Biochimica et Biophysica Acta, 334 (1974) 423–430

BBA 67148

SOME PROPERTIES AND SUSCEPTIBILITY TO INHIBITORS OF PARTIALLY PURIFIED ACID PROTEASES FROM *PLASMODIUM BERGHEI* AND FROM GHOSTS OF MOUSE RED CELLS

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(Received September 24th, 1973)

SUMMARY

1. An acid proteinase from erythrocytic stages of *Plasmodium berghei* was partially purified and some of its properties were compared with those of an acid protease from ghosts of mouse red cells.

2. The two enzymes gave similar elution patterns upon filtration through Sephadex G-100, had similar Michaelis constants towards hemoglobin, were inhibited by high concentrations of substrate, and degraded bovine serum albumin much more slowly than they did hemoglobin. The pH optimum was 3.0–3.6 for the parasite enzyme and 2.5–3.0 for the red cell enzyme. However, the pH-response curves were quite similar.

3. Both enzymes were inhibited by phenylmethane sulfonyl fluoride, and were unaffected by *p*-chloromercuribenzoate, iodoacetamide, dithiothreitol, and EDTA.

4. Both enzymes were completely inhibited by pepstatin, chymostatin, antipain and leupeptin. ID_{50}'s for the parasite enzyme were (in ng/ml) 0.25, 0.5, 70, and 200, respectively.

INTRODUCTION

The erythrocytic stages of the malarial parasite apparently derive the bulk of their amino acids from breakdown of host-cell haemoglobin [1–4]. Considerable degradation of parasite organelles also occurs following invasion by *Plasmodium* of the host red cell. Because proteolytic enzymes are presumably involved in the breakdown of both hemoglobin and the organelles, they would seemingly be important for normal growth and development of the parasite. The existence of an alkaline protease in *Plasmodium berghei* and in *Plasmodium knowlesi* has been reported [5]. The enzyme degraded globin much more rapidly than it did hemoglobin. An acid protease was also present, but it was relatively inactive and very unstable. It has also

Abbreviations: PMSF, phenyl methane sulfonyl fluoride; PCMB, *p*-chloromercuribenzoic acid.

been reported that cell free extracts of *Plasmodium gallinaceum* degraded hemoglobin very slowly at pH 6.5, while denatured globin was degraded much faster [6].

Recently, we reported that mouse red cells infected with *P. berghei* had a greatly elevated level of an acid proteinase [7]. The enzyme had a pH optimum of 2.5–3 when hemoglobin was used as substrate, was tightly bound to some subcellular structure, and exhibited structural-linked latency. It was indistinguishable from an acid protease from ghosts of host red cells. We have also noted the existence of acid proteases in *Plasmodium falciparum* and in *P. knowlesi* (Levy, M. R., Siddiqui, W. A. and Chou, S. C., in the press).

In this study, we report on some properties of partially purified acid proteases from P. *berghei* and from ghosts of mouse red cells. Both enzymes are unusual for acid proteases in that they are sensitive to phenyl methane sulfonyl fluoride (PMSF). In addition, both are extremely sensitive to a number of protease inhibitors from cultures of actinomycetes, and especially to pepstatin, which reportedly inhibits cathepsin D and pepsin-like enzymes [8–9]. The enzymes from the two sources share a number of other properties, including insensitivity to sulfhydryl reagents, similar Michaelis constants, retardation through Sephadex, and inhibition by high substrate concentrations. They differ slightly in pH response.

MATERIALS AND METHODS

P. berghei was maintained in female BALB/c mice by intraperitoneal injections. Animals were sacrificed when the parasitemia had reached 40–60%. Procedures for removal of leukocytes and collection of parasites have been described [7]. Proteinase assays were carried out as described elsewhere [7], except that the hemoglobin concentration was reduced to 10 mg/ml and, in some cases, reactions were carried out for 90–120 min.

Partial purification of enzyme was achieved in either of two ways. In the initial experiments, parasites or ghosts were thawed in potassium phosphate buffer and Triton X-100 was added to a final concentration of 0.6% (v/v). Insoluble material was removed by centrifugation and the supernatant fluid was adjusted to pH 3.9 by addition of acetate buffer. After 24 h the precipitate was collected by centrifugation. In the case of the parasites, the precipitate contained about 30% of the original enzyme, with a specific activity about 20 times that of the parasites, or about 100–200 times that of the infected red cells. When the ghosts were treated in the same manner, the enrichment was similar, but only about 10% of the enzyme was recovered.

