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The zymogen of plasmepsin V from *Plasmodium falciparum* is enzymatically active

Huogen Xiao^a, Brian C. Bryksa^a, Prasenjit Bhaumik^b, Alla Gustchina^c, Yoshiaki Kiso^d, Shao Q. Yao^e, Alexander Wlodawer^c, and Rickey Y. Yada^{a,*}

^aDepartment of Food Science, University of Guelph, 50 Stone Road East, Guelph, ON, Canada N1G2W1

^bDepartment of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Powai, Mumbai, India – 400076

^cProtein Structure Section, Macromolecular Crystallography Laboratory, National Cancer Institute, Frederick, MD 21702, USA

^dLaboratory of Peptide Science, Nagahama Institute of Bio-Science and Technology, Nagahama, Shiga 526-0829, Japan

^eDepartment of Chemistry, National University of Singapore, 3 Science Drive, Singapore 117543

Abstract

Plasmepsin V, a membrane-bound aspartic protease present in *Plasmodium falciparum*, is involved in the export of malaria parasite effector proteins into host erythrocytes and therefore is a potential target for antimalarial drug development. The present study reports the bacterial recombinant expression and initial characterization of zymogenic and mature plasmepsin V. A 484-residue truncated form of proplasmepsin (Glu37–Asn521) was fused to a fragment of thioredoxin and expressed as inclusion bodies. Refolding conditions were optimized and zymogen was processed into a mature form via cleavage at the Asn80–Ala81 peptide bond. Mature plasmepsin V exhibited a pH optimum of 5.5–7.0 with K_m and k_{cat} of 4.6 μM and 0.24 s^{-1} , respectively, at pH 6.0 using the substrate DABCYL-LNKRLHETQ-E(EDANS). Furthermore, the prosegment of proplasmepsin V was shown to be nonessential for refolding and inhibition. Unexpectedly, unprocessed proplasmepsin V was enzymatically active with slightly reduced substrate affinity (~2-fold), and similar pH optimum as well as turnover compared to the mature form. Both zymogenic and mature plasmepsin V were partially inhibited by pepstatin A as well as several KNI aspartic protease inhibitors while certain metals strongly inhibited activity. Overall, the present study provides the first report on the nonessentiality of the prosegment for plasmepsin V folding and activity, and therefore, subsequent characterization of its structure-function relationships of both zymogen and mature forms in the development of novel inhibitors with potential antimalarial activities is warranted.

*Corresponding author. Tel.: +1 519 824-4120 x58915; Fax: +1 519 824-0847; ryada@uoguelph.ca.

Keywords

plasmepsin; zymogen; refolding; prosegment; protease inhibitor; *Plasmodium falciparum*

1. Introduction

Malaria afflicts up to 500 million people annually of which 1–2 million cases are deadly [1]. It is primarily spread through the bite of *Anopheles* mosquitos which transmit protozoans of the genus *Plasmodium*, namely *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* [2]. *P. falciparum*, the most virulent malaria parasite, is responsible for nearly all malarial deaths [3]. After nearly a century of success in reducing its spread there has been a resurgence of malaria during the past two decades due to increased resistance of the parasites to available drugs and insecticides. Hence, there is an urgent need for engineering new antimalarial compounds having novel mechanisms of action.

Ten plasmepsins (PMs) have been reported from the genome of *P. falciparum*, four of which (PMs I, II, IV and histo-aspartic protease) have been shown to be involved in hemoglobin degradation in the food vacuole of the parasite [4–6]. These have been potential targets for developing novel antimalarials [4], however, it was recently reported that the endoplasmic reticulum aspartic protease PMV [7] could be a superior target for malaria control [8–10]. In order to survive and promote its virulence, the parasite must export hundreds of its proteins beyond an encasing vacuole and membrane into the host red blood cell [11,12]. These exported proteins are synthesized in the endoplasmic reticulum having the *Plasmodium* export element (PEXEL), a pentameric motif (R/KxLxE/Q/D) [11,12]. PMV is responsible for cleavage of PEXEL [8,9,13] and is thus required for parasite protein export mediation [8,9,13,14]. For this reason, PMV could be an important target in the development of novel, effective antimalarial drugs [8–10] particularly in consideration of recently published findings regarding the engineering of a PEXEL-mimetic inhibitor that was shown to effectively kill *P. falciparum* parasites via direct action against PMV *in vivo* [15], a potentially critical advance in the fight against malaria.

