

Identification and biochemical characterization of vivapains, cysteine proteases of the malaria parasite *Plasmodium vivax*

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Cysteine proteases play important roles in the life cycles of malaria parasites. Cysteine protease inhibitors block haemoglobin hydrolysis and development in *Plasmodium falciparum*, suggesting that the cysteine proteases of this major human pathogen, termed falcipains, are appropriate therapeutic targets. To expand our understanding of plasmodial proteases to *Plasmodium vivax*, the other prevalent human malaria parasite, we identified and cloned genes encoding the *P. vivax* cysteine proteases, vivapain-2 and vivapain-3, and functionally expressed the proteases in *Escherichia coli*. The vivapain-2 and vivapain-3 genes predicted papain-family cysteine proteases, which shared a number of unusual features with falcipain-2 and falcipain-3, including large prodomains and short N-terminal extensions on

the catalytic domain. Recombinant vivapain-2 and vivapain-3 shared properties with the falcipains, including acidic pH optima, requirements for reducing conditions for activity and hydrolysis of substrates with positively charged residues at P1 and Leu at P2. Both enzymes hydrolysed native haemoglobin at acidic pH and the erythrocyte cytoskeletal protein 4.1 at neutral pH, suggesting similar biological roles to the falcipains. Considering inhibitor profiles, the vivapains were inhibited by fluoromethylketone and vinyl sulphone inhibitors that also inhibited falcipains and have demonstrated potent antimalarial activity.

Key words: cysteine protease, haemoglobin, *Plasmodium*, vivapain.

INTRODUCTION

Malaria is one of the most important infectious diseases in the world, and the problem appears to be worsening [1,2]. Control efforts are seriously limited by the increasing resistance of malaria parasites to antimalarial drugs, inadequate control of mosquito vectors and the lack of effective vaccines. Considering these factors, new approaches to antimalarial chemotherapy are needed urgently. Among these approaches is the targeting of newly identified enzymes with essential roles in the parasite life cycle.

Proteases play crucial roles in the life cycles of malaria parasites [3,4]. They appear to be required for a number of important functions in erythrocytic parasites, including haemoglobin hydrolysis, erythrocyte rupture and erythrocyte invasion. The best characterized proteolytic function is the hydrolysis of haemoglobin, which provides amino acids for parasite protein synthesis and may serve other necessary functions [5]. In *Plasmodium falciparum*, the most virulent human malaria parasite, haemoglobinases have been identified from the aspartic [6–8], cysteine [9–11] and metallo [12] protease classes, but the specific roles of these enzymes in the sequential hydrolysis of haemoglobin have not yet been delineated. Treatment with cysteine protease inhibitors blocks haemoglobin hydrolysis and parasite development *in vitro* [9,13] and in murine models [14,15], suggesting that plasmodial cysteine proteases are appropriate new chemotherapeutic targets. Therefore improved characterization of plasmodial cysteine proteases is an important goal.

Papain-family cysteine proteases have been characterized in *P. falciparum* (falcipains) and murine malaria parasites (vinckepains) [10,11,16–18]. Falcipain-2 and falcipain-3 appear to be the key *P. falciparum* haemoglobinases [10,11]. Plasmodial cysteine proteases may also hydrolyse other erythrocyte proteins. In particular, falcipain-2 cleaves cytoskeletal proteins [19,20], potentially facilitating erythrocyte rupture by mature schizonts, and falcipain-1 appears to be required for the invasion of erythrocytes by merozoites [21].

Although less virulent than *P. falciparum*, *P. vivax* is the most widely distributed human malaria parasite, and it causes extensive morbidity [22]. These two parasites are responsible for more than 90 % of episodes of human malaria, totalling several hundred million cases annually. However, comprehensive studies of *P. vivax* have been limited due to technical shortcomings. Notably, unlike the case with *P. falciparum*, routine *in vitro* culture of *P. vivax* is not available, and animal models are limited to primates. It is essential that the development of drugs against plasmodial cysteine proteases considers targets in both of the two prevalent human parasites. In the present paper, we describe the identification and cloning of two cysteine protease genes (vivapain-2 and vivapain-3) from *P. vivax* and the biochemical characterization of the heterologously expressed gene products. We found that the cysteine proteases are apparent orthologues of falcipain-2 and falcipain-3, but that key differences in the biochemical properties of the plasmodial proteases warrant attention to the inhibition of each enzyme in the evaluation of antimalarial protease inhibitors.

Abbreviations used: ACC, 7-amino-4-carbamoylmethylcoumarin; AMC, 7-amino-4-methylcoumarin; DTT, dithiothreitol; E-64, *trans*-epoxysuccinyl-L-leuciloamido-(4-guanidino)butane; FMK, fluoromethylketone; IPTG, isopropyl-1-thio- β -D-galactopyranoside; Ni-NTA, Ni²⁺-nitrilotriacetate; Z, benzylloxycarbonyl.

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The nucleotide sequences reported in this paper have been submitted to the DDBJ, EMBL, GenBank[®] and GSDB Nucleotide Sequence Databases under the accession numbers AY208270 and AY211736.

EXPERIMENTAL

Materials

P. vivax genomic DNA was kindly provided by Dr John Barnwell (Centers for Disease Control and Prevention, Atlanta, GA, U.S.A.). Z-Phe-Arg-AMC (benzyloxycarbonyl-Phe-Arg-7-amino-4-methylcoumarin) was purchased from Bachem (Torrance, CA, U.S.A.) and Z-Leu-Arg-AMC was from Peptides International (Louisville, KY, U.S.A.). All other peptide substrates were a gift from Dr David Tew (GlaxoSmithKline, King of Prussia, PA, U.S.A.). FMK (fluoromethylketone) and vinyl sulphone inhibitors were gifts from Dr Robert Smith (Prototek, Dublin, CA, U.S.A.) and Dr James Palmer (Celera, South San Francisco, CA, U.S.A.) respectively. Restriction endonucleases and T4 DNA ligase were from New England Biolabs (Beverly, MA, U.S.A.). Antibodies for protein 4.1 were kindly provided by Dr Joel Chasis (University of California, San Francisco, CA, U.S.A.). All other reagents and antibodies were from Sigma or as mentioned in the text.