In more recent experiments, enzyme was solubilized by repeated freeze–thawing. After the insoluble residue was removed, the enzyme was precipitated by addition of ammonium sulphate, to a final concentration of 75%. This procedure gave a poorer enrichment of enzyme, but recoveries were greater than 95% for the parasite enzyme, and greater than 70% for that from the ghosts. No differences in pH response, K_m, or elution pattern throught Sephadex could be seen between the enzyme prepared in the two manners. Because we were interested in comparing the properties of the enzyme from ghosts and parasites, we used the procedure that gave the better recovery in most experiments.

RESULTS

Passage of either the ghost or parasite enzyme through Sephadex G-100 gave a single peak of activity (Fig. 1). The enzyme emerged slightly after hemoglobin, and therefore has a molecular weight of less than 68 000. The retardation was the same for the enzyme from each source, and was also the same whether the sample prepared by acid precipitation or by $(NH_4)_2SO_4$ precipitation.

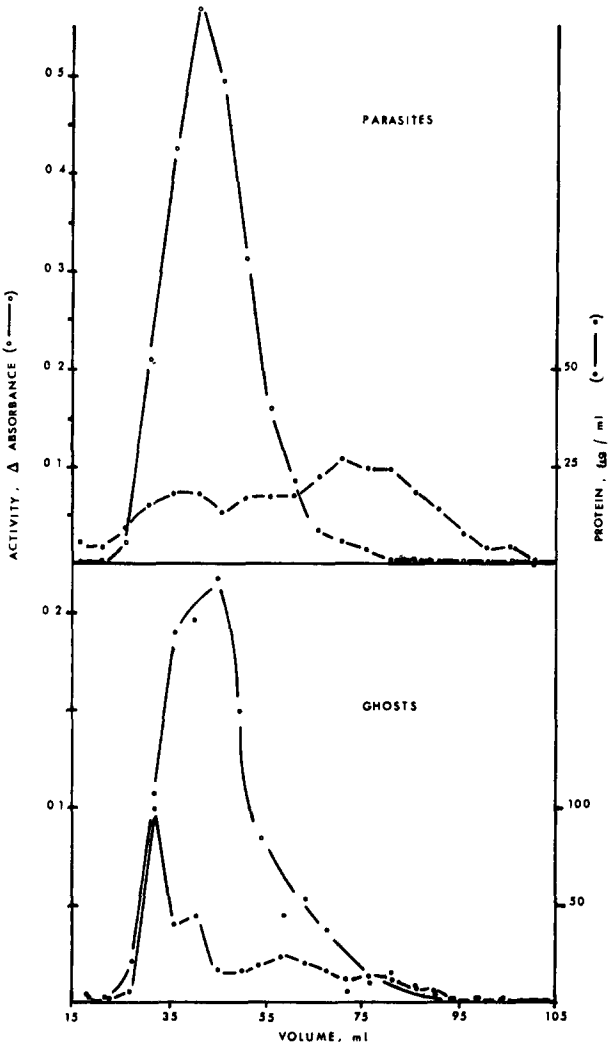

Fig. 1. Filtration of protease through Sephadex G-100. A sample of parasite or of ghost enzyme partially purified by solubilization and $(NH_4)_2SO_4$ precipitation was passed through a column of Sephadex G-100, 2.2 cm × 24 cm. Columns were equilibrated and developed with 0.01 M potassium phosphate buffer, pH 6.8 at a flow rate of about 15 ml/h. The void volume of the column was about 24–28 ml. The enzyme from the ghost fraction lost all activity within about 12 h after emerging from the column. Preparations of ghost or parasite enzyme prepared by acid precipitation, having a higher specific activity but which contained only 10 or 30% respectively of the original enzyme, gave elution patterns identical to those shown here.

A number of properties of the partially purified enzyme were tested. In the case of the parasite enzyme, the most active fractions emerging from the Sephadex column were combined and used. The enzyme from red cell ghosts tended to become very unstable as it became purified. For example, the samples shown in Fig. 1 had lost all activity when tested again several hours later. Hence, most of the properties of the enzyme from red cell ghosts were tested on the $(NH_4)_2SO_4$-insoluble material, which was purified about 4-fold over the original ghost fraction, or about 60-fold over the original red cells.

The effects of substrate concentration on enzyme activity are shown in Fig. 2. The ghost and parasite enzyme exhibited a similar response, in that both were inhibited by high substrate concentration and both exhibited very low activity towards bovine serum albumin. The K_m for substrate was about 2.5 mg/ml for the enzyme from each source. The values for the samples prepared by acid precipitation were very similar, about 2.0 mg/ml. The responses to high substrate concentrations and bovine serum albumin were also very similar.

