J. Biochem. **138**, 71–78 (2005)
DOI: 10.1093/jb/mvi105

Purification and Characterization of a Hemoglobin Degrading Aspartic Protease from the Malarial Parasite *Plasmodium vivax*

Arun Sharma[1,*], Alex Eapen[2] and Sarala K. Subbarao[1]

[1]Malaria Research Centre, 22 Sham Nath Marg, Delhi-110 054, India; and [2]Malaria Research Centre (Field Station), Anna Nagar Western Extention, Megappair, Chennai-600 050, India

Received December 13, 2004; accepted April 22, 2005

Aspartic proteases of human malarial parasites are thought to play key roles in essential pathways of merozoite release, invasion and host cell hemoglobin degradation during the intraerythrocytic stages of their life cycle. Therefore, we have purified and characterized *Plasmodium vivax* aspartic protease, to determine if this enzyme can be used as potential drug target/immunogen, and its inhibitors as potential antimalarial drug. The *P. vivax* aspartic protease has been purified by a combination of ion exchange and size exclusion chromatographies and HPLC. Its properties were examined in order to define a role in the hemoglobin degradation process. The purified enzyme migrated as a single band on native PAGE and SDS/PAGE with a molecular mass of 40 kDa. Gelatin zymogram analyses revealed a clear zone of proteolytic activity corresponding to the band obtained on native PAGE and SDS/PAGE. The enzyme has an optimal pH of 4.0 and exhibits its highest activity at 37°C. The enzyme is inhibited by pepstatin, but not by other inhibitors including *o*-phenanthroline, EDTA, PMSF or E-64, supporting its designation as an aspartic protease; its IC_{50} value was found to be 3.0 μM. A Lineweaver Burk double reciprocal plot with pepstatin shows that the inhibition is competitive with respect to the substrate. Ca^{2+} and Mg^{2+} ions enhance the protease activity, whereas Cu^{2+} and Hg^{2+} ions were found to be inhibitory. The pivotal role of aspartic protease in initiating hemoglobin degradation in *P. vivax* malaria parasite is also demonstrated.

Key words: aspartic protease, chloroquine, hemoglobin, malaria, plasmepsin, *Plasmodium vivax*.

Abbreviations: plasmepsin, *P. falciparum* and *P. vivax* pepsin-like aspartic protease activity; EDTA, ethylenediamine-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; E-64, L-*trans*-epoxy-succinyl-L-leucylamido-(4-guanidino)-butane; TNBSA, trinitrobenzene sulphonic acid; DTT, dithiothretol.

Malaria is a tropical mosquito-borne disease caused by the *Plasmodium* species. It is responsible for 1.5–2.7 million deaths and infects 300–500 million people annually worldwide (*1*). The disease has remained uncontrolled to date as the parasite rapidly develops resistance to existing antimalarial drugs, and also due to a very high degree of antigenic variation. Therefore, new targets unique to the parasite need to be identified, and a detailed characterization of these targets may lead to rational drug designs (*2*).

During the erythrocytic stage of its life cycle, the malaria parasite degrades hemoglobin as a major source of amino acids (*3*) for protein synthesis (*4–7*). The malaria parasite thus produces/posseses a large number of proteases (*8*, *9*). These proteases are essential for parasite survival since they play important roles in host cell invasion, nutrition and growth, and the processing of precursor proteins (*10*). These proteases are thus believed to be promising targets for antimalarial chemotherapy (*11*, *12*) as the inhibition of *P. falciparum* proteases blocks parasite development *in vitro* (*13*) and a *P. vinckei* protease has been shown to cure murine malaria infections *in vivo* (*14*).

In *Plasmodium* species, aspartic proteases are believed to be involved in the release of merozoites, invasion, antigen processing, and the digestion of hemoglobin. A key aspartic protease that plays a role in initiating hemoglobin degradation before other proteolytic activities (*15*) and that functions efficiently in food vacuoles (*16*) has been defined as Plasmepsin 4, and two plasmepsins have been identified in *Plasmodium falciparum* (*17–23*). Two aspartic proteases , plasmepsin I and II have been identified in hemoglobin degradation by *P. falciparum* and the rodent parasite *Plasmodium berghei* (*24*), and inhibitors apparently specific to plasmepsins (*25*) are capable of inhibiting the growth of *P. falciparum* (*26–28*). A family of allophenylnorstatine base compounds has also been evaluated for their potential to inhibit a wide spectrum of plasmepsins (*29*, *30*). The relative role of aspartic proteases has not been yet fully examined in *P. vivax* (*31*), and not much work on the characterization and role of plasmepsins in *P. vivax* has been done to date (*32*, *33*) because of the failure to develop an *in vitro* culture system for *P. vivax*.

The elucidation of the structure/activity and biochemical strategies of an enzyme is necessary to facilitate the development of a potent specific inhibitor for potential

*To whom correspondence should be addressed. Tel/Fax: +91-11-23979998, 23928804, Fax : 23943743, E-mail: sharmaaru20032003@yahoo.co.in

application as an antimalarial drug. In order to determine whether recombinant forms of plasmepsins are appropriate for use in systematic investigations into inhibitor drug design and development, it was considered vital to isolate, characterize and establish the properties of the naturally occurring enzymes in terms of activity and specificity to reflect their recombinant forms (*34*). Thus, the isolation, characterization and inhibition of hemoglobin catabolism catalyzed by aspartic proteases in *P. vivax* offers an attractive target for chemotherapeutic intervention studies if these classes of enzymes are to be exploited as drug targets. We have now purified and characterized *P. vivax* aspartic proteases, and have shown that they share properties similar to those of *P. falciparum* and may play a pivotal role in the development of new drugs or drug targets.

MATERIALS AND METHODS

Materials—All protease inhibitors (pepstatin, E-64, PMSF, leupeptin *etc.*) were from Boehringer Mennhein Corporation, USA; Acrylamide, Tris-HCL buffer, Triton X-100, TNBSA (Trinitrobenzene sulphonic acid), Cathepsin D were from Sigma Chemical Co. (St. Louis, MO); Centricon filters unit were from Amicon Corp. (Danvers, MA). DEAE-Sephacel was from Pharmacia Fine Chemicals (Piscataway, NJ); The Bio Gel HPHT HPLC column was from Biorad (USA). Hemoglobin was purified from normal peripheral blood as described (*35*) and was stored at –70°C for further use. All other chemicals used were purchased locally and were of analytical grade.