In order to facilitate the development of specific inhibitors with antimalarial activities, the elucidation of structure-function relationships of PMV, particularly with respect to its modes of proteolysis, inhibition and activation, are important starting points in the initial work towards a level of understanding that facilitates structure elucidation, and ultimately inhibitor design. The present study reports the recombinant expression, pH conditions for optimal activity, inhibitor testing, and most importantly the finding that the prosegment is apparently non-essential for obtaining proteolytic activity and ligand binding.

2. Materials and methods

2.1. Materials

pET32b(+) and pET19b(+) vectors, *E. coli* Rosetta-gami B (DE3)pLysS, BugBuster™ reagent and u-MAC™ cartridges were purchased from Merck KGaA (Darmstadt, Germany). A synthetic 44-residue peptide corresponding to the PMV prosegment

(ENKIDNVGKKIENVGKKIGDMENKNDNVENKNDNVGNKNDNVKN) was purchased from GenicBio (Shanghai, China). A quenched fluorescent synthetic peptide substrate (HRPII; 4-(4-dimethylaminophenyl) diazenylbenzoic acid (DABCYL)-LNKRLHETQ-E(5-[(2-Aminoethyl)amino]naphthalene-1-sulfonic acid) (EDANS), and L→A mutant HRPII DABCYL-LNKRLAHETQ-E(EDANS), was purchased from CanPeptide (Pointe-Claire, QC, Canada). All other chemicals and media were obtained from Fisher Scientific Canada (Nepean, ON, Canada) or Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cloning and construction of soluble expression vectors

The gene encoding for zymogenic PMV (proPMV) [9] was amplified from the genomic DNA of *Plasmodium falciparum* 3D7 (MR4/American Type Culture Collection, Manassas, VA, USA) using primers PMVF109 (5'GCACCATGGAAAATAAAATTGACAATGTTG) and PMVR1563 (5'AATCCATGGCTAATTAGATGGGCATTAGATTC). Product was digested with *Nco*I and ligated into pET32b(+) yielding pET32b-proPMV wherein *proPMV* was fused to a fragment of *thioredoxin* (*trx*).

2.3. Subcloning and construction of insoluble expression vectors

E. coli codon-optimized synthetic genes for mature PMV and proPMV were purchased from GenScript (Piscataway, NJ, USA). The former was amplified using primers *optVF241* 5'-GCACCATGGCGAGCTCTGATCTGTATAAATAC and *optVR1563* 5'AATCTCGAGTTAATGGTGATGGTGATGGTGTTACTCGGACACTTAGATTC, and subsequently subcloned into pET19b(+) at the *Nco*I and *Xho*I restriction sites to produce *pET19b-PMV* containing a C-terminal His₆ tag. *ProPMV* was amplified using primers *optVF109Htag* 5'GCACCATGGCTCATCATCATCATCATGAAAACAAGATCGATAACGTG and *optVR1563* 5'AATCTCGAGTTAGTTACTCGGACACTTAGATTC, and inserted at the *Nco*I and *Xho*I restriction sites yielding *pET19b-proPMV*. Control protein products were made as well by producing Asp118Ala mutants (Ala knockout of the critical catalytic Asp118) for both *PMV* and *proPMV*. The mutants were made using QuickChange™ Site-Directed Mutagenesis (Agilent Technologies, Santa Clara, CA, USA).