Cloning of the vivapain-2 and vivapain-3 genes

PCR was performed with 200 ng of *P. vivax* genomic DNA (*SaII* strain), *Taq* DNA polymerase (Invitrogen) and two degenerate oligonucleotide primers based on conserved amino acids of previously identified *Plasmodium* papain-family enzymes [5'-AATTGTGG(T/A)TC(A/C)TG(C/T)TGGGC(T/C)TTCAGCAC-3' and 5'-CCA(A/C)GA(A/G)TTCTT(T/C)A(G/C)AATGTAGT-AGTA-3']. Amplified products were gel-purified, ligated into the pCR2.1 vector and transformed into competent *Escherichia coli* TOP10 cells, using a TOPO TA Cloning Kit (Invitrogen). Sequencing of multiple clones revealed amplification of two different gene fragments (named vivapain-2 and vivapain-3). To complete the characterization of the vivapain genes, inverse PCR was performed by the following method: genomic *P. vivax* DNA (2 µg) was digested with *Bam*HI at 37 °C for 3 h and the digested DNA fragments were purified by ethanol precipitation. The precipitated DNA was suspended in distilled water, and ligation was performed by using T4 DNA ligase in a total volume of 500 µl at 15 °C overnight. The ligated DNA was purified by ethanol precipitation and suspended in 50 µl of distilled water. Inverse PCR was performed with inverted primers for vivapain-2 (PV2IF, 5'-GCAGAAGATGCCTACGATTTTGATACGAAA-3' and PV2IR, 5'-CTGTTGCTCACTTATGGAGACCAGCTGATT-3') and vivapain-3 (PV3IF, 5'-ATGGAAGAAATGTATGATGCC-ATGAGCCGA-3' and PV3IR, 5'-TTCTTGCTCACTTAGGGGA-GACTAACTCTTT-3') using the ligated DNA as a template. The amplified products were gel-purified, ligated into the pCR2.1 vector and transformed into competent *E. coli* TOP10 cells as described above. Clones were selected and sequenced in both directions. Nucleotide and deduced amino acid sequences were analysed with the SeqEd.V1.0.3 program and CLUSTAL of the Megalign program, a multiple-alignment program of the DNASTAR package (DNASTAR, Madison, WI, U.S.A.).

Expression, purification and refolding of recombinant vivapain-2 and vivapain-3

Fragments predicted to encode the mature regions and portions of the prodomains of vivapain-2 and vivapain-3 were amplified using primers specific for vivapain-2 (5'-GAGCTCGAGATG-CAACAGAGGTACCT-3', containing a 5' *Sac*I site and 5'-CTGC-AGCTAATCCACGAGCGCAACGA-3', containing a 5' *Pst*I site) and vivapain-3 (5'-GGATCCGAAATGCAACAGAGGTACCT-3',

containing a 5' *Bam*HI site and 5'-CTGCAGTCAAACCTTCG-TCAATCAAAG-3', containing a 5' *Pst*I site). The PCR products were purified, ligated into the pCR2.1 vector and transformed into competent *E. coli* TOP10 cells as described above. The resulting plasmid DNA was digested with corresponding restriction enzymes and ligated to pQE30 expression vectors (Qiagen, Valencia, CA, U.S.A.), predigested with the same enzymes. These plasmids were transformed into competent *E. coli* M15 (pREP4) cells (Qiagen), and positive clones were selected, grown overnight and treated with 1 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside) to induce protein expression. The bacteria were then suspended in 2 M urea/2.5% (v/v) Triton X-100, sonicated and centrifuged at 12 000 g for 20 min. Inclusion bodies were solubilized in lysis buffer (8 M urea/20 mM Tris/HCl/500 mM NaCl/10 mM imidazole, pH 8.0), and the recombinant proteins were purified by Ni-NTA (Ni²⁺-nitrilotriacetate; Qiagen) chromatography. Refolding of the purified recombinant enzymes was optimized by testing more than 100 different buffer combinations in a microplate format as described previously [23]. For large-scale refolding, 100 mg of Ni-NTA-purified vivapain-2 or vivapain-3 was reduced with 10 mM DTT (dithiothreitol), diluted 100-fold in 2 litres of ice-cold optimized refolding buffer and incubated at 4 °C for 20 h. The refolded sample was concentrated to 100 ml using a High-Performance Ultrafiltration Cell (model 2000; Amicon, Beverly, MA, U.S.A.) with a 10 kDa cut-off membrane. To allow processing to active enzyme, the pH of the refolded sample was adjusted to 5.5 with 3.5 M sodium acetate (pH 2.6); DTT was added to a final concentration of 5 mM, the precipitated material was removed (0.22 µm filter; Millipore, Billerica, MA, U.S.A.) and the sample was incubated at 37 °C for 2 h. The pH was then readjusted to 6.5 with 1 M Tris/HCl (pH 8.0) and the protein was applied to a Q-Sepharose column (Amersham Biosciences) pre-equilibrated with 20 mM Bis-Tris/HCl (pH 6.5) and maintained at 4 °C. The column was washed with 5–10 bed vol. of the same buffer, and the protein was eluted with a 0–0.4 M linear NaCl gradient for 30 min at a flow rate of 1.5 ml/min. Fractions containing enzyme activity were pooled and concentrated by membrane filtration (10 kDa cut-off Centriprep; Millipore). Enzyme activity was assayed fluorimetrically as the hydrolysis of Z-Leu-Arg-AMC, as described previously [10]. Briefly, 30 µl of enzyme solution was added to 320 µl of sodium acetate buffer (pH 5.5) containing 25 µM Z-Leu-Arg-AMC and 10 mM DTT, and the release of fluorescence (excitation 355 nm, emission 460 nm) for 20 min at room temperature was assessed with a Labsystems Fluoroskan II spectrofluorometer.

Characterization of biochemical properties of vivapain-2 and vivapain-3

Enzyme assays evaluated the hydrolysis of Z-Leu-Arg-AMC, as described above, with changes in buffers and reductants as described in the Figure legends. For substrate gel analysis, samples were mixed with SDS/PAGE sample buffer lacking 2-mercaptoethanol and electrophoresed on an SDS/polyacrylamide gel co-polymerized with 0.1% gelatin. The gel was washed twice with 2.5% Triton X-100 at room temperature for 30 min, incubated overnight at 37 °C in 100 mM sodium acetate, 10 mM DTT (pH 5.5), stained with Coomassie Blue and then destained to identify proteolytic activity as clear bands on the gel.

N-terminal amino acid sequencing

Purified processed enzymes were electrophoresed, transferred to PVDF membranes (Millipore), stained with Coomassie Blue and

destained. Enzyme bands were excised and sequenced by Edman sequencing at the UCSF Biomolecular Resource Center.

Enzyme kinetics

The concentrations of vivapain-2 and vivapain-3 were determined by titration with Mu-Leu-hPhe-FMK (where Mu stands for morpholine urea and hPhe is homophenylalanine). Rates of hydrolysis of peptide-AMC substrates were determined in the presence of constant enzyme concentrations for each substrate (0.077 nM vivapain-2 and 0.5 nM vivapain-3). Fluorimetric assays of the enzymes were performed in 100 mM sodium phosphate/5 mM DTT, pH 6.5 (vivapain-2) or 100 mM sodium acetate/6 mM DTT, pH 5.5 (vivapain-3) in a final volume of 0.35 ml. Fluorogenic substrates were added, and the release of AMC was monitored (excitation 355 nm, emission 460 nm) for 10 min at room temperature with a Labsystems Fluoroskan Ascent spectrofluorometer. Activities were compared in terms of fluorescence as a function of time. The kinetic constants K_m and V_{max} were determined using GraphPad software.