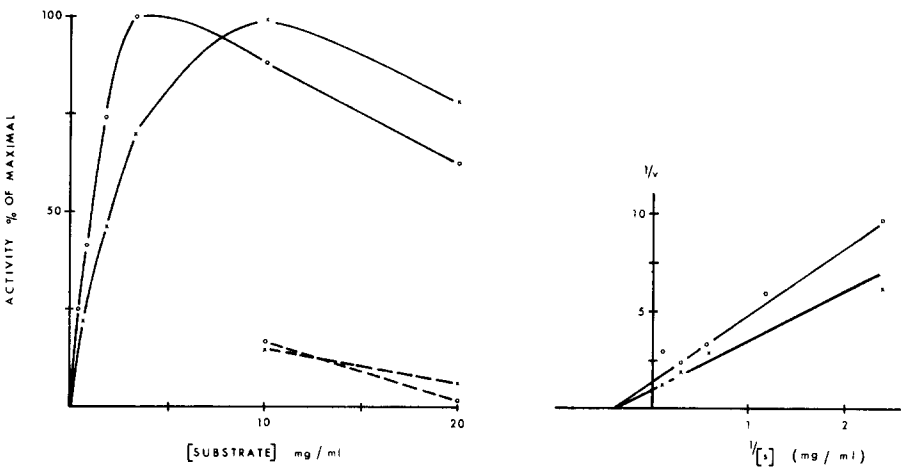

Fig. 2. Effects of substrate and substrate concentration. Preparations were those described for Fig. 3. (a) ○, enzyme from *P. berghei*; ×, enzyme from red cell ghosts; solid lines, activity towards hemoglobin; broken lines, activity towards bovine serum albumum (b) Double reciprocal plot.

The pH response of the enzyme from the two sources was similar (Fig. 3), although perhaps not identical. The parasite enzyme exhibited maximal activity at pH 3.0 to 3.6 while the optimum for that from the ghosts was 2–2.5. There was little or no activity in the alkaline range. These data are similar, although not identical to those reported from the enzyme from crude preparations [7].

The effects of several compounds on the acid protease from ghosts and parasites are shown in Table I. PMSF, which reportedly inhibits serine proteases, was the only compound that caused substantial inhibition, a concentration of 1.5 mM causing nearly complete inhibition of the partially purified enzyme from both sources. Interestingly, while the enzyme from crude preparations of ghosts from uninfected cells were insensitive to PMSF, the enzyme from crude preparations of ghosts from infected cells was inhibited. This phenomenon was also seen when a number of other

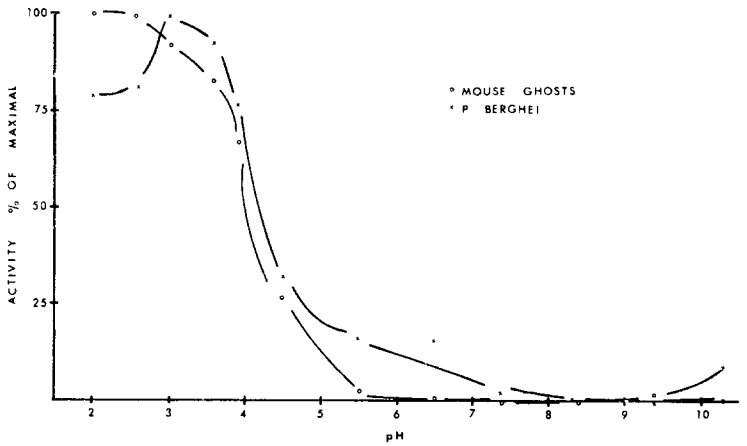

Fig. 3. pH response of enzyme from *P. berghei* and red cell ghosts. The ghost enzyme used was material insoluble in 75% $(NH_4)_2SO_4$ while the parasite enzyme was from the most active fraction emerging from filtration through Sephadex G-100.

TABLE I

EFFECT OF INHIBITORS ON ENZYME FROM *P. BERGHEI* OR RED CELL GHOSTS

The enzyme from *P. berghei* was purified by solubilization, acid precipitation, and gel filtration. The ghost enzyme was prepared by solubilization and $(NH_4)_2SO_4$ precipitation.

