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Recombinant expression and partial characterization of an active soluble histo-aspartic protease from *Plasmodium falciparum*

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Abstract

Malaria aspartic proteases are attractive drug targets for the treatment of malaria, however, recombinant expression of active histo-aspartic proteinase (HAP) to facilitate its characterization has proven elusive. The present study reports on the first recombinant expression of soluble, active histo-aspartic proteinase from *Plasmodium falciparum* as a thioredoxin fusion protein. A truncated form of HAP (77p-451) was fused to *thioredoxin* in the pET32b(+) vector and the fusion protein (Trx-tHAP) was expressed in *Escherichia coli* Rosetta-gami B (DE3)pLysS. The fusion protein was partially purified from the culture medium using a combination of anion exchange and Ni^{2+} affinity chromatography. Soluble tHAP was subsequently purified by enterokinase treatment and removal, followed by gel filtration chromatography. Although truncated HAP was incapable of autocatalytic activation, enterokinase digestion of partially purified fusion protein released the truncated prosegment yielding a mature form of tHAP (mtHAP). N-terminal sequencing of mtHAP indicated that enterokinase cleavage took place at Lys119-Ser120, four residues upstream of the native cleavage site (Gly123-Ser124). Initial activity tests showed that mtHAP was capable of hydrolyzing acid-denatured globin as well as cleavage of the synthetic substrate EDANS-CO-CH_2-CH_2-CO-ALERMFLSFP-Dap(DABCYL)-OH. Inhibition studies showed that the activity of mtHAP was completely inhibited by pepstatin A and to a lesser degree, PMSF. Using the synthetic substrate, mtHAP showed a pH optimum of 5.2, and $K_m = 3.4\,\mu M$ and $k_{cat} = 1.6 \times 10^{-3}\,s^{-1}$. The successful expression of active recombinant HAP from *E. coli* will accelerate the investigation of the structure–function relationships of HAP and facilitate the development of specific inhibitors with antimalarial activities.

Keywords: Malaria; Histo-aspartic protease; Soluble; Recombinant; Expressions

The plasmepsins produced by the *Plasmodium* parasite are aspartic proteases and have been recognized as attractive targets for the design of novel chemotherapeutic compounds for the control of malaria [1]. Currently, considerable advancements are being made in the development of potent plasmepsin inhibitors [2–10]. There are 10 plasmepsins reported in the genome of *Plasmodium falciparum*, PM I, II, and IV-X, and histo-aspartic protease (HAP)[2][11]. Four of them, PM I, PM II, HAP, and PM IV have been localized in the food vacuole of the parasite, and have been shown to be involved in hemoglobin degradation [11,12]. PM V was shown to be localized to the parasite's endoplasmic reticulum and was suggested to have a role in intra-erythrocytic biology that is distinct from that of PM I, PM II, HAP, and PM IV [13]. PM I, II, and IV are known to be classical aspartic proteases containing two catalytic aspartic acids, whereas, one of the catalytic aspartic acids is replaced with a histidine in HAP. In addition, there are unique HAP residues in a conserved flap region over the binding cleft, including Ser77 and Lys78, instead of Tyr77 and Val78 in PM II [11,14]. Native HAP is inhibited by both the aspartic protease inhibitor pepstatin A and the serine protease inhibitor PMSF, thus HAP represents a novel type of aspartic protease [12]. To date there have been no reports of

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[2] *Abbreviations used:* HAP, histo-aspartic proteinase; tHAP, truncated HAP; mtHAP, mature tHAP; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside.

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recombinantly expressed active HAP due to insolubility in vitro [14,12]. It has been postulated that HAP contains a type II membrane anchoring region which consists of approximately 19 hydrophobic residues in the prosegment and this, together with up to 80% AT gene content, may contribute to the difficulties of recombinant expression [14–16]. Thus, analyses of the structure and mechanism of native HAP have been limited to molecular modeling [14,17,18]. To overcome the difficulty of expressing malaria aspartic proteases, a 76 amino acid deletion of the membrane-binding domain has proven successful for in vitro expression of various plasmepsins [19,20]. In the present work, the soluble expression of a truncated form of HAP fused with thioredoxin from *Escherichia coli* and the activation of the fusion zymogen to produce an active recombinant HAP is reported for the first time.