Preparation of Samples—Blood samples were obtained from patients (with informed consent) from different geographical regions of India, and screened microscopically for the presence of malaria (*P. vivax* + *ve*) parasites. Age- and sex-matched control samples were obtained from the same areas. Blood samples from *P. vivax*–infected patients (0.05–0.5% parasitaemia) of either sex were collected in heparinized tubes. Blood was centrifuged at 1,500 × *g* for 10 min to separate the plasma and buffy coat, or the pellet was diluted with PBS and overlaid on Ficoll-paque for the removal of WBC, followed by centrifugation for 10 minutes in the cold at 1,500 × *g*.

Separation of Parasitized Red Cells on a Percoll Density Gradient—Percoll was adjusted to iso-osmolarity with 1.5 M PBS, pH 7.2 (9 parts Percoll:1 part PBS). Different concentrations, 30, 45, 50 and 65%, were prepared. The cell suspension was overlaid on 30% Percoll, followed by centrifugation at 1,500 × *g* for 15 min in the cold. The top layer was then aspirated off and washed twice with complete medium to obtain a 10% suspension. This suspension was overlaid on a 45, 50 and 65% Percoll gradient. The top and bottom layers were collected and checked for purity by microscopic examination (*36*). Parasitized red cells were washed three times with normal saline and then lysed with 5 mM phosphate buffer, pH 8.0. Thereafter the lysed suspension was washed and centrifuged at 1,500 × *g* for 30 min. The uppermost layer of the haemolysate was separated for enzymatic estimation. An erythrocyte lysate from normal patients was treated the same as the parasitized cells and served as a control. Isolated parasites were kept at –70°C for further biochemical analysis. The purity of the parasites was established as described previously (*37*). The parasites were isolated with little contamination by host red cell cytoplasmic materials.

The hemoglobin concentration was determined by the cyanomethemoglobin method (*38*). Glutamate dehydrogenase was measured by the method of Walter *et al.* (*39*). Most of the glutamate dehydrogenase activity, which is considered to be a marker for isolated parasites, was found to be concentrated in the isolated parasites. However, the catalase activity was found to be 17% of that present in the host cell cytoplasm (Table 1). Protein was estimated by the method of Lowry *et al.* (*40*), using BSA as a standard.

Parasite Extract Preparation—When sufficient parasites were accumulated, the parasite preparations were pooled, suspended in 10 mM Tris-HCl buffer, pH 7.0, containing 1 mM PMSF, 1 mM 1, 10, phenanthroline, 100 μM leupeptin, and sonicated for 15 s three times on ice. The sonicate was centrifuged at 100,000 × *g* for 1 h at 4°C. The pellet was then resuspended in 10 ml of 10 mM Tris HCl buffer, pH 7.0, with Triton X-100, 1 mM PMSF, 1, 10, phenanthroline and 100 μM leupeptin. Finally, the extract was centrifuged at 25,000 × *g* for 30 min, and the pellet was re-extracted with 5 ml of more buffer; the supernates were combined.

DEAE-Sephacel Chromatography—The parasite extract was passed through a 1.5 × 7.0 cm column of DEAE Sephacel equilibrated with 20 mM Tris-HCL, pH 7.0. The column was eluted with a step gradient of NaCl from 0.1 to 0.7 M in 0.1 M increments. Two 8 ml fractions were collected. The fractions were assayed for aspartic protease activity as described below. These fractions were pooled and concentrated on Centricon 10 ultra filtration membranes to ~1 ml, and 0.25 volume of 1 M sodium acetate buffer, pH 4.5 was added. The preparation was incubated on ice for 20 min and centrifuged at 4°C for 20 min at 14,000 rpm. The supernate was immediately neutralized with 0.125 volume of 1 M Tris HCL buffer, pH 8.8.

Hydroxylapatite Chromatography—The pH 4.5 supernate was diluted to 10 ml with 10 mM sodium phosphate buffer, pH 7.0, containing 10 μM $CaCl_2$, and loaded onto a ultrogel-HA column equilibrated with the dilution buffer. The column was eluted with a gradient of 0 to 0.15 M sodium phosphate buffer, pH 7.0, containing 10 μM $CaCl_2$. Active fractions were collected and again passed through the DEAE-Sephacel column, and all fractions were pooled (*18*).

Aspartic Acid Protease Purification by HPLC—Aspartic protease activity isolated from the extract of *P. vivax* parasites by electroelution was purified further by size exclusion HPLC on a Biogel HT hydroxyapatite column using a Shimadzu 10A HPLC apparatus (*19*). The column was eluted with a 0.01–0.35 M sodium phosphate gradient, pH 6.8. The flow rate was 0.8 ml/min, and 0.8 ml fractions were collected and assayed for aspartic protease activity. A 50 μl aliquot of the HPLC peak was subjected to electrophoresis in a 12% SDS-PAGE gel and stained with Coomassie Brilliant Blue. The specific activity of the peak obtained by HPLC was 252-fold that of the starting material, with a yield being 8%. This protease activity migrated as a single band corresponding to M_r 40,000 on SDS-PAGE and also on HPLC gel filtration chromatography (Fig. 1). Definitive assignment, however, requires

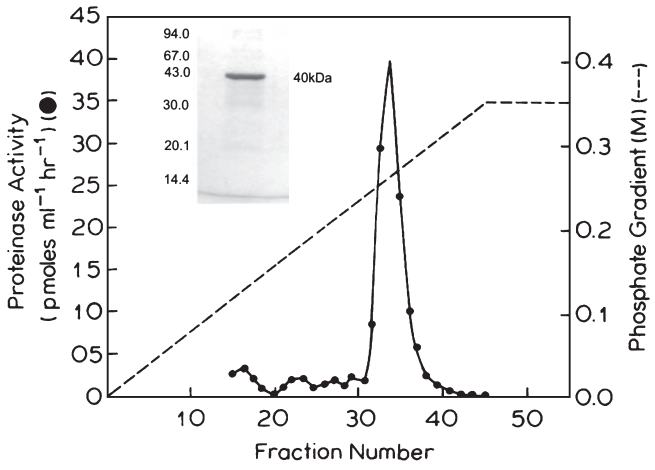

Fig. 1. **HPLC purification of aspartic protease on a Biogel HPHT column using a Shimadzu 10A apparatus.** The column was eluted with a 0.01 to 0.35 M gradient of sodium phosphate pH 6.8. Flow rate was 0.8 ml/min and 0.8 ml fractions were collected and assayed for aspartic protease activity as described in "MATERIALS AND METHODS." Inset: SDS-PAGE analysis. A 50 μl aliquot of the HPLC peak was subjected to electrophoresis in a 12% SDS-PAGE gel. The gel was stained with Coomassie Brilliant Blue.

purification to homogeneity and N-terminal sequencing to demonstrate that the protein shows homology with other aspartic proteases.