Determination of substrate specificity using positional scanning tetrapeptide libraries

Two synthetic combinatorial libraries were used to determine the substrate specificities of the S1–S4 subsites of vivapain-2 and vivapain-3 as described previously [24]. Briefly, a bifunctional fluorophore, ACC (7-amino-4-carbamoylmethylcoumarin), incorporating a site for peptide synthesis and another site for attachment to a solid support was used to prepare the fluorogenic substrates. By using an acid-labile Rink linker between the ACC group and the resin, Fmoc (9-fluorenylmethoxycarbonyl)-based solid-phase synthesis techniques were used to produce efficiently the substrate libraries, as described previously [25]. To determine P1 specificity, a P1 diverse library consisting of 20 sublibraries was used. In each sublibrary, the P1 position contained one native amino acid (cysteine was replaced by norleucine), and the P2, P3 and P4 positions were randomized with equimolar mixtures of amino acids (in each case, cysteine was omitted and methionine was replaced by norleucine) for a total of 6859 tetrapeptide substrates per sublibrary. Aliquots of 8.9×10^{-9} mol from each sublibrary were added to 20 wells of a 96-well Microfluor-1 U-bottom plate (Dyex Technologies, Chantilly, VA, U.S.A.) for a final concentration of 13 nM of each compound per well. To determine P2, P3 and P4 specificity, a P1-lysine fixed library was used. In this library, the P1 position was fixed with lysine and the P2, P3 or P4 positions were spatially addressed with 19 amino acids (cysteine was omitted and methionine was replaced by norleucine), whereas the remaining two positions were randomized. Aliquots of 9×10^{-9} mol from each sublibrary were added to 60 wells (361 compounds/well) for a final concentration of 250 nM of each compound per well. Hydrolysis reactions were initiated by the addition of 8.8 nM vivapain-2 or 7.5 nM vivapain-3 and monitored fluorimetrically with a Molecular Devices SpectraMax Gemini spectrofluorometer, with excitation at 380 nm and emission at 460 nm. Assays were performed at 37 °C in 100 mM sodium phosphate (pH 6.5) for vivapain-2 or 100 mM sodium acetate (pH 5.5) for vivapain-3, in each case also with 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.01 % Brij-35 and 1 % DMSO.

Haemoglobinase activity assays

To evaluate haemoglobinase activity, vivapain-2 and vivapain-3 were incubated with native human haemoglobin with different

reaction conditions as described in the Figure legends. For studies of protease inhibitors, proteases were preincubated with the inhibitors in 100 mM sodium acetate, 10 mM DTT (pH 5.5) for 15 min before adding haemoglobin. Reactions were stopped at appropriate time points by the addition of SDS/PAGE reducing sample buffer, and reaction products were analysed by SDS/PAGE. The remaining haemoglobin at each time point was densitometrically quantified as a percentage of the substrate density at the start of the reaction. As an alternative assay, haemoglobin hydrolysis was analysed based on spectrophotometric changes caused by proteolysis, as described previously [26].

Hydrolysis of erythrocyte membrane proteins

Erythrocyte ghosts were purified from fresh human blood by hypo-osmotic lysis of erythrocytes in 5 mM sodium phosphate (pH 8.0) and 5 mM $MgCl_2$, as described previously [27]. The purified ghosts were incubated with 200 nM vivapain-2 or vivapain-3 at pH 6.5, 7.0 or 7.5 at 37 °C for 3 h and then analysed by SDS/PAGE. For immunoblots, proteins were transferred on to nitrocellulose membranes (0.45 μm ; Bio-Rad Laboratories, Hercules, CA, U.S.A.). Membranes were blocked with PBST (0.05 % Tween 20 in PBS) containing 2 % (w/v) BSA for 1 h at room temperature, and then incubated with antibodies. The antibodies studied were monoclonal antibodies against spectrin (1:500), band 3 (1:30000), actin (1:1000) and glycophorin A (1:2000) and anti-peptide antibodies directed against two regions of protein 4.1, the spectrin–actin binding domain (KKRERLDGENIYIRHSNLMLE) and the C-terminus (HPDMSVTKVVVHQETEIADE). Blots were incubated with antibodies for 2 h at room temperature, washed with PBST, incubated with alkaline phosphatase-conjugated anti-mouse IgG (1:30000) or, for protein 4.1 antisera, alkaline phosphatase-conjugated anti-rabbit IgG (1:20000) for 2 h at room temperature, washed with PBST and incubated in freshly prepared substrate (5-bromo-4-chloroindol-3-yl phosphate/Nitro Blue Tetrazolium) for 10 min at room temperature. The reaction was stopped by washing the membrane with distilled water several times.

Inhibitor kinetics

For inhibitor studies, recombinant vivapain-2 and vivapain-3 were prepared as above, and falcipain-2 and falcipain-3 were prepared by similar methods, as described previously [10,11]. Inhibitor second-order binding constants were determined using the progress-curve method [28]. Briefly, the enzymes were incubated in the presence of peptide-AMC substrates and inhibitors under pseudo-first-order conditions (inhibitor concentration at least ten times the enzyme concentration, determined by active-site titration), and product formation was continuously monitored using a Labsystems Ascent spectrofluorometer. The resultant progress curves (fluorescence versus time) obtained in the presence and absence of inhibitor were analysed by non-linear regression analysis (GraphPad software) using the pseudo-first-order equation

$$y = A[1 - \exp(-k_{obs}t) + B],$$

where y is the fluorescence at time t , A the amplitude of the reaction and B the offset. If k_{obs} varied linearly with $[I]$, the association constant k_{ass} was determined by linear regression using

$$k_{ass} = (k_{obs}/[I])(1 + [S]/K_m),$$

where $[S]$ is the substrate concentration.