Materials and methods

Cloning of HAP and construction of expression plasmid

The HAP gene was isolated from the genomic DNA of *Plasmodium falciparum* strain 3D7 (MR4/American Type Culture Collection, Manassas, VA, USA) using polymerase chain reaction (PCR) with primers HAPFW (5′-AAGGA TCCCATGAATTTAACCATTAAAG-3′) and HAPRV (5′-AATGGATCCTTATAAATTTTTGGCTAAAGCAA ATCC-3′). The PCR products were cloned into vector pUC-19 to produce plasmid construct pUC19-*HAP*. The sequence of the HAP gene was confirmed by sequencing with M13 primers using Dye Terminator Cycle Sequencing on an ABI PRISM Model 377 (Guelph Molecular Super-Center, University of Guelph). The transmembrane region of the propeptide was then removed by amplification from pUC19-HAP using primer HAP-H228 (5′-CGGGATCCA AAATATTCGACAGTAGGATTT-3′) and HAPRV to give the truncated *HAP* (*tHAP*). Amplified *tHAP* product was digested with *Bam*HI and cloned into pET32b(+) to give rise to pET32b-*tHAP* in which *tHAP* was fused to *thioredoxin*. The DNA sequence of the expression construct was confirmed by sequencing with T7 promoter and T7 terminator primers.

Expression of the fusion protein

The expression construct pET32b-tHAP was transformed into *E. coli* Rosetta-gami B (DE3)pLysS cells. Transformants were cultured in 1 L of LB medium containing 12.5 μg/ml tetracycline, 15 μg/ml kanamycin, 34 μg/ml chloramphenicol, and 50 μg/ml ampicillin until the OD_{600} reached 1.0, then induced with isopropyl-β-D-thiogalactopyranoside (IPTG) as suggested in the pET System Manual (Novagen, Madison WI, USA). IPTG concentrations and expression temperatures were optimized for maximal solubility of tHAP. Cells were collected by centrifugation (2500*g* for 15 min).

Purification of the fusion protein

Cell pellets were resuspended in BugBuster™ reagent (Novagen, Madison WI, USA) and incubated at room temperature for 20 min with gentle shaking. The sample was then centrifuged at 16,000*g* for 20 min at 4 °C. The supernatant and the insoluble cell debris were subsequently collected and analyzed using SDS–PAGE and Western blot. The soluble fraction obtained above was dialyzed overnight in 20 mM Tris–HCl pH 7.5 to remove the detergents and salts contained in the BugBuster™ reagent and applied to a 23 ml Source 15Q column on an AKTA™ FPLC system (GE Healthcare, Uppsala, Sweden). The dialysate was fractionated using a 20 column volume gradient from 0 to 1 M NaCl in 20 mM Tris–HCl pH 7.5 and tHAP was detected by Western blot with anti-thioredoxin primary antibody. The fractions containing the highest amount of fusion protein were combined and further purified by Ni^{2+} affinity chromatography using a HisSelect™ (Sigma-Aldrich, Oakville ON, Canada) column; 50 mM sodium phosphate/0.3 M NaCl/10 mM imidazole pH 8.0 wash buffer and 50 mM sodium phosphate/0.3 M NaCl/250 mM imidazole pH 8.0 elution buffer.

Activation, purification, and N-terminal sequence of the active recombinant HAP

Purified thioredoxin-tHAP (Trx-tHAP) was digested using 7.5 μg enterokinase (Sigma-Aldrich, Oakville ON, Canada) at 37 °C to yield mature tHAP (mtHAP). Enterokinase cleavage was done in 50 mM sodium acetate pH 5.7 at 37 °C for two days. The digestion reaction was applied to an EK capture kit (Sigma-Aldrich, Oakville ON, Canada) to remove enterokinase. mtHAP was further purified by gel filtration with a Superdex™ 75 10/300 GL (GE Healthcare, Uppsala, Sweden) column equilibrated with 50 mM MES/150 mM NaCl pH 5.7 to obtain pure mtHAP. mtHAP protein was subjected to SDS–PAGE and electroblotted to a PVDF membrane for N-terminal sequence analysis by the Nucleic Acid–Protein Service Unit at the University of British Columbia (Vancouver, British Columbia, Canada).