Protease Assay—The protease activity of the *P. vivax* parasite extracts was measured by casein/gelatin hydrolysis assay as described previously (*41*). A modified version of the method included in the Pierce Quanticleve Protease assay kit II (Pierce Chemical Company) was used for the protease assays. Briefly, 100 μg of succinylated casein/gelatin in a 100 μl volume of 40 mM Tris HCl buffer, pH 7.4 (1 mg/ml) was added to the wells in the left half of the plate and an equal (100 μl) amount of buffer was added to the wells in the right half. A different amount of sample (dissolved in 40 mM Tris-HCl buffer, pH 7.4) was added to each well. Equal amounts of samples were added to the blank buffer wells to subtract the background contributed by proteins present in the sample. The wells with buffer and substrate (succinylated casein/gelatin) served as a control. The samples were incubated at 37°C for 60 min, and then 50 μl of (0.03%) trinitrobenzene sulfonic acid (TNBS) was added to each well, and the plates were incubated further for 20 min at room temperature. Color development was measured at 450 nm. Blanks were prepared containing buffer and proteases, buffer and substrate, and buffer only. An inhibitor cocktail containing 1 mM PMSF, 1, 10, phenanthroline and 100 μM leupeptin *etc.* was also added to the routine assays.

PAGE and Gelatin PAGE—The molecular weight of the purified aspartic protease protein was determined by SDS-PAGE according to the method of Laemmli (*42*). For gelatin PAGE, 0.1% gelatin was copolymerized with acrylamide. After electrophoresis, the gels were washed in 2.5% Triton X-100 (three washes for 30 min each) to remove SDS. The gels were then incubated in glycine buffer (0.1 M glycine, 0.002 M $CaCl_2$, pH 7.0) at 37°C for 72 h to allow the digestion by the proteases before the gels were stained with Commassie Brilliant Blue. Protease activity was visible as a clear band against a blue background. Aspartic protease activity (40 kDa) was electroeluted out of the gel and reconstituted in sample buffer for further analysis.

Table 1. **Percentage of total recovered glutamate dehydrogenase and catalase in isolated *P. vivax* parasites (values are mean ± SD).**

Enzyme	% of recovered parasites (parasite/ parasite + red cells supernatant × 100)
Protein	5.62 ± 1.60
Glutamate dehydrogenase	78.30 ± 10.82
Catalase	0.97 ± 0.32

Inhibitor Studies and Enzyme Specificity—Different concentrations of pepstatin (2–12 μM) were used to inhibit aspartic proteases. Purified parasite extracts was incubated for 4 h with pepstatin, and gelatin PAGE was carried out as described above. Parasite homogenates were also incubated with varying concentrations of other inhibitors of cysteine, metallo, and serine proteases to ascertain the specificity of the aspartic protease activity.

Kinetic Analysis of the Aspartic Protease Activity—To understand the kinetics of the inhibition of aspartic protease activity by pepstatin we conducted experiments with increasing concentrations of pepstatin (2, 5, 10 μM) and plotted the inhibition kinetics by Lineweaver Burk double reciprocal plot.

Hemoglobin Degradation—To compare the fragments generated by hemoglobin degradation by the purified aspartic protease with those of the fragments produced by the parasite *in vivo*, we carried out experiments with purified hemoglobin. Hemoglobin was incubated for 30 min with our isolated aspartic protease and also in presence and absence of pepstatin, and then analyzed on SDS-PAGE.

Effects of the Antimalarials—The effects of antimalarial drugs on the enzyme activity were also investigated. The enzyme was incubated with varying concentrations of each drug for 15 min at 30°C, followed by the measurement of the residual activity under standard assay conditions.

RESULTS

Isolation of Parasites—Parasites were isolated from the blood of patients infected with *P. vivax* by lysis with 5 mM phosphate buffer (pH 8.0) and extensive washing to remove all host cell cytosolic materials. Uninfected control erythrocytes did not exhibit much glutamate dehydrogenase activity. The parasites were isolated with relatively little contamination from the host cell cytoplasmic materials as already published (*37*). Most of the glutamate dehydrogenase activity, which is considered to be a marker for parasites, was found to be concentrated in the isolated parasites. However, the specific activity of catalase was found to be about 17% of that present in the host cell cytoplasm (Table 1). We have found that parasite preparations have the same hemoglobin degrading enzyme activity with either lysis or mechanical disruption, and despite the presence of host cell membranes in samples isolated by lysis, suggesting that the activity is

Table 2. **Purification profile of *Plasmodium vivax* aspartic protease.**

Purification step	Total protein (mg)	Activity (nmol/min)	Specific activity (nmol/min/mg)	Purity	Yield (%)
1. Triton extract	0.758	5.56	0.142	1	100
2. DEAE extract	0.139	6.92	3.250	23	124
3. pH 4.5 cut	0.034	2.61	6.960	49	47
4. Hydroxyl apatite	0.013	0.97	23.660	167	17
5. HPLC	0.006	0.44	40.150	282	8

due to parasites proteases and not to latent host cell proteases that may have been activated by the parasites (*37*).