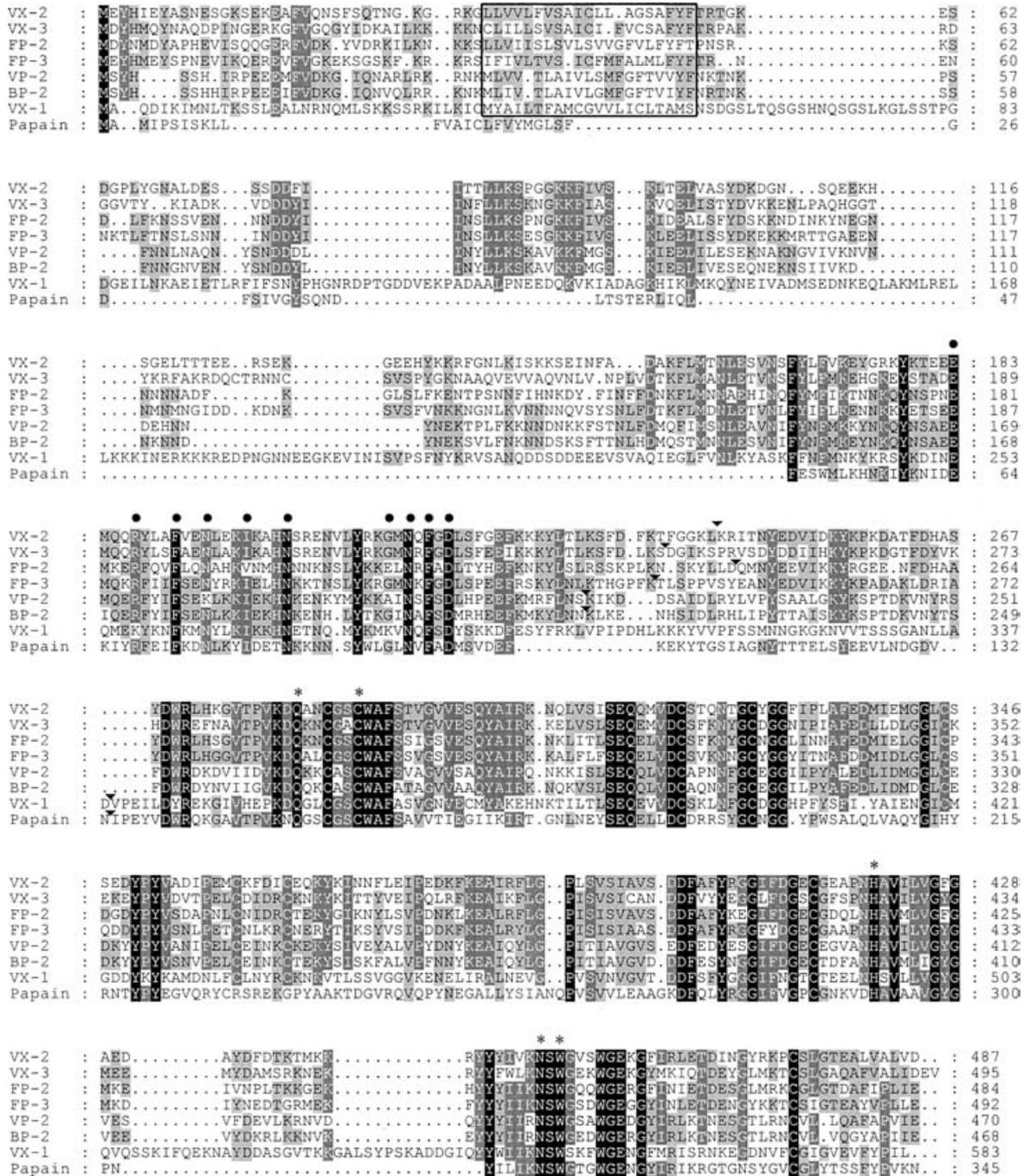


Figure 1 Sequence alignment

Deduced amino acid sequences of vivapain-1 (VX-1), vivapain-2 (VX-2), vivapain-3 (VX-3), falcipain-2 (FP-2), falcipain-3 (FP-3), berghepain-2 (BP-2), vinckeapain-2 (VP-2) and papain were aligned using the DNASTAR program. Dashes represent gaps introduced to maximize alignment. The shading represents degree of homology. Predicted transmembrane domains are boxed. Amino acids representing ERFNIN and GNFD-conserved prodomain motifs are labelled with filled circles. Asterisks (*) indicate conserved active-site residues. The positions of confirmed or predicted mature domain processing sites are indicated by arrows. Percentage of identity among the sequences is represented as shading: black (> 88%), dark grey (75–88%), light grey (37.5–75%) and no shading (< 37%).

If k_{obs} varied hyperbolically with $[I]$, then non-linear regression was performed [29] to determine the inactivation constant k_{inact} and the inhibition constant K_i , using the equation

$$k_{obs} = (k_{inact}[I]) / ([I] + K_{i,app}),$$

where $K_{i,app} = K_i(1 + [S]/K_m)$.

RESULTS

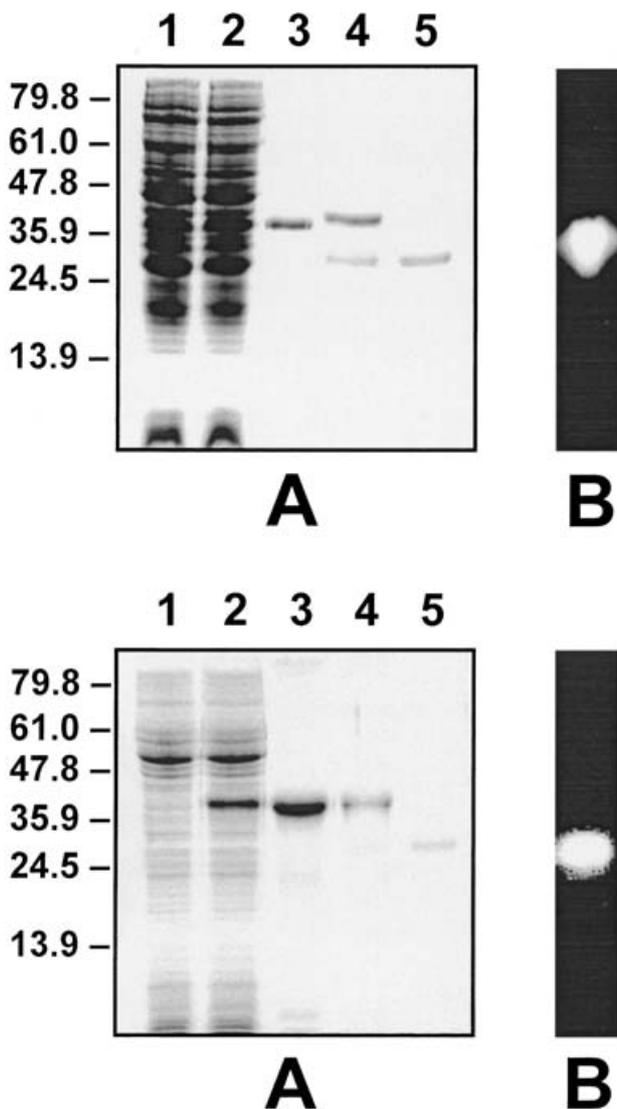
Cloning and sequence analysis of vivapain-2 and vivapain-3

Degenerate oligonucleotide primers based on other plasmodial cysteine protease genes were used to amplify portions of homologous genes, namely vivapain-2 and vivapain-3, from *P. vivax* genomic DNA. Amplification of the remainder of these genes

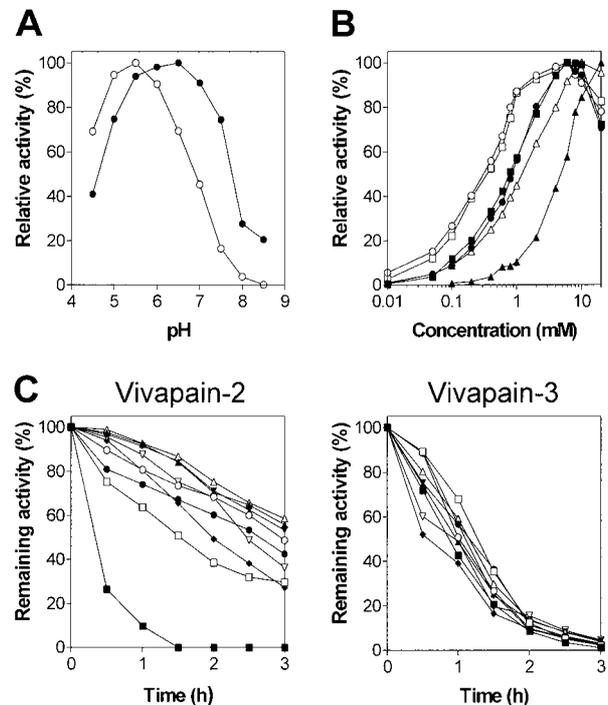
Table 1 Percentage identities of mature domains of plasmodial cysteine proteases

VX-1, vivapain-1; VX-2, vivapain-2; VX-3, vivapain-3; FP-2, falcipain-2; FP-3, falcipain-3; BP-2, berghepain-2 and VP-2, vinckepain-2.