Protein concentration determinations

Enzyme and protein concentrations were determined in triplicate using Coomassie protein microplate assay (Pierce, Rockford IL, USA) with bovine serum albumin standards.

Human globin degradation

Human globin was isolated from human hemoglobin (Sigma-Aldrich, Oakville ON, Canada) as described by Rossi et al. [21]. The degradation assay was performed as per Banerjee et al. (2002). Three micrograms of human globin was incubated with 50 nM mtHAP in 100 mM

citrate-phosphate pH 5.7. Reactions were terminated by heating at 95 °C in sample buffer containing SDS. The degradation of globin was detected by Coomassie brilliant blue R-250 stained SDS–PAGE.

pH optimum determination, enzyme kinetic assays, and inhibition studies

The pH optimum of mtHAP was determined using internally quenched fluorescent synthetic peptide substrate EDANS-CO-CH2-CH2-CO-ALERMFLSFP-Dap-(DABCYL)-OH (2837b) (AnaSpec Inc., San Jose CA, USA), as described previously by Istvan and Goldberg [22], at various pH values between 3.5 and 7.0 in buffers 100 mM sodium acetate pH 3.5–6.5 and 100 mM Tris–HCl pH 7.0. All assays contained 50 nM mtHAP and 3 μM substrate 2837b.

Inhibition assays on the activity of mtHAP were done with the aspartic protease inhibitor pepstatin A (1 μM), and serine protease inhibitors PMSF (1 mM) and leupeptin (10 and 100 μM). The reaction was carried out in 100 mM sodium acetate pH 5.2 using 50 nM mtHAP and 3 μM peptide substrate 2837b.

Kinetic parameters were determined using substrate 2837b. The reaction was carried out in 100 mM sodium acetate pH 5.2 using 50 nM mtHAP and 0.1–8.0 μM substrate. The assays were performed using a Victor 2 1420 multilabel counter (Perkin Elmer, Woodbridge ON, Canada) with excitation at 335 nm and emission at 535 nm. The initial reaction rates were determined by calculation of the slope of the linear portion of the curve (fluorescence/min). The observed fluorescence units were converted to moles per second using a conversion factor derived from a standard curve for the complete digestion of the substrate by commercial yeast proteinase A (Sigma-Aldrich, Oakville ON, Canada) at four substrate concentrations. Nonlinear regression with the Michaelis–Menten model was used to derive K_m, and k_{cat} was calculated from $k_{cat} = V_{max}/[E]$.

Results and discussion

Soluble expression of Trx-tHAP fusion protein

Membrane proteins are notoriously difficult to express recombinantly since most currently used expression strategies have been developed for soluble proteins [23,15]. Specific hurdles for the expression of genes encoding key malaria enzymes from *P. falciparum* have also been attributed to an 80% AT content that limits expression in heterologous systems [16]. Therefore, the recombinant expression and subsequent structural and functional characterization of malaria enzymes presents a difficult problem. Common problems that were hypothesized for expression were overcome with a dual-approach strategy by first truncating the membrane-binding domain of HAP and subsequently linking tHAP to a highly soluble protein, thioredoxin. The successful soluble expression of truncated HAP (77p-451) fused with thioredoxin (Trx-tHAP) in *E. coli* Rosetta-gami B (DE3)pLysS was confirmed, although only a small proportion of total tHAP was soluble in the cell lysate supernatant as detected by SDS–PAGE (Fig. 1A) and the corresponding Western blot (Fig. 1B). The initial expression yielding soluble fusion protein was done under standard conditions of 37 °C and 1 mM IPTG.

