

IDENTIFICATION OF THREE STAGE-SPECIFIC  
PROTEINASES OF *PLASMODIUM FALCIPARUM*

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The human malaria parasite *Plasmodium falciparum* has a 48-h asexual cycle in human erythrocytes, during which the intracellular parasites develop from ring forms into larger and more metabolically active trophozoites, and then into multinucleated schizonts. Mature schizonts rupture their host erythrocytes and release merozoites, which rapidly invade other erythrocytes to reinitiate the cycle. Parasite proteinases appear to have important roles at several different stages of the erythrocytic cycle of malaria parasites: (a) trophozoites degrade host erythrocyte hemoglobin to provide amino acids for protein synthesis (1); (b) schizont and merozoite proteins are proteolytically processed during the final stages of schizogony (2, 3); (c) the proteinase inhibitors chymostatin and leupeptin prevent the rupture of erythrocytes by mature schizonts (4, 5); and (d) chymostatin inhibits the invasion of erythrocytes by merozoites (4).

We have attempted to identify proteinases that are involved in the differentiation and multiplication of malaria parasites. We collected homogeneous populations of specific life cycle stages from highly synchronized parasites, and have assayed for proteinase activity using gelatin substrate polyacrylamide gel electrophoresis (PAGE). Three proteinases were identified at neutral pH, and each was active at a different life-cycle stage. The stage-specific activity and inhibitor sensitivity of these proteinases suggest potential functional roles for each enzyme.

Materials and Methods

*Culture and Synchronization of Parasites.* Cloned parasites from a Brazilian isolate (It) of *P. falciparum* were cultured (6) in human erythrocytes at a 2% hematocrit. Leukocytes and platelets were removed from the erythrocytes before use for culture by pouring the cells over glass beads and a microcrystalline cellulose/ $\alpha$ -cellulose (Sigma Chemical Co., St. Louis, MO) column (7), and then centrifuging the eluant on a 90:80:40 Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) gradient (8,000 g, 20 min), and collecting the erythrocyte pellet. To obtain highly synchronized parasites, infected erythrocytes were treated sequentially with 0.5% (wt/vol) gelatin (300 Bloom; Sigma Chemical Co.) in RPMI 1640 (Gibco Laboratories, Grand Island, NY) (8), and 5% sorbitol (Sigma Chemical Co.) (9). Erythrocyte rupture occurred only during the 6-h interval between gelatin suspension and sorbitol treatment.

*Collection of Different Life Cycle Stages.* Ring-infected erythrocytes were obtained by suspending mature schizonts in 0.5% gelatin (8) and then allowing the schizonts to rupture in the presence of a small number of erythrocytes (three erythrocytes per schizont). A

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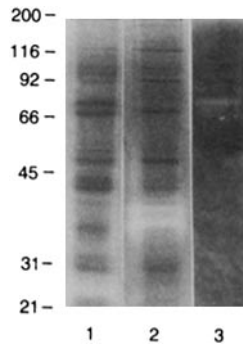


FIGURE 1. Gelatin substrate PAGE of extracts from *P. falciparum* trophozoites (lane 1), schizonts (lane 2), and merozoites (lane 3). The gels (12.5% acrylamide) were washed with Triton X-100 after electrophoresis to remove SDS, and were then incubated in glycine buffer with 10 mM cysteine at pH 7.0 for 18 h. After staining with Coomassie Blue proteinase activity was identified as a clear area against the diffusely stained background of the gel. Proteinase activity (arrows) was detected at 28 kD with trophozoites, 35–40 kD with schizonts, and 75 kD with merozoites. Molecular mass markers (kD) are indicated on the left.

parasitemia of 25% was achieved. Trophozoite-infected erythrocytes (28–34 h after erythrocyte rupture) were enriched to 75% parasitemia with 0.5% gelatin (8). Mature schizont-infected erythrocytes were obtained by adding chymostatin (100  $\mu\text{g}/\text{ml}$ ; Sigma Chemical Co.) to early schizonts (mostly 2–4 nuclei/parasite), incubating for 8–12 h, and then enriching the schizonts with 0.5% gelatin. Merozoites were collected by an adaptation of the technique of Miller et al. (10). Briefly, anti-human erythrocyte antibody (Cooper Biomedical, West Chester, PA) was added to erythrocytes containing mature schizonts, the erythrocytes were lysed by aspiration through a 26-gauge needle, and the mixture was poured over a protein A–Sepharose 4 B column (Pharmacia Fine Chemicals). Merozoites and residual bodies were obtained in the void volume.

**Sample Preparation.** Infected erythrocytes were washed in PBS, and erythrocyte membranes were lysed by incubation with 0.1% (wt/vol) saponin (Sigma Chemical Co.) in PBS. Merozoites were not treated with saponin. Samples were frozen (for <24 h,  $-70^{\circ}\text{C}$ ) before electrophoresis to allow the simultaneous assay of samples from different life cycle stages. Immediately before electrophoresis, the samples were solubilized with nonreducing electrophoresis sample buffer (5% SDS, 62.5 mM Tris HCl, pH 6.8).