2.4. Expression, solubilization, refolding, and purification

E. coli Rosetta-gami B (DE3)pLysS (for soluble expression) transformed with expression vector constructs were cultured, induced and harvested as per the manufacturer's instructions. Frozen cell pellets were resuspended in BugBuster™ cell lysis reagent and incubated at room temperature for 20 min with gentle shaking. Soluble and insoluble materials were separated by centrifugation at 16,000 × g for 20 min at 4°C. Insoluble protein was solubilized in 50 mM Tris-HCl pH 9.0 buffer containing 8 M urea and 10 mM β-mercaptoethanol. To optimize refolding conditions, solubilized protein solution was diluted 20-fold in various refolding buffers having different pH, ratio of oxidized:reduced glutathione, as well as addition of varying concentrations of urea, NaCl, glycerol, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), MgSO₄, Tween-20, Triton X-100, sucrose, arginine, 2-(Cyclohexylamino)ethanesulfonic acid (CHES) and KCl. Refolding efficiency was assessed as per Russo *et al.* [9].

Refolded protein was purified by size-exclusion chromatography using a Superose12 10/300 GL™ column (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) in 50 mM Tris-HCl pH 8.0 followed by MonoQ™ HP anion exchange pre-equilibrated with 50 mM Tris-HCl pH 8.0, and eluted with a linear gradient to 1 M NaCl. Fractions were screened for target protein by non-reducing SDS–PAGE and enzyme activity assays. Fractions containing monomers with relatively high activity were pooled and further purified by cobalt affinity chromatography using a 1 mL u-MAC™ cartridge. Eluent was dialyzed against 50 mM Tris-HCl pH 8.0 containing 10 mM EDTA at 4°C (10,000-fold dilution) and was subsequently stored at –20°C.

2.5. Activation of PMV

In order to determine optimal activation pH, 2 µg of purified Trx-proPMV were incubated in pH 5.0–9.5 buffers at 37°C for 12 h. Activation was determined by SDS-PAGE band-shifting.

2.6. SDS-PAGE and N-terminal sequencing

SDS-PAGE was performed according to the method of Laemmli [16] using a Mini-Protean II electrophoresis cell (Bio-Rad, Hercules, CA, USA). N-terminal sequence analysis was done by the Advanced Protein Technology Centre (Toronto, ON, Canada).

2.7. Proteolytic activity pH-optimum

pH-optima were determined using 10 nM enzyme, 5 µM substrate HRPII at 37°C and pH 3.0–9.0. Assay detection was performed using a Victor2™ 1420 Multilabel Counter (Perkin Elmer, Woodbridge, ON, Canada) with $\lambda_{\text{excitation}}$ at 335 nm and $\lambda_{\text{emission}}$ at 500 nm [17].

2.8. Enzyme kinetics and inhibition

Kinetic parameters were determined at 37°C in 50 mM MES buffer pH 6.0 containing 0.005% Tween-20 using 10 nM PMV or proPMV and 0.10–12 µM HRPII peptide substrate. Initial reaction rates were determined by converting the slope of the linear portion of the curve (fluorescence·min^{–1}) to µmol·min^{–1} using a conversion factor of 845,582 µM^{–1} derived from a standard curve for HRPII substrate fully digested by commercial yeast proteinase A (Sigma-Aldrich, Oakville, ON, Canada). Non-linear regression analysis using the Michaelis-Menten model was applied for the determination of kinetic parameters. Inhibition assays tested various protease inhibitors and cations at 37°C in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) pH 6.0 with 0.005% Tween-20. Ten nM enzyme was pre-incubated with inhibitor or cation for 5 min prior to the addition of 5 µM substrate. K_i values for pepstatin A, kenostatin (KNI)-10742 and KNI-10743 were determined using the equation of Cheng and Prusoff [18].