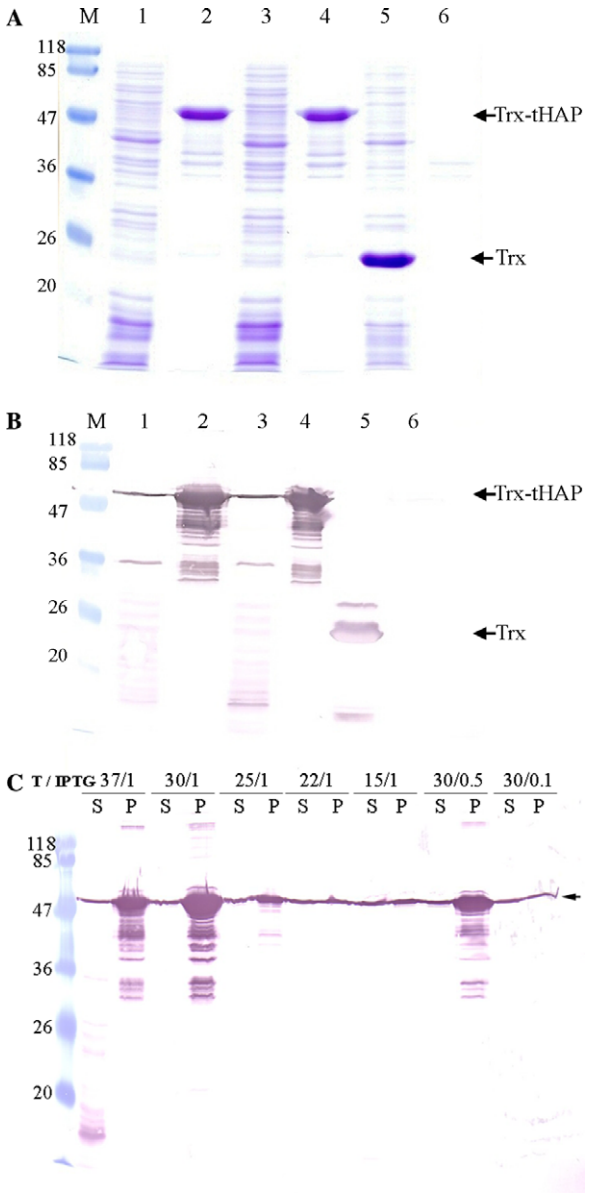

Fig. 1. Expression of Trx-tHAP fusion protein from *E. coli* Rosetta-gami B (DE3)pLysS. (A) CBB-stained 15% SDS–PAGE and (B) anti-thioredoxin Western blot of expression cultures induced with IPTG. Lanes 1 and 3; soluble fractions of clones 1 and 2, respectively, lanes 2 and 4; insoluble fractions of clones 1 and 2, respectively, lanes 5 and 6; soluble and insoluble fractions of the lysate from control vector pET32b(+) expressing only thioredoxin. (C) Anti-thioredoxin Western blot showing the effects of IPTG concentration and temperature on recombinant Trx-tHAP fusion protein expression in *E. coli* Rosetta-gami B (DE3)pLysS. Soluble (S) and insoluble (P) fractions are shown for various temperatures (T) and IPTG concentrations (mM).

A greater amount of insoluble fusion protein was detected in the cell lysate pellet (Figs. 1A and B) suggesting that a large proportion of the fusion protein was likely improperly folded [15]. A control strain of *E. coli* transformed with the pET-32b(+) without insert expressed all detectable thioredoxin in a soluble form (Figs. 1A and B). To optimize the expression and solubility of the Trx-tHAP fusion protein, various temperatures and IPTG concentrations were tested. It was hypothesized that slower expression of the fusion protein under conditions of lower temperature and/or lower inductant concentration may result in a higher yield of soluble tHAP. Western blot using anti-Trx primary antibody (Invitrogen, Burlington ON, Canada) showed that induction with 1 mM IPTG at 30 °C produced more soluble fusion protein as compared to 37 °C (Fig. 1C). Decreasing the expression temperature below 30 °C improved the proportion of soluble to insoluble fusion protein by decreasing the relative amount of insoluble product, however, an increase in soluble product was not observed (Fig. 1C). Decreasing IPTG concentration by 2-fold and 10-fold did not improve the solubility of tHAP-Trx fusion protein (Fig. 1C). Thereafter, the expression of the Trx-tHAP fusion protein was induced with 1 mM IPTG at 30 °C for maximal production of soluble Trx-tHAP. Thirteen percent of the total Trx-tHAP fusion protein was expressed in the soluble form as determined by densitometric analysis using Syngene GeneTools™ software for Coomassie stained SDS–PAGE (data not shown). The greater proportion of insoluble recombinant Trx-tHAP produced relative to soluble fusion protein highlights the difficulties of expressing not only this malarial aspartic proteinase, but of membrane-associated proteins in general.