	VX-2	VX-3	FP-2	FP-3	BP-2	VP-2	Papain
VX-1	33.6	32.5	35.3	34.6	32.5	30.4	26.3
VX-2	—	65.5	65.5	69.3	53.4	53.1	39.0
VX-3	—	—	60.3	61.4	53.8	53.1	39.7
FP-2	—	—	—	66.6	54.1	53.8	42.4
FP-3	—	—	—	—	52.1	52.8	39.0
BP-2	—	—	—	—	—	80.0	33.4
VP-2	—	—	—	—	—	—	35.2

**Figure 2** Expression and purification of vivapain-2 (upper panels) and vivapain-3 (lower panels)

Expression constructs were transformed into *E. coli* and products were evaluated by SDS/PAGE and staining with Coomassie Blue (A) and by gelatin substrate SDS/PAGE (B). The lanes show uninduced cells (lane 1), cells induced with IPTG (lane 2) and recombinant protein purified by Ni-NTA affinity chromatography (lane 3), refolded (lane 4) and additionally purified by anion-exchange chromatography after processing at acidic pH (lane 5). The positions of molecular-mass standards are indicated in kDa.

**Figure 3** Biochemical properties of vivapain-2 and vivapain-3

Activities were measured at 37 °C against the fluorogenic substrate Z-Leu-Arg-AMC. (A) pH optimum: the activities of vivapain-2 (●) and vivapain-3 (○) were assayed in 100 mM sodium acetate (pH 4.5–5.5), 100 mM sodium phosphate (pH 6.0–6.5) or Tris/HCl (pH 7.0–8.5), in each case with 1 mM DTT. (B) Effect of reducing agents: activities were assayed in 100 mM sodium acetate (pH 5.5), with different concentrations of reducing agents [vivapain-2: DTT (●), L-cysteine (▲) and GSH (▲); vivapain-3: DTT (○), L-cysteine (□) and GSH (△)]. (C) Enzyme stability: vivapain-2 (left-hand panel) and vivapain-3 (right-hand panel) were incubated in the buffers noted for (A), and residual enzyme activity was assayed in 100 mM sodium acetate (pH 5.5), 1 mM DTT at the indicated time points. ■, pH 4.5; □, pH 5.0; ●, pH 5.5; ○, pH 6.0; ▲, pH 6.5; △, pH 7.0; ▼, pH 7.5; ▽, pH 8.0 and ◆, pH 8.5.

Table 2 Substrate hydrolysis kinetics for plasmodial cysteine proteases

Values for vivapain-2 and vivapain-3 are means for a representative experiment performed in duplicate; individual results varied by <10%. Values for falcipain-2, falcipain-3 and vinckepain-2 were reported previously [10,11,19].

	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)				
	VX-2	VX-3	FP-2	FP-3	VP-2
Z-Phe-Arg-AMC	NH*	NH*	4.51×10^4	1.20×10^2	9.70×10^1
Z-Leu-Arg-AMC	7.34×10^5	8.62×10^4	1.06×10^5	5.90×10^2	5.26×10^3
Z-Val-Arg-AMC	NH*	NH*	5.44×10^3	2.60×10^1	1.40×10^2
Z-Leu-Leu-Arg-AMC	4.02×10^6	3.46×10^5	1.86×10^5	3.02×10^4	7.16×10^4
Z-Val-Leu-Arg-AMC	2.67×10^6	2.66×10^5	6.19×10^5	2.24×10^3	3.68×10^4
Z-Val-Val-Arg-AMC	1.15×10^6	5.81×10^3	4.03×10^3	1.99×10^2	3.23×10^4

* NH, no hydrolysis.

was accomplished using inverse PCR. The deduced amino acid sequences of vivapain-2 and vivapain-3 show similar degrees of identity (60–70%) with each other and with falcipain-2 and falcipain-3, and much lower identity with other papain-family cysteine proteases, including the more distantly related *P. vivax* protease vivapain-1 (Table 1). Papain-family active-site residues and prosequence motifs are well conserved in vivapain-2 and vivapain-3 (Figure 1). The *P. vivax* proteases also share with falcipain-2, falcipain-3 and related plasmodial proteases several features that are unusual for papain-family enzymes. These