Aspartic Acid Protease Purification—An aspartic protease activity was isolated from the extract of *P. vivax* parasites by conventional chromatography on DEAE, hydroxylapatite, and gel filtration columns (Table 2). The resultant peak of activity by purification with HPLC was 252-fold that of the starting material, and the recovery was approximately 8%. This protease activity migrates as a single band of M_r 40,000 on SDS-PAGE and also by HPLC gel filtration chromatography (Fig. 1). The zymogram of protease activity staining also revealed a clear zone of proteolytic activity against a blue background. The protease activity was found to be inhibited by various concentrations of pepstatin confirming it to be an aspartic protease (Fig. 2a). Both the protein and activity showed a molecular mass of 40 kDa. It was also revealed that the isolated protein retains protease activity as shown in the zymogram in Fig. 2b. Definitive assignment will however, require purification to homogeneity and N-terminal sequencing to demonstrate that protein shows homology to other aspartic proteases.

pH Profile of the Aspartic Protease—The pH profile of the aspartic protease activity for the degradation of hemoglobin was determined over the pH range 3–7 using saturating amounts of substrate. The pH optimum of the enzyme was found to be 4.0 (Fig. 3).

Inhibition of Protease Activity by Pepstatin—The effect of the protease inhibitor pepstatin on the activity showed

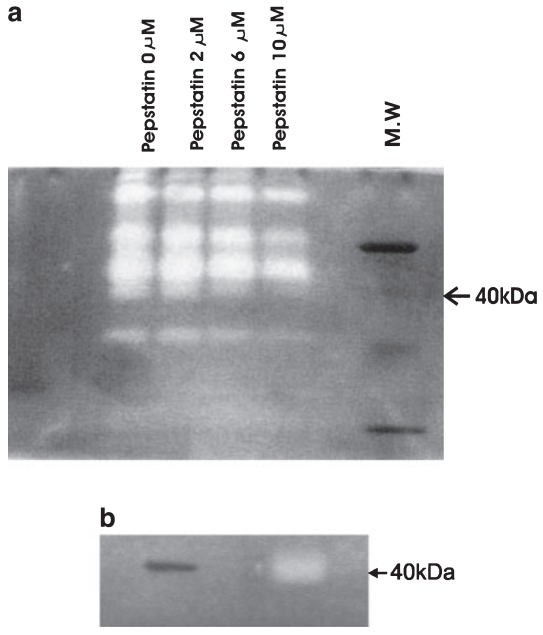

Fig. 2. **a: Zymogram (Gelatin gel PAGE) showing the aspartic protease activity (40 kDa) and its inhibition by different concentrations of Pepstatin (2, 6, 10 μM).** Purified parasite extracts were incubated for 4 h with pepstatin, and gelatin gel PAGE was carried out by copolymerizing 0.1% gelatin with acrylamide. Protease activity was visible as a clear band against a blue background. **b: SDS-PAGE and Zymogram (Gelatin gel PAGE) showing the purified aspartic protease protein and protease activity (40 kDa).** SDS-PAGE analysis. A 50 μl aliquot of the HPLC peak was subjected to electrophoresis in a 12% SDS-PAGE gel. The gel was stained with Coomassie Brilliant Blue. Zymogram analysis: The HPLC-purified protein was electrophoresed in an SDS-PAGE gel with the acrylamide copolymerized with 0.1% gelatin. Protease activity is visible as a clear band against a blue background.

Table 3. **Effect of heavy metal ions and protease inhibitors on the activity of aspartic protease from *P. vivax*.**

Compound	Concentration (mM)	Relative activity (%)
Control		100
Metal ions		
Ca^{2+}	1.0	124
Mg^{2+}	1.0	115
Cu^{2+}	1.0	13
Hg^{2+}	1.0	11
Zn^{2+}	1.0	85
Co^{2+}	1.0	94
Mn^{2+}	1.0	91
Protease inhibitors		
PMSF	1.0	95
o-Phenanthroline	1.0	85
	2.5	97
	5.0	98
EDTA	5.0	81
	10.0	80
	50.0	86

Table 4. **Effect of protease inhibitors on the activity of aspartic protease from *P. vivax*.**

Compound	Concentration (μM)	Relative activity (%)
Control		100
Pepstatin	2.0	75
	5.0	45
	10.0	15
E-64	1.0	98
	2.5	96
	5.0	92
p-Chloromercuribenzoate	1.0	92
SDS	5.0	90
2-Mercaptoethanol	2.5	88
Dithiothreitol	2.5	83
Urea	10.0	90

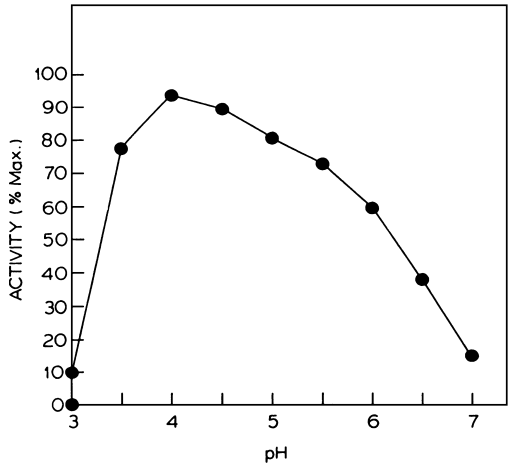

Fig. 3. **Influence of pH on the activity of purified aspartic protease.** Protease activity was determined over a pH range of 3–7 using a saturating amount of substrate as described in "MATERIALS AND METHODS."

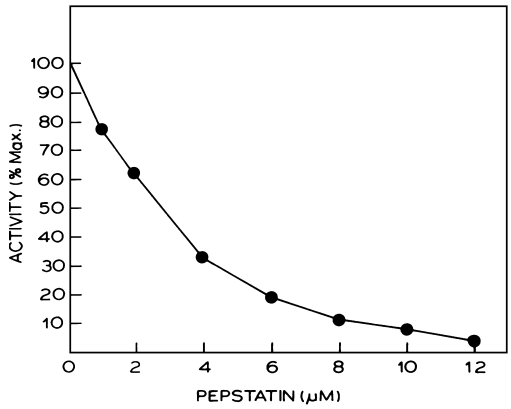

Fig. 4. **Inhibition of the aspartic protease activity by pepstatin.** Protease activity was determined in presence of varing concentrations of pepstatin (2–12 μM) using saturating amounts of substrate as described under "MATERIALS AND METHODS."

an IC_{50} value of ~3.0 μM, indicating that the enzyme belongs to the aspartic protease family (Fig. 4). The enzyme activity was found to be totally inhibited by pepstatin (Fig. 5) whereas the other inhibitors of thiol groups (*p*-chloromercuribenzoate), metalloproteases (*o*-phenanthroline), and cysteine (E-64 and leupeptin) and serine proteases (PMSF) had no effect on the enzyme activity (Fig. 6 and Table 4). Kinetic analyses of the inhibition of the aspartic protease activity by Lineweaver Burk double reciprocal plot of various concentrations of pepstatin demonstrate that the inhibition is competitive with respect to substrate (Fig. 7).