3. Results and Discussion

3.1. Identification of recombinant mature PMV

The ability to recombinantly produce substantial quantities of pure, active PMV is critical to the elucidation of its biochemical characteristics, and eventual determinations of its structure

and modes of inhibition. Bacterial expression as inclusion bodies was previously reported for establishing PMV's PEXEL-cleaving role [9]; however, soluble expression and biochemical characterization of PMV remained to be conducted. A dual-approach was attempted for soluble expression such that a truncated PMV sequence (Glu37–Asn521) was used in combination with deletion of both the N- and C-terminal membrane-binding domains. This PMV form was then fused to a highly soluble thioredoxin fragment and fusion protein was subsequently expressed in *E. coli* Rosetta-gami B (DE3)pLysS. Fusion protein was identified by Western blotting using anti-thioredoxin primary antibody (Invitrogen, Burlington, ON, Canada) as well as N-terminal sequence analysis.

Soluble expression of fusion protein in usable quantities was unsuccessful (data not shown), therefore, inclusion bodies were then refolded and purified for subsequent analyses. Various conditions that were previously reported to aid in refolding [21,22] were screened, and assessment of refolding efficiency was based on HRPII substrate activity and amount of aggregation detected on non-reducing SDS-PAGE. The best combination for refolding was 50 mM Tris-HCl pH 9.0 containing 0.5 mM oxidized glutathione, 1.25 mM reduced glutathione and 250 mM arginine (data not shown). The addition of arginine was essential for refolding PMV, an ingredient whose mode of action is attributed to its ability to suppress aggregation [23,24].

The refolded Trx-proPMV solution was subjected to size-exclusion chromatography (Fig. 1A) and fractions were analysed by non-reducing and reducing SDS-PAGE (Fig. 1B and 1C, respectively). The results showed that fractions 2–4 consisted of higher molecular weight aggregates whereas fractions 6–9 contained the principal monomeric form. The latter were pooled and subsequently incubated overnight at 37°C at various pH values to determine optimal activation conditions. Trx-proPMV underwent slow activation between pH 6.0 – 9.5 as evidenced by a band shift from 73 kDa (Trx-proPMV) to 56 kDa (proPMV), and then further to 51 kDa (PMV) (Fig. 1D). N-terminal sequencing revealed that cleavage occurred where the fusion partner enterokinase recognition sequence (underlined) joins proPMV (italized) (...DDDDKAMENK...). Subsequent cleavage between Asn80-Ala81 within proPMV resulted in release of 51 kDa PMV. The activation process was slow and did not go to completion even after three days at 37°C, but rather led to degradation (data not shown).

3.2. Characterization of recombinant mature PMV

Although the preceding method for obtaining active soluble PMV did produce active enzyme, it did not result in useable amounts of pure sample for biochemical characterization or structure determination. To characterize mature length PMV, Ala81-Asn521 (i.e., no prosegment) was directly expressed as inclusion bodies, solubilized and re-folded as outlined in the Materials and Methods section. In addition to desirable refolded monomers, non-reducing SDS-PAGE indicated that higher molecular-weight disulfide bond multimers were formed (data not shown) which were separated by anion exchange and size exclusion chromatography and were shown to have virtually no activity with synthetic HRPII peptide substrate. Purity of PMV was verified by both reducing and non-reducing SDS-PAGE (Fig. 2A). To exclude contaminant proteolytic activity, inactive Asp118Ala PMV mutant was

expressed, refolded and purified as a negative control and was verified to show no activity (data not shown).

Recombinant PMV did not hydrolyze synthetic peptide EDANS-CO-CH₂-CH₂-CO-ALERMFLSFP-Dap(DABCYL)-OH, a substrate specific for food vacuolar PMI, PMII, PMIV and HAP [17,19,20]. This lack of activity for a standard PM cleavage site is consistent with previous reports that PMV is highly specific for the PEXEL sequence R/KxLxE/Q/D [8,9,13]. A pH-activity profile was generated based on hydrolysis of HRPII peptide (Fig. 2B). Similar to that reported for natural-source wild-type PMV [8], recombinant PMV showed a pH optimum between 5.5–7.0. The pH-stability of the recombinant form was also examined by 1 h incubation at 37°C (pH 3.0–11.0) followed by assaying for activity at pH 6.0 using 5 µM HRPII substrate. Results showed that the enzyme was stable between pH 5.0–9.0, but stability was markedly reduced in more acidic (pH 3.0) and alkaline (pH 10.0–11.0) conditions (Fig. 2C). In addition, PMV proteolysis was verified to fit the Michaelis-Menten model; the K_m and k_{cat} obtained at optimal pH were $K_m = 4.6 \pm 0.4 \mu\text{M}$ and $k_{cat} = 0.24 \pm 0.07 \text{ s}^{-1}$, respectively (Fig. 2D).