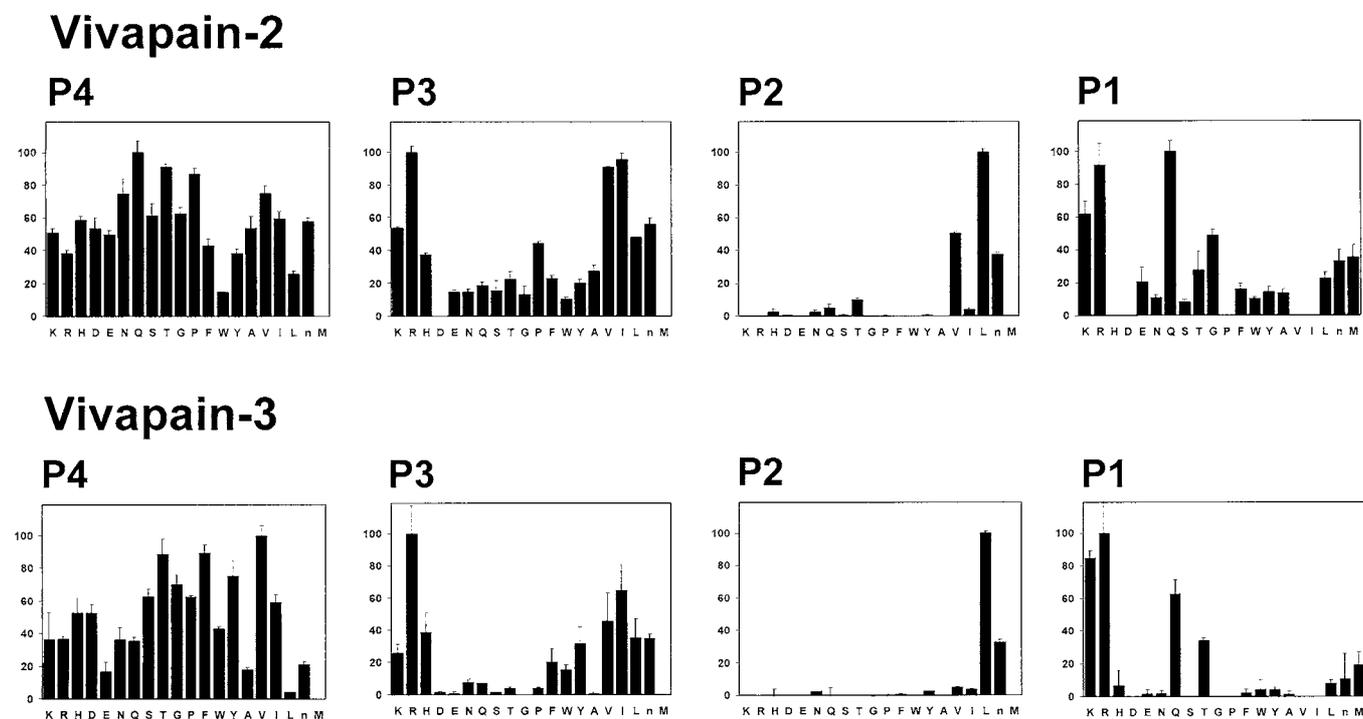


Figure 4 Substrate specificity profile using positional-scanning tetrapeptide substrate libraries

A P1 diverse library, and P2, P3 and P4 sublibraries of the P1-lysine fixed library were used to determine specificities. Activities were displayed as percentages of the maximum for each position. Amino acids are represented by the single-letter code (n is norleucine). Error bars represent the S.D. from the results of three separate experiments.

features include unusually large prosequences (albeit not as large as that of vivapain-1), predicted membrane-spanning domains near the N termini, predicted N-terminal extensions to the catalytic domains on the mature proteases (not found in vivapain-1 or falcipain-1), and an insert between highly conserved residues near the C-terminus [10,11,18]. These findings support the assignment of the falcipain-2/falcipain-3 subfamily as a distinct subset of proteases in human, primate and rodent malaria parasites [30].

Expression and refolding of vivapain-2 and vivapain-3

Constructs encoding portions of the prodomains and all of the mature domains of vivapain-2 and vivapain-3 were amplified, cloned into the pQE-30 expression vector and transformed into *E. coli*. Both enzymes were expressed as insoluble proteins (Figure 2). Inclusion bodies were solubilized, and the recombinant proteins were purified by Ni-NTA affinity chromatography and then refolded under alkaline conditions. Optimization of refolding conditions utilized a systematic microplate format [23]. Refolding, measured as the ability to hydrolyse the proteolytic substrate Z-Leu-Arg-AMC, was optimal with recombinant vivapain-2 in 100 mM Tris/HCl, 1 mM EDTA, 20% sucrose, 250 mM L-arginine, 5 mM GSH and 1 mM GSSG (pH 8.0) and with vivapain-3 in 100 mM Tris/HCl, 1 mM EDTA, 250 mM L-arginine, 1 mM GSH and 0.5 mM GSSG (pH 8.0). The refolded proteases were processed to enzymically active species of the sizes predicted for the mature proteases on exposure to an acidic buffer (Figure 2). The N-terminal sequences of the processed enzymes were KRITN for vivapain-2 and DGIKS for vivapain-3 (Figure 1). This finding supports the conclusion that, as is the case with other members of the falcipain-2/falcipain-3 subfamily, mature

vivapain-2 and vivapain-3 contain N-terminal extensions. This extension plays a key role in the folding of falcipain-2 [31], and has not been described in proteases from organisms other than plasmodia.

Biochemical properties of vivapain-2 and vivapain-3

Vivapain-2 and vivapain-3 had some typical properties of papain-family cysteine proteases, including acidic pH optima, requirements for a reducing environment for maximum activity (Figures 3A and 3B) and inhibition by cysteine protease inhibitors [*trans*-epoxysuccinyl-L-leuciloamido-(4-guanidino)butane (E-64) and leupeptin], but not inhibitors of other proteolytic classes (results not shown). The activities of the vivapains differed in that vivapain-3 was more active at acidic pH and more unstable (except at pH 4.5) than vivapain-2 (Figure 3C). The substrate specificities of vivapain-2 and vivapain-3 were characterized with several fluorogenic peptide substrates (Table 2). Vivapain-2 and vivapain-3 readily hydrolysed Z-Leu-Arg-AMC, consistent with activities of other plasmodial cysteine proteases. However, in contrast with most of the studied papain-family enzymes, including homologous plasmodial proteases, the vivapains did not hydrolyse dipeptide substrates with Val or Phe at the P2 position. Among the tested substrates, the vivapains were most active against tripeptide substrates with Leu at the P2 position and showed surprisingly good activity against Z-Val-Val-Arg-AMC, despite a lack of activity against Z-Val-Arg-AMC. For all tested substrates, vivapain-2 showed approx. 10-fold greater activity than did vivapain-3. To analyse substrate specificities more broadly, the activities of vivapain-2 and vivapain-3 against two positional scanning tetrapeptide substrate libraries were assessed (Figure 4). Patterns of hydrolysis were similar for the two