Effects of Heavy Metals, Chelating Agents and Sulfhydryl Reagents—The effects of chemical treatments of the protease with heavy metal ions, chelating agents and sulfhydryl agents are summarized in Table 3. The proteases were treated for 30 min with a given chemical, after which the protease activity was determined. The enzyme activity was found to be activated by Ca^{2+} and Mg^{2+} ions, and to be inhibited by Hg^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, and Mn^{2+}

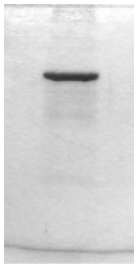

Fig. 5. **SDS-PAGE showing the aspartic protease protein (40 kDa) from *P. vivax* purified by conventional chromatography and HPLC techniques.** A 50 μl aliquot of the HPLC peak was subjected to electrophoresis in a 12% SDS-PAGE gel. The gel was stained with Coomassie Brilliant Blue.

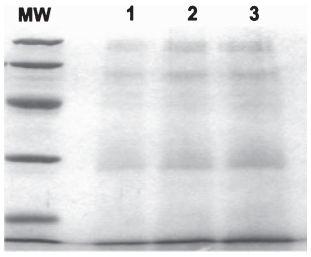

Fig. 6. **SDS-PAGE analysis of the inhibition of aspartic protease activity by other inhibitors.** Lane 1, thiol group (*p*-chloromercuribenzoate), Lane 2, metalloprotease (*o*-phenanthroline) and Lane 3, cysteine protease (E-64). A 50 μl aliquot of the purified parasite extract was subjected to electrophoresis in a 12% SDS-PAGE gel in presence of other inhibitors as described above. The gel was stained with Coomassie Brilliant Blue.

(Table 3). The protease activity was also found to be sensitive to *p*-hydroxymercuribenzoate, suggesting that the protease may possess an essential sulfhydryl group (Table 3). The activity was found to be insensitive to E-64, PMSF and *o*-phenanthroline, which inhibit cysteine, serine and metalloproteases, respectively. The protease activity was found to be sensitive to pepstatin, thereby confirming the aspartic protease nature of the activity. The effects of chelating agents are generally weak, and EDTA had little effect on the activity. SDS, 2-mercaptoethanol and urea also did not have any effect on the activity of this protease (Table 4).

Inhibition of Protease Activity by Antimalarials—The aspartic protease activity was examined for its sensitivity to a series of antimalarial drugs at pH 4.0. The protease activity was found to be insensitive to known classes of antimalarials. Quinine, Chloroquine and Primaquine had essentially no effect at concentrations up to 1 mM. In comparison, 1 mM mefloquine inhibited the enzyme activity to some extent (Table 5).

Table 5. **Inhibition of aspartic protease from *P. vivax* by antimalarial drugs.**

Drug	Concentration (mM)	% Activity
Quinine	1.0	
Chloroquinine	1.0	100
Primaquine	1.0	85
Mefloquine	1.0	65

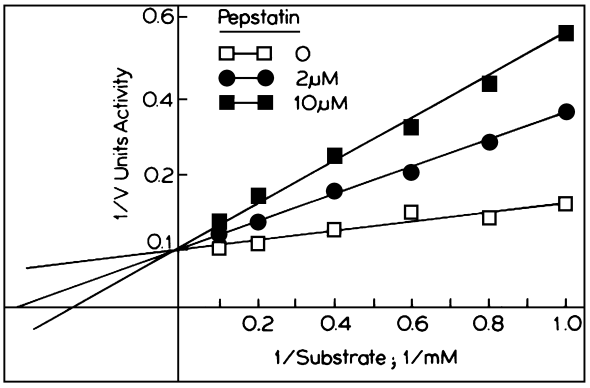

Fig. 7. **Kinetic analysis of the inhibition of aspartic protease activity Lineweaver Burk double reciprocal plot of various concentrations of pepstatin (0, 2, and 10 μM).**

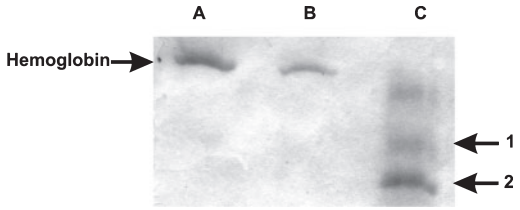

Fig. 8. **SDS-PAGE of the proteolysis products: Hemoglobin (4 μM) was incubated with *P. vivax* malaria aspartic protease (0.14 pmol/min) for 30 min, pH 4.0, in presence or absence of the aspartic protease inhibitor, pepstatin.** An 8–25% gradient gel was run under denaturing reducing conditions. Hemoglobin control (Lane A), hemoglobin + aspartic protease + 10 μM pepstatin (Lane B) and hemoglobin + aspartic protease (Lane C). Arrows 1 and 2 mark the two peptide fragments.

Hemoglobin Degradation In Vitro—To define the substrate specificity of the purified *Plasmodium vivax* aspartic protease towards its natural substrate, hemoglobin, and the enzyme was incubated with hemoglobin for varying periods of time in the presence and absence of pepstatin. After 30 min incubation in the absence of pepstatin, a profile distinguishable from the zero time point was obtained. Arrows 1 and 2 in Fig. 8 mark the two peptides fragments. With increasing time the two primary cleavage products accumulated and can be seen in the absence of pepstatin as fractioned by SDS-PAGE (Fig. 8). The hydrolysis was found to be abolished by the inclusion of pepstatin in the reaction mixture.