Inhibitor	Concentration (mM)	Percentage of control				
		P. berghei		Ghosts, normal		Ghosts, infected crude
		Crude	Sephadex G-100	Crude	$(NH_4)_2SO_4$-insoluble	
PMSF	0.5	88	47	—	62	59
	1.0	90	34, 0*	96	—	35
	1.5	—	0*	—	14	—
	4	—	—	94	—	—
PCMB	0.5	114	92	100	—	114
	1.0	96	84	91	102	110
Iodoacetamide	2.0	117	93	99	104	117
	5.0	99	93	101	101	106

* Tested on a different preparation.

inhibitors were tested (see Fig. 4 and Table II). The enzymes probably do not contain essential sulfhydryl groups, as neither *p*-chloromercuribenzoate (PCMB) nor iodoacetamide produced substantial inhibition. We reported earlier that EDTA and dithiothreitol were also without effect on the partially purified enzyme [7], and this was also the case for all of the preparations used here.

The effects of a number of protease inhibitors isolated from actinomycetes cultures were also tested. These compounds are extremely potent and are reportedly specific for certain types of proteases [10, 12]. The enzyme from both sources was completely inhibited by all four inhibitors, at all stages of purification (Fig. 4). This

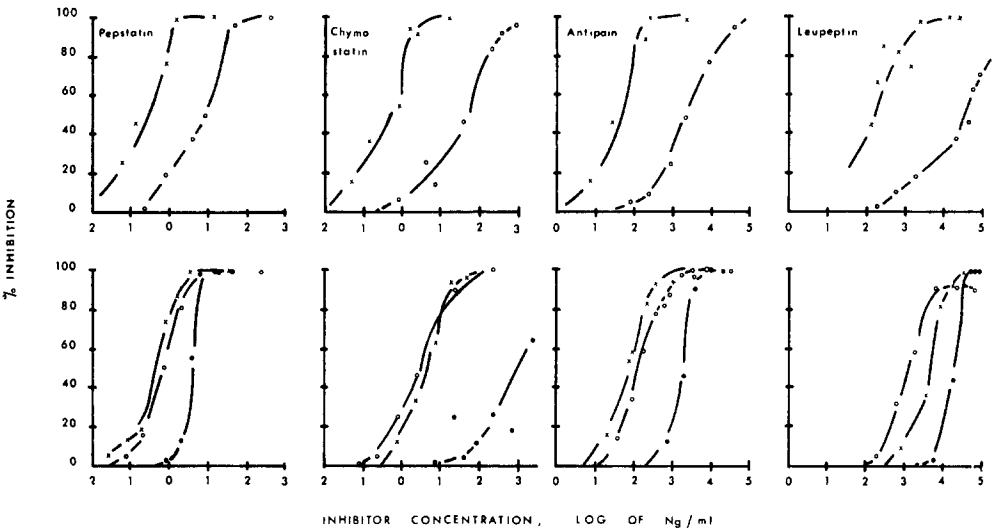

Fig. 4. Effects of protease inhibitors on proteolytic activity. Top: Parasite enzyme. ○, crude homogenate; ×, enzyme purified by solubilization, acid precipitation, and gel filtration. Bottom: Ghost enzyme. ●, ghosts from normal cells, crude homogenate; ×, same, after solubilization and precipitation with $(NH_4)_2SO_4$; ○, ghosts from infected cells, crude homogenate.

TABLE II

INHIBITOR CONCENTRATIONS NEEDED FOR 50% INHIBITION

Taken from Fig. 4.

	ID_{50} (μg/ml)				
	P. berghei		Red cell ghosts		
	Crude	Partially purified	Normal crude	Normal, partially purified	Infected cells, crude
Pepstatin	0.010	0.00025	0.003	0.0005	0.0006
Chymostatin	0.050	0.0005	1.0	0.005	0.003
Antipain	2.5	0.070	2.0	0.15	0.07
Leupeptin	40	0.20	20	5.0	1.5

is quite surprising, in view of the reported specificity of these compounds. Pepstatin, an inhibitor of acid proteinases such as cathepsin D and pepsin, was the most potent inhibitor, as concentrations of only 0.25 and 0.5 ng/ml (about $3.5 \cdot 10^{-10}$ and $7 \cdot 10^{-10}$ M) were required for 50% inhibition of the enzyme from parasites and ghosts, respectively (Table II). Chymostatin, which is most effective against chymotrypsin and cathepsin B, was also quite effective, especially against the parasite enzyme. Antipain and leupeptin were also relatively potent inhibitors, as the concentrations needed for 50% inhibition of the parasite enzyme are actually lower than those reported in the literature for inhibition of papain, trypsin, and cathepsin B, which are the proteases most sensitive to these compounds.

It is of interest that the sensitivity of the protease from ghosts of infected cells

was much greater than that of ghosts from normal cells, and that solubilization and $(NH_4)_2SO_4$ precipitation increased the sensitivity of the latter to approximately that of the enzyme from the infected ghosts (Fig. 4 and Table II). The difference is especially marked in the case of chymostatin, where the change was more than two orders of magnitude.