Recombinant soluble HAP can not undergo autocatalytic activation

Partial purification of Trx-tHAP from the cell lysate supernatant was accomplished by FPLC using anion-exchange and Ni^{2+} affinity chromatography (Figs. 2A and B). The supernatant was first applied on Source 15Q column on an AKTA FPLC system (GE Healthcare, Uppsala, Sweden). Fractions eluted with a linear gradient (0%–100%) of 20 mM Tris–HCl pH 7.5/1 M NaCl were detected by Western blot with anti-Trx primary antibody. The fractions containing the highest amount of fusion protein were combined and further purified by Ni^{2+} affinity chromatography using a HisSelect™ (Sigma-Aldrich, Oakville ON, Canada) column (Figs. 2A and B).

For the removal of thioredoxin, one hour incubation with enterokinase (EK) at pH 7.5 resulted in near complete digestion of Trx-tHAP fusion protein producing zymogenic tHAP (42.5 kDa) as observed by band-shift on SDS–PAGE (data not shown). However, 3 h EK digestion resulted in the appearance of a 37 kDa band, consistent with mature tHAP, and by 20 h all tHAP was present in the apparent mature form. Post-EK tHAP was tested for activity with substrate 2837b, however, no activity was detected. Likely,

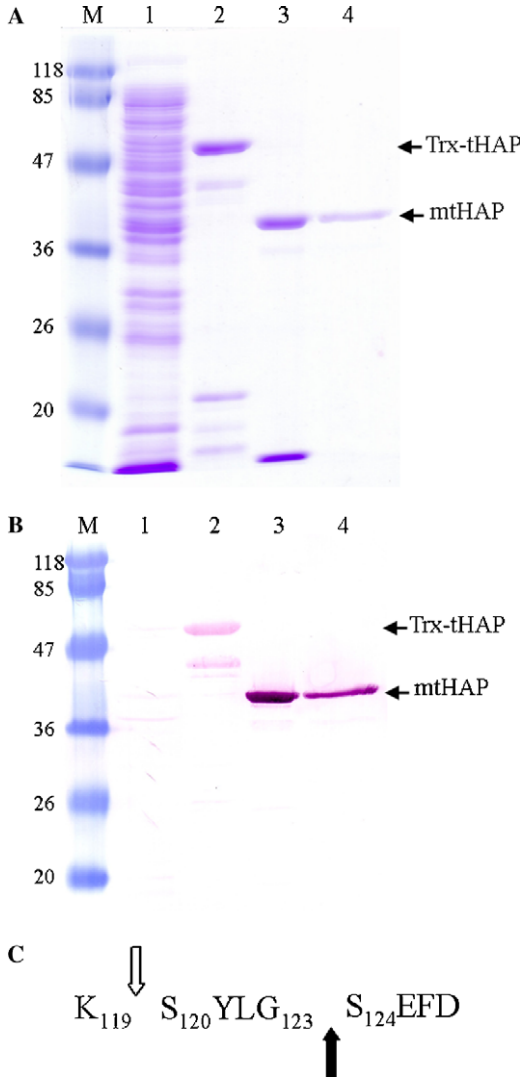

Fig. 2. (A) SDS–PAGE and (B) corresponding anti-HAP Western blot for mtHAP purification steps. Lane 1, lysate supernatant; lane 2, post-source Q and Ni^{2+} affinity chromatography; lane 3, mtHAP following enterokinase cleavage; lane 4, pure mtHAP following EK removal and gel filtration. (C) The cleavage site of tHAP by EK is indicated with the unfilled arrow as determined by N-terminal sequencing of the band. The cleavage site of the native protein is marked with the filled black arrow.

the mature form was denatured in the neutral pH EK cleavage buffer [12], therefore, pH 5.7, a value closer to the pH optimum of HAP and a value at which EK retained activity was subsequently used for EK cleavage. In terms of autoactivation, neither acidification of partially purified Trx-tHAP fusion protein nor tHAP zymogen obtained by EK digestion, yielded mtHAP as determined by band shift on SDS–PAGE and Western blot (data not shown).