DISCUSSION

Among the targets being explored for chemotherapeutic development are families of proteases that play key roles in the life cycle of protozoan parasites (*11*). Hemoglobin proteolysis in the digestive vacuoles of malaria parasites is an ordered process (*43*), presumably due to the limited capacity of the parasite to synthesize amino acids *de novo* (*4–7*). The success of aspartyl protease inhibitors as chemotherapy for HIV has stimulated interest in small molecular weight inhibitors as drugs (*12*, *29*, *44*). An aspartic protease activity is required to act on the intact hemoglobin before further degradation by other proteases can occur (*26*). The acidic nature of the food vacuole is well established, although the details of the mechanism of acidification are not known (*45*). In view of the acidic pH of the food vacuole, we can anticipate that the parasite would utilize hemoglobin degrading proteases with acidic pH optima. Aspartic proteases have been isolated and purified from *P. chabaudi* (*15*), *P. falciparum* (*45*) and *P. lopurae* (*46*), although the functions and intracellular locations of these enzymes have not yet been defined. A membrane bound aspartic protease has been isolated from *P. falciparum* (*19*), and a comparison of the active site properties of cloned plasmepsins from *P. falciparum, P. vivax and P. malariae* has suggested strategies for drug design to inhibit critical enzymes of the parasite (*12*, *32*, *33*).

In this paper, we describe the isolation, purification and properties of an aspartic protease enzyme from *Plasmodium vivax* (*25*). The assignment of the enzyme as aspartic protease is tentative and based on the molecular size (M_r 40,000), aggregation in the presence of SDS, broad substrate specificity, and, most importantly, acidic pH optimum in the range of 4.5–5.0 (*47*, *48*), as well as its high sensitivity to pepstatin (*49*). The lack of inhibition by other classes of protease inhibitors, which is a characteristic of most aspartic proteases (*50*), strongly supports its assignment as an aspartic protease. The data pertaining to the studies of its relationship to other proteases in *P. falciparum* and confirming its aspartic protease mechanism will come from the N-terminal sequencing, cloning and expression studies that are currently in progress in our laboratory.

This paper describes the characterization of an enzyme that is believed to play a key role in the catabolic pathway of hemoglobin. We have studied the protease specificity with its natural substrate, and the substrate properties of this aspartic protease is noteworthy (*31*, *33*, *51*) because it is evident from substrate specificity studies/kinetic analysis (*34*) and structural information that plasmepsins show unique binding characteristics for native and recombinant enzymes (*23*). The ability of aspartic proteases to catalyse the hydrolysis of native hemoglobin (*52*) is consistent with the observation that native hemoglobin is a major form of protein in food vacuoles (*16*, *53*). The possibility, however, exists that oxidized forms of hemoglobin might exist (*54*) and may also be substrates undergoing proteolysis in the food vacuole. Purified enzyme preparations were capable of cleaving hemoglobin, and residues 1 and 2 were extraneous hydrolysis products cleaved by pepstatin-insensitive proteases (*18*, *55*).

The mode of action of chloroquine may be due in part to a diversion of ferriprotoporphyrin IX from its normal processing into the malaria pigment by forming a complex. The acidic pH of the food vacuole appears to be a prime factor contributing to the accumulation of chloroquine (*56*). The antimalarial activity may be a result of the inhibition of hemoglobin degrading proteases by free ferriprotoporphyrin IX by its complex with chloroquine, primaquine etc (*57*). The present study, therefore, provides additional support for this model by demonstrating that the hemoglobin degrading proteases are located in the same place in food vacuole where the malaria pigment is produced.

Our results, therefore, strengthen the idea that proteases are present in *P. vivax* and are involved in plasmodial development. The presence of aspartic proteases in *P. vivax* opens up new avenues to chemotherapy through the design or screening of specific inhibitors that may block essential metabolic pathways of hemoglobin degradation by parasites. Further studies of the malaria aspartic protease should afford a better understanding of the protease–substrate interactions, and may allow rational drug design for a new class of antimalarials.

Thanks are due to Dr. C.R. Pillai, Incharge, Malaria Parasite Bank, Malaria Research Centre, Delhi, for providing *P. falciparum* and *P. vivax* isolates for *in vitro* studies. We wish to thank Mr. Bhanu Arya, Technical Officer, and Mrs. Poonam Gupta, Techical Assistant, for excellent technical help during the course of this study. Thanks are also due to Mr. Tarun Malhotra for excellent artistic work during the preparation of this manuscript.