The cleavage site specificity of PMV was verified by incubation with HRPII at 37°C for 20 h and analyzed by mass spectrometry (see Fig. 3A for control and Fig. 3B for PMV-hydrolyzed HRPII). As expected, the results indicated that cleavage resulted in two fragments: LNKRL and HETQ. A control substrate (L→A mutant HRPII) [8] (Fig. 3C) was also tested and indicated that it was not cleaved (Fig. 3D). Together, the above results demonstrated that the observed activity was the result of hydrolysis by recombinant PMV and not potential contaminant proteases.

3.3. PMV inhibition

Several compounds (e.g., inhibitors, cations) were tested as an initial screening for PMV inhibition mode(s) (Table 1). Compounds tested included several inhibitors from the KNI series which were originally designed and developed for the purpose of inhibiting HIV-1 protease [25]. KNI compounds were subsequently designed as effective inhibitors for proteases expressed in *Plasmodium* parasites [26–28]. PMV was partially inhibited by the generic aspartic protease inhibitor pepstatin A as well as by KNI inhibitors (Table 1 and Fig. 4). IC₅₀ values for pepstatin A, KNI-10743 and KNI-10742 were 22, 44 and 108 µM, respectively (Fig. 4). The relatively weak inhibition by pepstatin A is similar to mammalian aspartic proteases renin and BACE1 [29]. Furthermore, despite its effectiveness against PMI, PMII, PMIV, HAP as well as parasite growth in infected red blood cells [30], the G16 compound did not inhibit PMV. The HIV protease inhibitor saquinavir weakly inhibited PMV while the serine protease inhibitor PMSF exhibited no inhibition (Table 1). The observed low-level inhibition by saquinavir was in agreement with the previous observation that *P. falciparum*-infected erythrocytes incubated with HIV inhibitors lopinavir, nelfinavir, ritonavir and saquinavir showed mildly reduced PEXEL cleavage [9]. Taken together, these results suggest that PMV and HIV-1 protease may share some similarities [9] in their substrate binding motifs and binding pocket structures.

Examination of the effects of cations on PMV activity indicated that all had an inhibitory effect (Table 1 and Fig. 4). Cu²⁺ and Hg²⁺ showed markedly better inhibition than other

cations tested with IC_{50} values of 0.4 μ M and 0.5 μ M, respectively. Addition of the chelator EDTA after addition of Cu^{2+} or Hg^{2+} greatly reduced their inhibitory effects (Table 2) indicating reversibility for their mode(s) of action. Inhibition by Cu^{2+} was non-competitive (data not shown), and subsequent addition of DTT resulted in 19% recovery of activity whereas addition of DTT after addition of Hg^{2+} resulted in full recovery. These findings implicate involvement of cysteine residue(s) in the inhibition of PMV by Cu^{2+} and Hg^{2+} . Similarly, HIV-1 protease is inhibited by approximately stoichiometric concentrations of copper or mercury ions as the result of their binding to cysteine residues [31]. Copper has been shown to interfere with malarial parasite growth in both normal and G6PD-deficient erythrocytes [32], and a new class of Cu^{2+} nanohybrid solid has been reported to inhibit PMII [33]. Further exploration of copper and its derivatives in antimalarial chemotherapy is thus a potentially important future avenue for PMV inhibitor-mediated anti-malarial compound research.