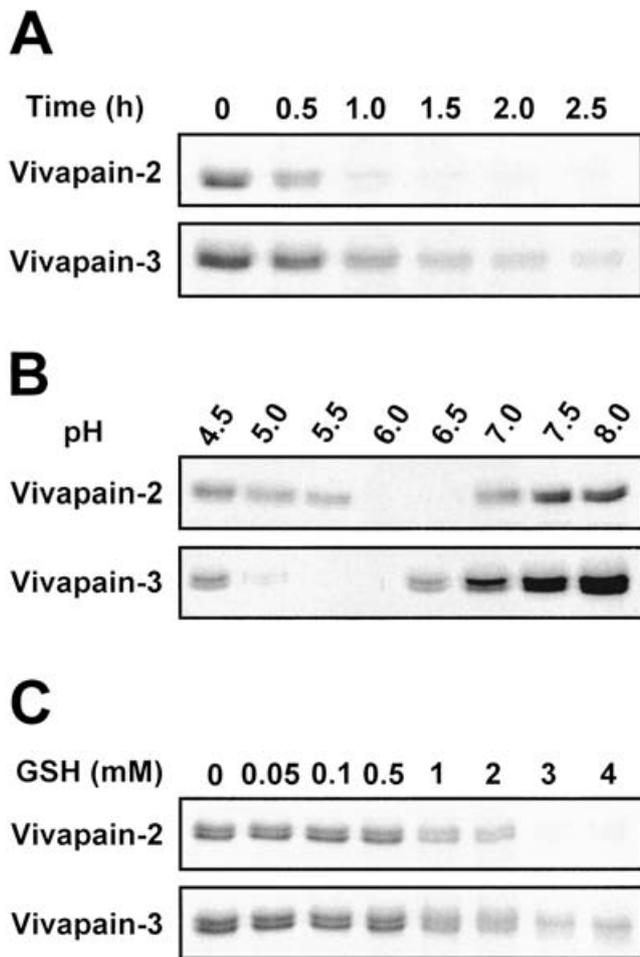


Figure 5 Haemoglobin hydrolysis by vivapain-2 and vivapain-3

For all experiments, native human haemoglobin (2 μ g) was incubated with enzymes at 37 °C, and at the completion of the reaction, products were resolved by SDS/PAGE and stained with Coomassie Blue. **(A)** Time course: haemoglobin was incubated with 200 nM vivapain-2 or vivapain-3 in 100 mM sodium acetate (pH 5.5), 1 mM DTT and aliquots were withdrawn at indicated time points. **(B)** Effect of pH: vivapains (200 nM) were incubated with haemoglobin in 100 mM sodium acetate (pH 4.5–5.5), 100 mM sodium phosphate (pH 6.0–6.5), or Tris/HCl (pH 7.0–8.5) and 1 mM DTT for 2 h. **(C)** Effect of reducing strength: vivapains (150 nM) were incubated with haemoglobin in 100 mM sodium acetate (pH 5.5) containing different concentrations of GSH for 2 h.

enzymes, with preference for positively charged residues and Gln at P1, Leu at P2 and less strict specificity at P3 and P4. A relative preference for P2 and P3 Val in vivapain-2 was consistent with much greater relative activity of this enzyme, compared with vivapain-3, against Z-Val-Val-Arg-AMC.

Haemoglobin hydrolysis

Both recombinant vivapains hydrolysed native human haemoglobin under physiologically relevant conditions (acidic pH; 1–2 mM GSH, Figure 5). Haemoglobin was maximally hydrolysed at lower pH by vivapain-3, consistent with results for the peptide substrate Z-Leu-Arg-AMC. Vivapain-2 was somewhat more active, with hydrolysis of native substrate about twice as rapid as that for vivapain-3. Similar results were obtained in an alternative assay, which measured spectrophotometric changes caused by haemoglobin disruption, with similar relative activities and pH requirements for the two vivapains (results not shown).

Hydrolysis of erythrocyte membrane proteins

Although haemoglobin hydrolysis is the best-characterized activity against natural substrates for the falcipain-2/falcipain-3 subfamily of plasmodial proteases, falcipain-2 has also been shown to hydrolyse some erythrocyte cytoskeletal proteins [19,20]. The activities of recombinant vivapains against proteins from erythrocyte ghosts were studied (Figure 6). Some hydrolysis of spectrin, protein 3, actin, glycophorin A and protein 4.1 was identified, with generally greater activity against all substrates for vivapain-2. However, for action against erythrocyte cytoskeletal proteins, only activities seen at neutral pH are probably biologically relevant. In this regard, marked activity was seen for both vivapains against protein 4.1 over the pH range 6.5–7.5. Using two antisera specific for different portions of protein 4.1, we found that both proteases cleaved the protein to small intermediates, with the accumulation of stable fragments that were recognized by antisera against the spectrin–actin binding domain, but not the C-terminus of the protein.

Inhibition of the vivapains by peptidyl protease inhibitors

The effects of E-64, and peptidyl vinyl sulphone and FMK inhibitors on vivapain-2, vivapain-3 and homologous falcipains were determined using the progress curve method. The second-order rate constants (k_{ass}) indicated similar trends in sensitivity to the inhibitors for vivapain-2 and vivapain-3, although vivapain-2 was consistently much more sensitive (Table 3). A comparison of sensitivities between the vivapains and falcipains revealed similar trends and also some important differences. Notably, vivapain-2 was much more sensitive to E-64 than were falcipain-2 and falcipain-3. In all cases, inhibitors with Leu at the P2 position were preferred to corresponding peptides with Phe at this position. However, for the vivapains, fairly good activity was seen for FMKs with Phe at P2, despite the absence of activity of the enzymes against substrates having this sequence.

DISCUSSION

The present study offers the first functional characterization of cysteine proteases of the human malaria parasite *P. vivax*. Two proteases, vivapain-2 and vivapain-3, were functionally expressed and characterized. The vivapains share many biochemical features with the falcipain proteases of *P. falciparum*, but important differences have also been identified. As is the case with the falcipains, the vivapains demonstrate hydrolysis of host molecules that are probably their biological substrates. The characterization of *P. vivax* proteases is important, as homologous proteases from *P. falciparum* are considered potential chemotherapeutic targets, and new antimalarial drugs should target both *P. falciparum* and *P. vivax*. Indeed, differences between these target cysteine proteases argue that the activity of putative new inhibitors must be evaluated against each enzyme to be sure that broad antiplasmodial activity is achieved.

Vivapain-2 and vivapain-3 are the apparent orthologues of falcipain-2 and falcipain-3, but each of these proteases differs biochemically, and it is not yet possible to assign precise functions among the plasmodial species. Vivapain-2 had a less acidic pH optimum and was more stable at neutral pH than vivapain-3, suggesting, as for falcipain-2, action of vivapain-2 against erythrocyte cytoskeletal targets in addition to activity against haemoglobin. Despite some differences, it is clear that vivapain-2 and vivapain-3 fit into the falcipain-2/falcipain-3 subfamily, which now includes members from the two most important human malaria parasites in addition to other primate and rodent parasites