REFERENCES

1. Walsh, J.A. (1989) Disease problems in the third world. *Ann. N. Y. Acad. Sci.* **569**, 1–16
2. Olliaro, P., Cattani, J., and Writh, D. (1996) Malaria the submerged diseases. *JAMA* **275**, 230–233
3. Kamchonwongpaisan, S., Samoff, E., and Meshnick, S.R. (1997) Identification of hemoglobin degradation products in *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **86**, 179–186
4. Scheibal, L.W. and Sherman, I.W. (1988) Plasmodial metabolism and related organellar function during various stages of the life cycle: Proteins, lipids, nucleic acids and vitamins in *Malaria: Principals and practice of Malariology* (Wernsdofer, W.H. and McGregerrds, eds.) pp. 219–252, Churchill Livingstone, Edinburgh
5. Mckerrow, J.H., Sun, E., Rosenthal, P.J., and Bouvir, J. (1993) Proteases and pathogenicity of parasitic protozoa. *Ann. Rev. Microbiol.* **17**, 821–853
6. Rosenthal, P.J. and Meshnick, S.R. (1996) Hemoglobin catabolism and Fe utilization by malaria parasites. *Mol. Biochem. Parasitol.* **83**, 131–139
7. Francis, S.E., Sullivan, D.G. Jr., and Goldberg, D.E. (1997) Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum*. *Annu. Rev. Microbiol.* **51**, 97–123
8. Sherman, I.W. (1979) Biochemistry of plasmodium (malaria parasite). *Microbiol. Rev.* **43**, 453–494
9. Sherman, I.W. and Tanigoshi, L. (1970) Incorporation of ^{14}C-amino acids by malaria (*Plasmodium lophurae*) IV. *In vivo* utilization of host cell hemoglobin. *Int. J. Biochem.* **1**, 635–637
10. Salas, F., Fichmann, J., Lee, G.K., Scott, M.D., and Rosenthal, P.J. (1995) Functional expression of falcipain, *Plasmodium falciparum* cysteine proteinase supports its role as a malarial hemoglobinase. *Infect. Immun.* **63**, 2120–2125
11. Coombs, G.H., Goldberg, D.E., Klemba, M., Berry, C., Kay, J., and Mottram, J. (2001) Aspartic proteases of *Plasmodium falciparum* and other parasitic protozoa as drug targets. *Trends Parasitol.* **17**, 532–537
12. Boss, C., Richard-Bildstein, S., Weller, T., Fischli, W., Meyer, S., and Binkert, C. (2003) Inhibitors of the *Plasmodium falciparum* parasite protease plasmepsin II as potential antimalarial agents. *Curr. Med. Chem.* **10**, 883–907
13. Rosenthal, P.J., Mckerrow, J.H., Aikawa, M., and Leach, J.H. (1988) A malarial cysteine proteinase necessary for hemoglobin degradation by *Plasmodium falciparum*. *J. Clin. Invest.* **82**, 1560–1566
14. Rosenthal, P.J., Ring, C.S., Chen, X., and Cohen, F.E. (1993) Characterization of a *Plasmodium vivax* cysteine proteinase gene identifies uniquely conserved amino acid that may mediate the substrate specificity of malarial hemoglobinases. *J. Mol. Biol.* **241**, 312–316
15. Martins, T.M., Novo, C., do Rosario, V.E., and Domingos, A. (2003) Aspartic proteases from *Plasmodium chabaudi*: a rodent model for human malaria. *Acta Trop.* **89**, 1–12
16. Dame, J.B., Yowell, C.A., Omara-Opyene, L., Carlton, J.M., Cooper, R.A., and Li, T. (2003) Plasmepsin 4, the food vacuole aspartic proteinase found in all *Plasmodium* spp. infecting man. *Mol. Biochem. Parasitol.* **130**, 1–12
17. Berry, C., Dame, J.B., Dunn, B.M., and Kay, J. (1995) Aspartic proteinases from the human malaria parasite *Plasmodium falciparum*. *Adv. Exp. Med. Biol.* **362**, 511–518
18. Goldberg, D.E., Slater, A.F.G., Beavis, R., Chait, B., Cerami, A., and Henderson, G.B. (1991) Hemoglobin degradation in the human malaria pathogen *Plasmodium falciparum*: A catabolic pathway initiated by a specific aspartic protease. *J. Exp. Med.* **173**, 961–969
19. Vander Jagt, D.L., Hunsaker, L.A., Campos, N.M., and Scaletti, J.V. (1992) Localization and characterization of hemoglobin-degrading aspartic proteinases from the malarial parasite *Plasmodium falciparum*. *Biochim. Biophy. Acta* **1122**, 256–264
20. Dame, J.B., Reddy, G.R., Yowell, C.A., Dunn, B.M., Kay, J., and Berry, C. (1994) Sequence, expression and modeled structure of an aspartic proteinase from the human malaria parasite *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **64**, 177–190
21. Gluzman, I.Y., Francis, S.E., Oksman, A., Smith, C.E., Duffin, K.L., and Goldberg, D.E. (1994) Order and specificity of the *Plasmodium falciparum*: Hemoglobin degradation pathway. *J. Clin. Invest.* **93**, 1602–1608
22. Hill, J., Tyas, L., Phylip, L.H., Kay, J., Dunn, B.M., and Berry, C. (1994) High level expression and characterization of plasmepsin II, an aspartic proteinase from *Plasmodium falciparum*. *FEBS Lett.* **352**, 155–158
23. Tyas, L., Gluzman, I., Moon, R.P., Rupp, K., Westling, J., Ridley, R.G., Kay, J., Goldberg, D.E., and Berry, C. (1999) Naturally-occurring and recombinant forms of the aspartic proteinases plasmepsin I and II from the human malaria parasite *Plasmodium falciparum*. *FEBS Lett.* **454**, 210–214
24. Humphreys, M.J., Moon, R.P., Klinder, A., Fowler, S.D., Rupp, K., Bur, D., Ridley, R.G., and Berry, C. (1999) The aspartic proteinase from the rodent parasite *Plasmodium berghei* as a potential model for plasmepsin from the human malaria parasite, *Plasmodium falciparum*. *FEBS Lett.* **463**, 43–48
25. Johansson, P.O., Chen, Y., Belfrage, A.K., Blackman, M.J., Kvarnstrom, I., Jansson, K., Vrang, L., Hamelink, E., Rosenquist, A., and Samuelson, B. (2004) Design and synthesis of potent inhibitors of the malaria aspartyl proteases plasmepsin I and II. Use of solid phase synthesis to explore novel statine motifs. *J. Med. Chem.* **47**, 3353–3366
26. Wyatt, D.M. and Berry, C. (2002) Activity and inhibition of plasmepsin IV, a new aspartic proteinase from the malaria parasite *Plasmodium falciparum*. *FEBS Lett.* **513**, 159–162
27. Francis, S.E., Gluzman, I.Y., Oksman, A., Knickerbocker, A., Muelber, R., Bryant, M.L., Sherman, D.R., Rusell, D.G., and Goldberg, D.G. (1994) Molecular characterization and inhibition of a *Plasmodium falciparum* aspartic hemoglobinase. *EMBO J.* **13**, 306–317
28. Moon, R.P., Tyas, L., Certa, U., Rupp, K., Bur, D., Jacquet, C., Matile, H., Loetscher, H., Grueninger-Leitch, F., Kay, J., Dunn, B.M., Berry, C., and Ridley, R.G. (1997) Expression and characterization of plasmepsin I from *Plasmodium falciparum*. *Eur. J. Biochem.* **244**, 552–560
29. Nezami, A. and Freire, E. (2002) The integration of genomic and structural information in the development of high affinity plasmepsin inhibitors. *Int. J. Parasitol.* **32**, 1669–1676
30. Nezami, A., Luque, I., Kimura, T., Kiso, Y., and Freire, E. (2002) Identification and characterization of allophenylnorstatine-based inhibitors of plasmepsin II, an antimalarial target. *Biochemistry* **41**, 2273–2280
31. Na, B.K., Lee, E.G., Lee, H.W., Cho, S.H., Bae, Y.A., Kong, Y., Lee, J.K., and Kim, T.S. (2004) Aspartic proteases of *Plasmodium vivax* are highly conserved in wild isolates. *Korean J. Parasitol.* **42**, 61–66