**Gelatin PAGE.** Equivalent numbers of rings, trophozoites, and schizonts were electrophoresed in individual experiments ( $1-5 \times 10^8$  parasites per lane). Merozoite preparations were obtained from three times this number of schizonts because of the low yield of merozoite collection. Polyacrylamide (12.5%) was copolymerized with 0.1% gelatin (300 Bloom) (11). After SDS-PAGE, the gels were washed in 2.5% Triton X-100 detergent (Sigma Chemical Co.) (two 30-min washes) to remove SDS. They were then incubated in glycine buffer (0.1 M glycine, 0.002 M  $\text{CaCl}_2$ , pH 7.0) at  $37^{\circ}\text{C}$  for 18 h to allow digestion of gelatin by the proteinase before staining the gel with Coomassie blue.

**Inhibitor Studies.** Proteinase inhibitors (Sigma Chemical Co.) were incubated with samples for 10 min before addition of electrophoresis sample buffer, and were also added to the glycine buffer in which the gels were incubated after electrophoresis. Concentrations used were: phenylmethylsulfonyl fluoride (PMSF), 1 mM; *N*-ethylmaleimide (NEM), 5 mM; *L*-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane (E-64), 100  $\mu\text{g}/\text{ml}$ ; pepstatin, 100  $\mu\text{g}/\text{ml}$ ; and diisopropyl fluorophosphate (DIFP), 1 mM. The inhibition of proteinase activity was quantitated by scanning the gels with a Helena Laboratories (Beaumont, TX) Quick-Scan densitometer. The areas of troughs under the gel density base line (representing proteinase activity) were calculated by cutting and weighing chart tracings.

## Results

**Proteinase Activity at Different Life Cycle Stages.** Proteinase activity was not detected with gelatin PAGE gels of *P. falciparum* rings incubated at pH 7.0 (not shown). A clear band indicating proteinase activity was seen with trophozoite preparations at 28 kD (Fig. 1). Activity (the size and intensity of the clear band) was markedly increased when 10 mM cysteine was added to the glycine buffer in which the gel was incubated after electrophoresis. Proteinase activity was

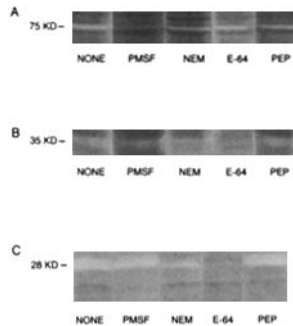


FIGURE 2. Inhibition of *P. falciparum* proteinases by 1 mM PMSF, 5 mM NEM, 100  $\mu$ g/ml E-64, and 100  $\mu$ g/ml pepstatin (PEP) compared to controls (NONE). (A) 75 kD proteinase of merozoites. (B) 35–40 kD proteinase of mature schizonts. (C) 28 kD proteinase of trophozoites. Proteinase inhibitors were added to parasite extracts before electrophoresis and to the glycine buffer with 10 mM cysteine (pH 7.0), in which the gels were incubated after electrophoresis.

TABLE I  
Inhibition of Proteinase Activity by Proteinase Inhibitors

Inhibitors	Percent inhibition of proteinase activity:		
	Trophozoites (28 kD)	Schizonts (35 kD)	Merozoites (75 kD)
NEM	67.8	82.6	36.6
E-64	100	100	47.8
PMSF	0	0	87.0
DIFP	ND	ND	100
Pepstatin	0	0	54.0

Percent inhibition determined by densitometry. The areas of troughs under the gel-density base line (representing proteinase activity) were calculated by cutting and weighing chart tracings. Parasites incubated with inhibitors were compared to controls without inhibitors.

detected at 35–40 kD with late schizont preparations. The activity usually appeared as a doublet or triplet and was also increased by incubation with cysteine. Proteinase activity was seen in merozoite preparations at 75 kD; this activity was not affected by cysteine. When cultures were not tightly synchronized, all three proteinase activities were sometimes seen in the same sample. Uninfected erythrocyte controls (treated with saponin) had no proteinase activity. Electrophoresis of chymostatin (used to prepare schizonts and merozoites) showed no contaminating proteinase activity.

**Inhibitor Studies.** Class-specific proteinase inhibitors were added to parasite preparations before electrophoresis, and to the glycine buffer in which the gels were incubated after electrophoresis. The 28 kD (trophozoite) and the 35–40 kD (schizont) activities were both inhibited by NEM and E-64 (Fig. 2 and Table I). The 75 kD (merozoite) proteinase activity was partially inhibited by all inhibitors tested, but was most markedly inhibited by PMSF (87%) and DIFP (100%, gel not shown).