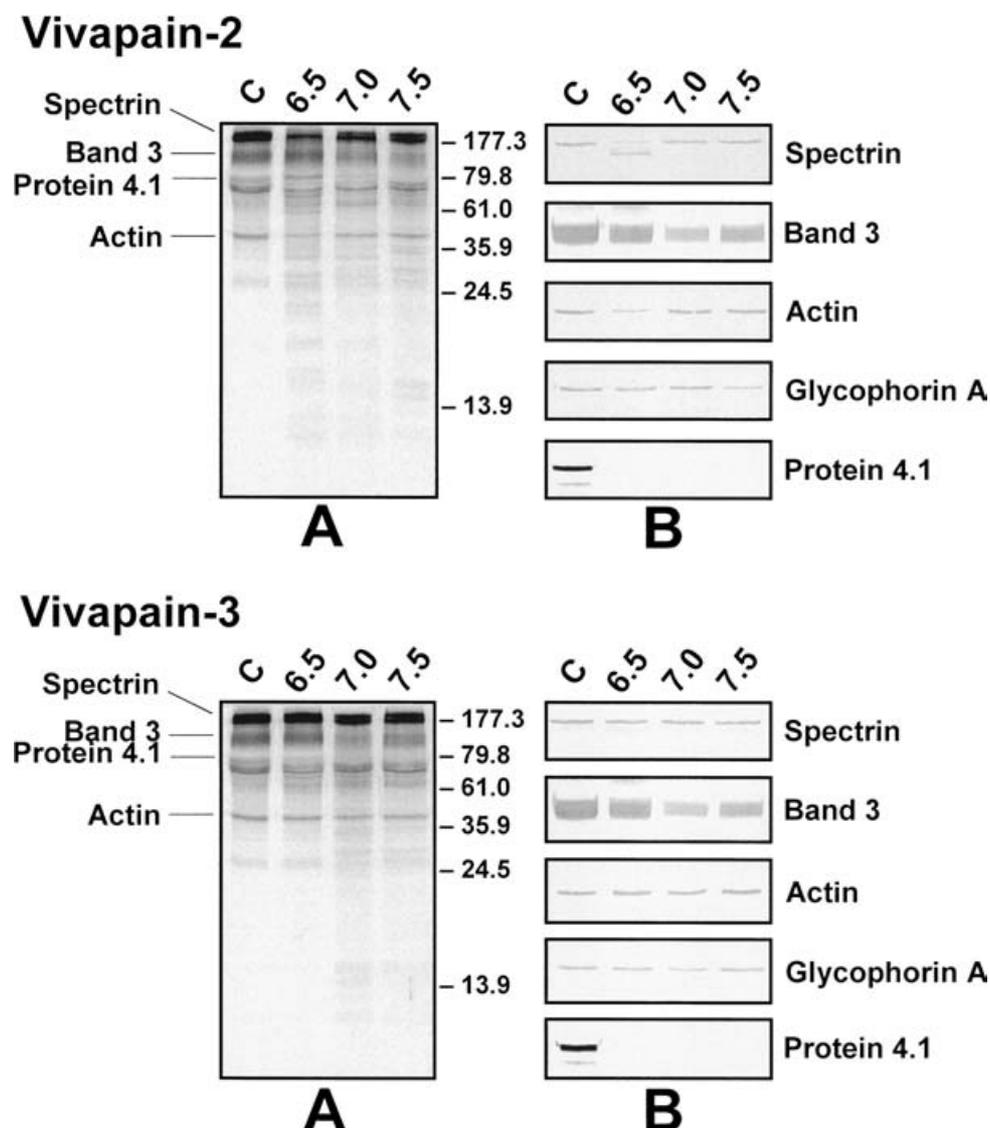


Figure 6 Degradation of erythrocyte membrane proteins by vivapain-2 and vivapain-3

Ghosts from fresh erythrocytes were incubated with vivapain-2 or vivapain-3 in 50 mM sodium phosphate at the indicated pH values at 37 °C for 3 h. **(A)** SDS/PAGE: prominent bands and the positions of molecular-mass standards are shown. **(B)** Immunoblots: reaction mixtures were transferred on to nitrocellulose membranes and probed with specific antibodies against human erythrocyte spectrin, band 3, actin, glycophorin A and protein 4.1. C, control without enzyme.

Table 3 Comparison of inhibitor kinetics for vivapains and other plasmodial cysteine proteases

Mu, morpholine urea; hPhe, homophenylalanine; Nmetpip, *N*-methylpiperazine urea; VSPH, phenylvinylsulphone.

	k_{ass} ($\text{M}^{-1} \cdot \text{s}^{-1}$)*			
	VX-2	VX-3	FP-2	FP-3
E-64	232 000 ± 17 700	5890 ± 588	14 200 ± 475	5870 ± 428
Z-Phe-Arg-FMK	47 900 ± 5090	3680 ± 218	241 000 ± 20 100	60 200 ± 6740
Mu-Phe-hPhe-FMK	61 700 ± 6180	540 ± 60	215 000 ± 5760	526 000 ± 59 300
Mu-Leu-hPhe-FMK	450 000 ± 16 800	79 200 ± 6490	924 000 ± 26 200	5 850 000 ± 15 800
Mu-Phe-hPhe-VSPH	267 ± 14	NA†	4890 ± 231	2060 ± 41
Mu-Leu-hPhe-VSPH	88 200 ± 470‡	4970 ± 370	102 000 ± 2530	22 000 ± 1420
Nmetpip-Phe-hPhe-VSPH	96 ± 5	15 ± 0.04	1230 ± 136	1900 ± 43
Nmetpip-Leu-hPhe-VSPH	53 600 ± 5450	2130 ± 158	26 300 ± 3700	11 900 ± 762

* Geometric mean ± S.E.M.

† No appreciable activity.

‡ k_{inact}/K_i .

[29]. These proteases share unusual features for papain-family enzymes, including large prodomains, predicted membrane-spanning sequences, an N-terminal extension to the mature region, which is required for folding by falcipain-2 [30], and a C-terminal insertion. These features suggest unique biological functions for the plasmodial proteases, including unique mechanisms of protein folding and enzyme targeting.

Vivapain-2, vivapain-3 and the falcipains all hydrolyse haemoglobin at acidic pH, suggesting that the principal function of this family of proteases is the hydrolysis of erythrocyte haemoglobin in acidic food vacuoles. This is supported by the demonstration that cysteine protease inhibitors block the hydrolysis of haemoglobin by erythrocytic *P. falciparum* parasites [9,13]. Differences in the substrate specificities of these enzymes probably allow them, in association with other enzymes including aspartic (plasmepsins) and metallo (falcilysin) proteases, to hydrolyse haemoglobin efficiently. Cysteine proteases also seem to be required for the rupture of erythrocytes by mature parasites, as cysteine protease inhibitors block this process [32–34]. Of interest in this regard, vivapain-2, vivapain-3 and falcipain-2 [19,20] hydrolyse the erythrocyte cytoskeletal protein 4.1 at neutral pH, potentially disrupting the erythrocyte cytoskeleton to allow egress of parasites. Thus inhibitors of vivapains and falcipains may elicit antimalarial activity by inhibiting multiple processes. Consistent with this conclusion, stage-specific effects of cysteine protease inhibitors were seen against cultured parasites both during the trophozoite stage, when haemoglobin hydrolysis is maximal, and in mature schizonts, the stage preceding erythrocyte ruptures [35].

Considering substrates and inhibitors, the vivapains had similar, but not identical specificities, but vivapain-3 was less active against all tested peptide substrates and against haemoglobin. With a range of peptidyl inhibitors, the vivapains showed greater inhibition by E-64, but lower activity of FMK and vinyl sulphone inhibitors, than did the falcipains. Vivapain-2 was much more sensitive to peptidyl inhibitors. In some cases, differences between the inhibition of vivapains and falcipains were large, suggesting that potent *P. falciparum* antimalarials might fail against *P. vivax*. In other cases, compounds (e.g. Nmetpip-Leu-hPhe-VSPH, where Nmetpip is *N*-methylpiperazine urea and VSPH is phenyl-vinylsulphone) that have already demonstrated *in vitro* efficacy against *P. falciparum* and *in vivo* efficacy against murine malaria [15,36] were also effective inhibitors of the *P. vivax* proteases, suggesting the possibility for broadly active antimalarial cysteine protease inhibitors.

Our studies allow us to extend advances made in the understanding of plasmodial cysteine proteases to *P. vivax*, a second critically important human malaria parasite. It is essential that new antimalarial drugs have to be effective against both *falciparum* and *vivax* malarias. As *P. vivax* is very difficult to study biologically, our biochemical assessment offers initial reassurance that cysteine protease inhibitors can be developed to target both organisms, but also the warning that attention to the inhibition of key proteases of both plasmodial species is needed.

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