EK digestion of Trx-tHAP fusion protein at pH 5.7 for a prolonged incubation of 48 h yielded product with an apparent molecular weight consistent with mtHAP (37 kDa). The EK reaction was treated with an EK removal kit, then applied to a Superdex 75 FPLC column to remove thioredoxin and the cleaved prosegment. The result of EK digestion and the subsequent purification of the fusion protein by gel filtration at pH 5.7 are shown in Figs. 2A and B

and the purification steps are summarized in Table 1. N-terminal sequencing of the band labeled as mtHAP in Fig. 2 confirmed the identity of mtHAP and indicated that cleavage by EK occurred between amino acids Lys119 and Ser120 (see Fig. 2C), four residues upstream of the native cleavage site at Gly123 - Ser124 identified by Banerjee et al. (2003) [24]. The yield for mtHAP was 0.65 mg/L of cell culture. The inability of mtHAP to autoactivate was in agreement with Banerjee et al. (2002) in that HAP may not possess the ability to autoactivate when expressed recombinantly [12,14]. This phenomenon differs from PMI and PMII which autoactivate at pH 4.7 at a position 12 residues upstream of the wild-type N-terminus [20,25].

pH optimum, globin digestion, inhibition, and kinetic studies

The pH-activity profile for mtHAP was determined over the pH range 3.5–7.0 using internally quenched fluorescent peptide substrate EDANS-CO-CH_2-CH_2-CO-Ala-Leu-Glu-Arg-Met-Phe-Leu-Ser-Phe-Pro-Dap-(DABCYL)-OH (2837b), which mimics the hemoglobin α33–34 cleavage site previously described by Istvan and Goldberg [22] (Fig. 3A). The pH optimum of recombinant mtHAP was pH 5.2 (Fig. 3A), similar to that previously determined for native HAP, pH 5.7 [12]. In vivo, globin is hydrolyzed by plasmepsins I, II, IV, and HAP [12]. Incubation of mtHAP with human globin for 12 and 24 h confirmed degradation of globin as detected by SDS–PAGE (Fig. 3B), yielding similar results to those previously shown for native HAP [12].

Inhibition assays showed that enzyme activity was completely inhibited by 1 μM pepstatin A ($P \leqslant 0.05$) (Fig. 3C). Serine protease inhibitor PMSF inhibited mtHAP activity by approximately 25% at 1 mM concentration ($P \leqslant 0.05$), whereas leupeptin at 10 and 100 μM showed no significant difference ($P > 0.05$) versus the control reaction (Fig. 3C). The above inhibition results were in agreement with those

Table 1
Summary of the purification of soluble recombinant tHAP from *E. coli* Rosetta-gami B (DE3)pLysS

Purification step	Concentration (mg/mL)	Total protein (mg)	Amount of Trx-tHAP/tHAP (mg/ml)	Fold enrichment	Percentage yield (%)
Supernatant	22.1	1104	25.7	1.00	100
Post-anion exchange/Ni^{2+} affinity	2.57	10.3	4.84	20.2	0.93
Post-enterokinase removal and gel filtration	0.332	0.651	0.623	41.1	0.06

Values represent the mean of three replicates.

