to a specific proteolytic cleavage of a lysine residue(s).

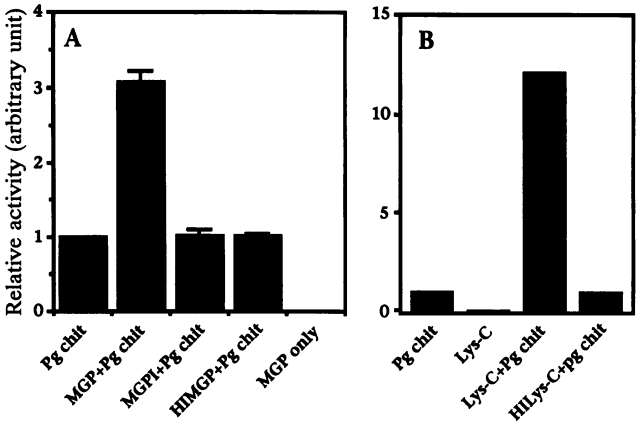

FIG. 3. Activation of *P. gallinaceum* chitinase (Pg chit) by mosquito midgut protease (*A*) and endoproteinase Lys-C (*B*). (*A*) Culture supernatants were assayed for chitinase activity before or after treatment with mosquito midgut protease (MGP), heat-inactivated midgut protease (HIMGP), or DFP-treated midgut protease (MGPI). (*B*) Chitinase activity without or after incubation with Lys-C or heat-inactivated Lys-C (HILys-C). The Lys-C preparation was also assayed for chitinase activity as control. Each experiment was performed at least three times.

The data presented here suggest that malaria parasite chitinase undergoes a postsecretory modification by mosquito midgut protease, which activates the enzyme by cleaving C-terminal to a lysine residue. The relationship between this zymogenic property of *Plasmodium* chitinase and the parasite's ability to traverse the PM is unclear. Although the mechanism by which the parasite only focally disrupts the PM around the penetrating ookinete (5) is not yet known, at least three factors may contribute to the highly localized action of parasite chitinase. First, the chitinase substrate is solid rather than in solution, keeping the enzyme–substrate complex close to the place of secretion by the parasite. Second, mosquito midgut trypsin activity is localized mainly within the PM (16), resulting in focal activation of the parasite chitinase. Third, the ratio of parasite chitinase to mosquito chitin synthase would be highest immediately adjacent to the parasite, allowing degradation of the PM by the parasite chitinase that is faster than repair by mosquito chitin synthase. Whatever the mechanism, *Plasmodium* chitinase appears to be a potentially important target for blocking transmission of malaria by impeding the egress of parasites from the blood meal. Whether the protease(s) that activates chitinase is also a target remains to be determined.

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