### Discussion

Highly synchronized asexual parasites were studied with gelatin substrate PAGE to identify proteinase activity of trophozoite, schizont, and merozoite stages of *P. falciparum*. Proteinase activity was detected at 28 kD with trophozoites, at 35–40 kD with schizonts, and at 75 kD with merozoites. These proteinases are of parasite rather than host cell origin, since no proteinase activity was detected with uninfected erythrocytes and each proteinase was detected with

a specific life cycle stage. The 28 and 35–40 kD proteinases had the characteristics of cysteine (thiol) proteinases: each was stimulated by a reducing agent (cysteine), inhibited by standard inhibitors of cysteine proteinases (NEM and E-64), and not affected by inhibitors of aspartic (pepstatin) or serine (PMSF) proteinases (Table I). The 75 kD proteinase had the characteristics of a serine proteinase, i.e., it was markedly inhibited by the serine proteinase inhibitors PMSF (87%) and DIFP (100%). The inhibitor studies therefore suggest that the 28 and 35–40 kD proteinases of *P. falciparum* are cysteine proteinases, probably similar to cathepsin B or L, because they are inhibited by the arginine-containing peptide E-64 (12). Proteinase activity that cleaved peptides at an arginine residue, consistent with the substrate specificity of a cathepsin-like cysteine proteinase, was identified with *Plasmodium berghei* (13). The molecular mass and stage specificity of the *P. berghei* proteinase was not reported. The 75 kD proteinase, in contrast, is most likely of the serine class. This proteinase may be analogous to a proteinase detected with mature schizonts and merozoites of *P. knowlesi* by David et al., which was also inhibited by DIFP (3). Additional malarial proteinases may not have been detected in our experiments if the enzymes were not active at neutral pH, if they were irreversibly denatured by SDS, if they were unable to degrade the gelatin substrate, if the incubation solution lacked an essential cofactor, or if an inadequate quantity of parasites was assayed (as might have been the case with rings).

The biological functions of the three proteinases we identified have not been determined. However, their stage-specific expression and their inhibition profiles allow us to speculate on potential functions. The 28 kD proteinase of trophozoites was inhibited by E-64. Both E-64 and leupeptin (which also inhibits cysteine proteinases) inhibited the degradation of erythrocyte cytoplasm and hemoglobin within the food vacuole of *P. falciparum* trophozoites (14, and P. J. Rosenthal, unpublished observations). The correlation of the effect of cysteine proteinase inhibitors on hemoglobin degradation with their inhibition of the 28 kD trophozoite proteinase suggests that the 28 kD proteinase may have an important role in hemoglobin degradation. The pH optimum of this enzyme was 6.0 (14), which is near the pH of 5.3 calculated for the malarial food vacuole (15, 16), where hemoglobin is degraded (17). Proteinases capable of degrading hemoglobin have previously been identified with *Plasmodium lophurae* (18) and *P. falciparum* (19, 20). Their pH optima were 4.5 or lower, and they were inhibited by pepstatin, indicating that they were of the aspartic proteinase class. Proteinases active at such low pHs may not have been detectable in our experiments at neutral pH, and we cannot exclude the possibility that multiple proteinases may act together to degrade hemoglobin in malarial trophozoites.

The 35–40 kD proteinase and the 75 kD proteinase were detected with mature schizonts and merozoites, but not with trophozoites. This stage-specific activity suggests that the proteinases are involved in the final stages of differentiation of the intracellular parasite or in the complex events of erythrocyte rupture and erythrocyte invasion. Potential functions for these proteinases include the processing of schizont and merozoite surface proteins (2, 3), and the degradation of erythrocyte membrane or cytoskeletal proteins during rupture or invasion. It is intriguing that the rupture of erythrocytes by mature schizonts was inhibited by

chymostatin (an inhibitor of serine proteinases) and leupeptin (an inhibitor of cysteine proteinases) (4, 5), and that the invasion of erythrocytes by merozoites was inhibited by chymostatin (4). These results suggest that cysteine and/or serine proteinases have roles in rupture or invasion. However, determination of the precise biological functions of the 35–40 kD cysteine and 75 kD serine proteinases we identified may require highly specific nontoxic inhibitors and monospecific antibodies. Specific inhibitors of the proteinases might provide new means of antimalarial chemotherapy.

### Summary

We have identified and characterized three stage-specific proteinases of *Plasmodium falciparum* that are active at neutral pH. We analyzed ring-, trophozoite-, schizont-, and merozoite-stage parasites by gelatin substrate PAGE and characterized the identified proteinases with class-specific proteinase inhibitors. No proteinase activity was detected with rings. Trophozoites had a 28 kD proteinase that was inhibited by inhibitors of cysteine proteinases. Mature schizonts had a 35–40 kD proteinase that also was inhibited by cysteine proteinase inhibitors. Merozoite fractions had a 75 kD proteinase that was inhibited by serine proteinase inhibitors. The stage-specific activity of these proteinases and the correlation between the effects of proteinase inhibitors on the isolated enzymes with the effects of the inhibitors on whole parasites suggest potential critical functions for these proteinases in the life cycle of malaria parasites.

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