32. Westling, J., Cipullo, P., Hung, S.H., Saft, H., Dame, J.B., and Dunn, B.M. (1999) Active site specificity of plasmepsin II. *Protein Sci.* **8**, 2001–2009
33. Westling, J., Yowell, C.A., Majer, P., Erickson, J.W., Dame, J.B., and Dunn, B.M. (1997) *Plasmodium falciparum*, *P. vivax*, and *P. malariae*: a comparison of the active site properties of plasmepsins cloned and expressed from different species of malaria parasite. *Exp. Parasitol.* **87**, 185–193
34. Luker, K.E., Francis, S.E., Gluzman, I.Y., and Goldberg, D.E. (1996) Kinetic analysis of plasmepsins I and II, aspartic proteases of the *Plasmodium falciparum* digestive vacuole. *Mol. Biochem. Parasitol.* **79**, 71–78
35. Fagan, J.M., Waxman, L., and Goldberg, A.L. (1986) Red blood cells contain a pathway for the degradation of oxidant-damaged hemoglobin that does not require ATP or ubiquitin. *J. Biol. Chem.* **261**, 5705–5713
36. Biswas, S., Saxena, Q.B., Roy, A., and Sharma, V.P. (1988) Isolation of different erythrocytic stages of *Plasmodium falciparum* and synchronization in culture. *Ind. J. Malariol.* **25**, 7–10
37. Sharma, A. (1993) Subcellular distribution of superoxide dismutase and catalase in human malaria parasite *Plasmodium vivax*. *Ind. J. Exp. Biol.* **31**, 275–277
38. Dacie, J.V. and Lewis, S.M. (1968) *Practical Haematology*, 4th ed., Churchill, London
39. Walter, R.D., Nordmeyer, J.P., and Konigk, E. (1974) NADP specific glutamate dehydrogenase from *P. chabaudi*. *Hoppe Seyler's Z. Physiol. Chem.* **355**, 495–500
40. Lowry, O.H., Roseborough, N.J., Fare, A.L., and Randall, R.L. (1951) Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275
41. Hatakeyama, T., Kohzaki, H., and Yamasaki, N. (1992) Microassay for proteases using succinyl casein as a substrate. *Annal. Biochem.* **204**, 181–184
42. Laemmli, U.K. (1970) Cleavage of structuaral proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
43. Goldberg, D.E., Slater, A.F.G., Cerami, A., and Henderson, G.B. (1990) Hemoglobin degradation in the malaria parasite *Plasmodium falciparum*: An ordered process in a unique organelle. *Proc. Natl Acad. Sci. USA* **87**, 2931–2935
44. Moyle, G.J., Youle, M., Higgs, C., Monaghan, J., Prince, W., Chapman, S., Cleudenin, N., and Nelson, M.R. (1998) Safety, .pharmacokinetics and antiretrovial activity of the potent specific human immunodeficiency virus protease inhibitor nelfinavir: results of a phase I/II trial and extended follow-up in patients infected with human immunodeficiency virus. *J. Clin. Pharmacol.* **38**, 736–743
45. Vander Jagt, D.L., Hunsaker, L.A., and Campos, N.M. (1986) Characterization of hemoglobin-degrading, low molecular weight protease from *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **18**, 389–400
46. Sherman, I.W. and Tanigoshi, L. (1983) Purification of *Plasmodium lophurae* cathepsin D and its effects on erythrocyte membrane proteins. *Mol. Biochem. Parasitol.* **8**, 207–226
47. Dunn, B.M., Valler, M.J., Rolph, C.E., Jimenez, M., and Kay, J. (1987) The pH dependence of the hydrolysis of chromogenic substrates by selected aspartic proteinases. *Biochim. Biophys. Acta* **913**, 122–130
48. Xie, D., Gulnik, S., Collins, L., Gustchina, E., Suvorov, L., and Erickson, J.W. (1997) Dissection of the pH dependence of inhibitor binding energetics for an aspartic protease: Direct measurement of the protonation states of the catalytic aspartic acid residues .*Biochemistry* **36**, 16166–16172
49. Bailly, E., Jambou, R., Savel, J., and Jaureguiberry, G. (1992) *Plasmodium falciparum*: Differential sensitivity *in vitro* to E-64 (cysteine protease inhibitor) and Pepstatin A (aspartyl protease inhibitor). *J. Protozool.* **39**, 593–599
50. Ring, C.S., Sun, E., Mckerrow, J.H., Lee, G.K., Rosenthal, P.J., Kuntz, L.D., and Cohen, F.E. (1993) Structure-based inhibitor design by using protein models for the development of antiparasite agents. *Proc. Natl Acad. Sci. USA* **90**, 3583–3587
51. Kay, J. and Dunn, B.M. (1992) Substrate specificity and inhibitors of aspartic proteinases. *Scand. J. Clin. Lab. Invest.* **52**, 23–30
52. Kolakovich, K.A., Gluzman, I.Y., Duffin, K.L., and Goldberg, D.E. (1997) Generation of hemoglobin peptides in the acidic digestive vacuole of *Plasmodium falciparum* implicates peptide transport in amino acid production. *Mol. Biochem. Parasitol.* **87**, 123–135
53. Semenov, A., Olson, J.E., and Rosenthal, P.J. (1998) Antimalarial synergy of cysteine and aspartic protease inhibitors. *Antimicrobiol. Agents Chemother.* **42**, 2254–2258
54. Goldberg, D.E. (1992) Plasmodial hemoglobin degradation: An ordered pathway in specialized organelle. *Infect. Agents Dis.* **1**, 207–211
55. Beavis, R.C. and Chait, B.T. (1990) Rapid sensitive analysis of protein mixtures by mass spectrometry. *Proc. Natl Acad. Sci. USA* **87**, 6873–6877
56. Yayon, A., Cabantchik, Z.I., and Ginsburg, H. (1984) Identification of the acidic compartment of *Plasmodium falciparum* infected human erythrocytes as the target of the antimalarial drug chloroquine. *EMBO J.* **3**, 2695–2700
57. Krogstad, D.J., Schlesinger, P.H., and Gluzman, I.Y. (1985) Antimalarials increase vesicle pH in *Plasmodium falciparum*. *J. Cell Biol.* **101**, 2302–2309