DISCUSSION

The present data indicate that the major hemoglobin-degrading enzyme in *P. berghei* is an acid protease with a pH optimum of about 3–3.6. While acid proteases are generally insensitive to compounds that inhibit serine proteases, the enzymes reported here are unusual in that they are inhibited by PMSF.

The response of these enzymes to the inhibitors from actinomycetes cultures is also unusual. Thus, these enzymes seem to be unique in that they are sensitive to all four of the inhibitors tested. This behavior is not readily explainable, since the inhibitors are reportedly specific [10, 12]. Enzymes reported to be sensitive to pepstatin for example, are not inhibited by the other three compounds, while enzymes inhibited by any of the other three are not sensitive to pepstatin. A response identical to that seen here was observed for the proteases from *P. knowlesi* and *P. falciparum* and from the ghosts of red cells from their respective hosts, Rhesus and Aotus monkeys (Levy, M. R., Siddiqui, W. A. and Chou, S. C., in the press). It is also of interest that the sensitivity to all four of the inhibitors seems to be the greatest yet reported, even though purified preparations of enzymes were used in many of the other studies. However, since the molar ratio of enzyme to inhibitor is important in determining the sensitivity, at least in the case of pepstatin [13], it may be that the amount of enzyme present in these samples is less than that used in other studies. Nevertheless, the sensitivities to pepstatin and chymostatin are especially remarkable, with only a fraction of 1 ng/ml being required for 50% inhibition. While concentrations of 3–10 μg/ml of pepstatin reportedly inhibit pepsin and cathepsin D by 50% [8, 9], the concentration of chymostatin needed to produce the same inhibition was 500-fold or more greater [12]. Also, chymotrypsin, which is the protease reportedly most sensitive to chymostatin, is more than two orders of magnitude less sensitive than the parasite protease. The unusual sensitivity of the enzymes to the actinomycetes inhibitors and the sensitivity to PMSF could perhaps be related. Thus, chymostatin, leupeptin, and antipain, which do not generally inhibit acid proteases, but which do inhibit serine proteases [12, 14], could perhaps be acting at the same site as PMSF, presumably a serine residue.

It is of interest that for three of the protease inhibitors, as well as for PMSF, the enzyme from crude preparations of ghosts from normal cells was much less sensitive than a similar preparation from ghosts of infected cells. The enzyme from crude preparations of red cell ghosts from either Aotus or Rhesus monkeys is much more sensitive to the inhibitors than the enzyme from mouse red cell ghosts (Levy, M. R., Siddiqui, W. A. and Chou, S. C., in the press).

The enzyme described here seems to be distinct from those described by Cook et al. [5], which had pH optima of 4 and 8 when tested against denatured globin. The acid protease was very unstable, losing most of its activity overnight at 2 °C,

while the alkaline protease was inhibited by EDTA. The acid protease described here is quite stable and is not affected by EDTA.

The data do not permit an unequivocal statement concerning the origin of the parasite enzyme. There is a slight difference in pH response between the enzyme from ghosts and parasites, and the former seems to become unstable as it is purified. However, they are quite similar with respect to a number of properties, including the unusual susceptibility to inhibitors. Thus, it remains to be determined if the protease found in the parasites was taken up from the red cells, perhaps together with hemoglobin, or if the parasites synthesize a protease that is very similar to that of the host cells. In the case of *P. knowlesi* in Rhesus monkeys, the latter seems to be the case, as the two enzymes differ markedly in pH response. The infected mouse red cells contain 5 or 10 times as much protease activity as the normal cells [7] and it does seem unlikely that the red cells would be capable of synthesizing the extra enzyme.

The extreme sensitivity of the parasite enzyme to the protease inhibitors raises the possibility that these could be used as potential antimalarial agents, since the parasite apparently derives most of its amino acids from breakdown of hemoglobin. It is therefore of interest that pepstatin, chymostatin, and leupeptin do indeed inhibit growth of *P. knowlesi* cultured in vitro in red blood cells (unpublished experiments, done in collaboration with Dr W. A. Siddiqui, Department of Tropical Medicine, University of Hawaii School of Medicine).

ACKNOWLEDGEMENTS

The excellent technical assistance of Mr Ronald Heu is acknowledged. This work was performed in the Pharmacology Department, University of Hawaii School of Medicine, during the tenure of a Sabbatical leave to the senior author. Pepstatin, chymostatin, antipain, and leupeptin were generously provided by Dr H. Umezawa of the Microbial Research Foundation.

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