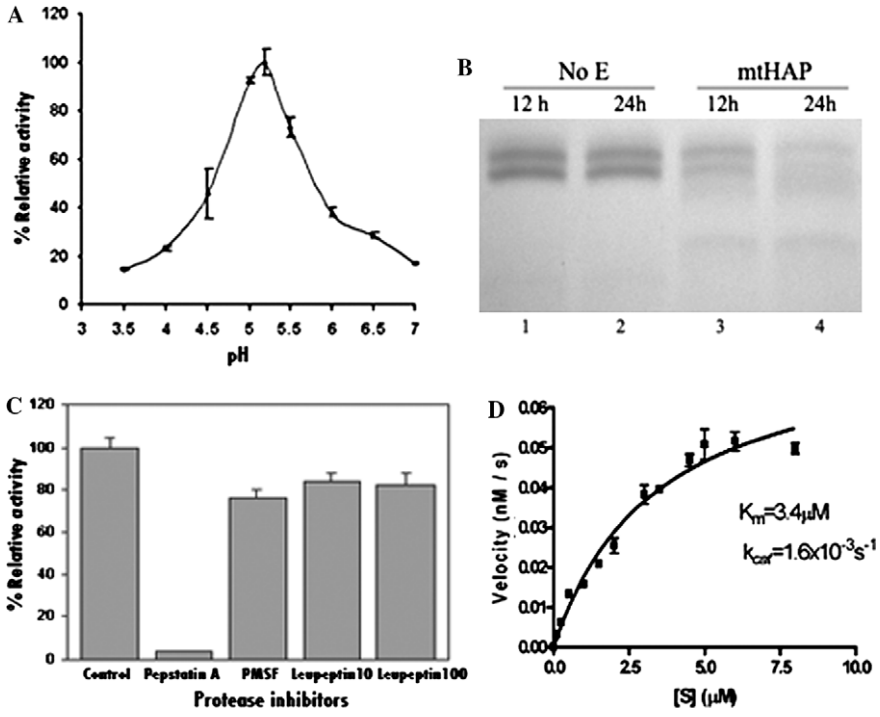

Fig. 3. (A) Effect of pH on mtHAP activity in 100 mM sodium acetate pH 3.5–6.5 and 100 mM Tris–HCl pH 7.0 using 50 nM mtHAP and 3 μM peptide substrate 2837b. Percent activity is expressed relative to the highest observed activity, averaged from triplicate determinations. (B) mtHAP degradation of acid-denatured human globin shown on CBB-stained SDS–PAGE. Globin was incubated without enzyme (No E) or with 50 nM recombinant mtHAP for 12 or 24 h in 100 mM sodium acetate pH 5.7. (C) mtHAP inhibition assays for 1 μM pepstatin A, 1 mM PMSF, and 10 μM, and 100 μM, leupeptin incubated in 100 mM sodium acetate pH 5.2, 50 nM mtHAP, and 3 μM peptide substrate 2837b. Control reaction was as above without inhibitor. Each data point represents the mean of three determinations. (D) mtHAP kinetic analysis for internally quenched fluorescent peptide substrate 2837b. Kinetic assays were completed over the substrate range of 0.1–8 μM using 50 nM mtHAP in 100 mM sodium acetate pH 5.2 with 10% glycerol.

for native HAP in that they were both completely inhibited by pepstatin A and not by leupeptin [12]. However, unlike recombinant mtHAP, native HAP was completely inhibited by PMSF [12]. Differences in inhibition profiles between malaria protease forms were also previously reported for the recombinantly expressed truncated PMI and PMII, relative to the native enzymes [26]. The apparent differences may be attributable to a proportion of misfolded mtHAP [26].

Kinetic parameters for mtHAP were determined using the quenched fluorescent substrate 2837b previously used for the kinetic characterization of PMII [22]. Analysis using the Michaelis–Menten model (Fig. 3D) yielded a K_m of 3.4 μM and a turnover number, k_{cat}, of 0.0016 s^{-1}. Recombinant mtHAP affinity for the substrate was moderately decreased relative to native HAP for which a K_m of 0.29 μM was reported [12]. Recombinant mtHAP also had decreased turnover relative to native HAP, previously reported as 0.05 s^{-1} [12]. Differences in catalytic parameters between native and recombinant forms were expected since native and recombinant plasmepsins I and II also showed kinetic differences [26].

In utilizing a gene-fusion expression system we were able to produce soluble active mtHAP with minimal purification steps. This methodology provides a solution to surmount difficulties with the use of alternate expression systems that produced insoluble HAP as inclusion bodies with no activity [12,14]. The successful expression of active recombinant HAP in *E. coli* provides a means to accelerate the investigation of the structure–function relationships of HAP with the ultimate goal being the development of specific inhibitors with antimalarial activities.

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