3.4. The prosegment has no apparent function in PMV refolding and inhibition

Most aspartic proteases are synthesized as zymogens containing an N-terminal prosegment domain that is proteolytically removed during activation. The prosegment can be important in processes such as initiation of correct folding, protein stability, active site accessibility blockage, pH-dependence of activation and zymogen intracellular sorting [34]. Prosegment-catalyzed folding has been reported for serine proteases [35–40] in addition to the aspartic protease pepsin [41]. Prosegments as well as synthetic peptides derived from prosegments have also been shown to be potent and specific inhibitors of their associated proteases [42–48]. In the present study, potential roles in refolding and inhibition of the PMV prosegment were investigated by adding synthetic prosegment under different refolding conditions. Refolding rates in the presence of prosegment were not significantly different ($P>0.05$) from refolding in its absence (Fig. 5). Although the prosegment does not catalyze the refolding of PMV, we do not rule out other possible effects by its presence *in vivo* in the prepro form of PMV from translation to final active product. In terms of inhibition, PMV prosegment did not inhibit its parent enzyme (Fig. 6). The above findings are consistent with the previously reported conclusion that the PMV prosegment has no strong structural or functional constraints due to poor interspecies sequence conservation [7].

3.5. The zymogen form of PMV is enzymatically active

Since the PMV prosegment does not inhibit PMV activity, and Klemba and Goldberg (2005) did not find evidence of prosegment release [7], it seemed reasonable to postulate that the zymogenic form of PMV might be catalytically active as is the case for the aspartic protease BACE1 [49]. ProPMV (E37–N521) was thus expressed as inclusion bodies, refolded and purified in a manner similar to that used above for mature PMV. Purified proPMV was shown to be monomeric by non-reducing SDS-PAGE (Fig. 7A), and zymogen catalytic activity was confirmed. A negative control active site mutant (Asp118Ala proPMV) was also tested to exclude substrate proteolysis arising from contaminant sources (data not shown). Similar to mature PMV, proPMV was optimally active between pH 5.5–7.0 (Fig. 7B), and it was strongly inhibited by cations Cu^{2+} and Hg^{2+} , partially inhibited by pepstatin A and not inhibited by PMSF, leupeptin and EDTA (Fig. 7C). The K_m and k_{cat} values obtained for proPMV at pH 6.0 were $7.9 \pm 0.7 \mu$ M and $0.24 \pm 0.02 s^{-1}$, respectively. Thus, zymogen and

mature length PMV turnover numbers were similar, however, binding affinity was modestly lower (~2-fold) for the zymogen ($P < 0.01$). The possibility that the observed activity arose from autoactivation of the zymogen during proteolytic assays was negated by the observations that proPMV incubation in various pH conditions did not result in proenzyme processing (data not shown). Furthermore, the proPMV reaction curve (Fig. 7D) is monophasic, and therefore, does not support a second source of catalysis.

If the structural mechanism for PMV zymogen-derived catalysis is similar to human BACE1 then proteolysis results from an open-flap state of the zymogen structure, a distinct structural form from its accompanying closed-flap zymogen and mature enzyme [50,51]. Zymogenic BACE1 exhibits moderate activity relative to its mature form and is inhibited by its propeptide [50]. Prorenin, another catalytically active zymogen, has a low intrinsic activity (~3%) relative to the mature form, and this activity is attributed to partial unfolding of the prosegment [52,53]. To characterize the structural phenomena associated with proteolysis by the zymogen of PMV, detailed structural data will be critical in order to understand its catalytic mechanism.

In conclusion, the present study was an important follow-up investigation in the context of Klemba and Goldberg [7] who did not detect a size shift *in vivo* indicative of PMV prosegment processing (i.e., there was as yet no evidence that the prosegment of PMV is removed upon activation). The authors suggested that the prosegment may be non-inhibitory and therefore there would be no requirement for its removal for the parent enzyme's activation. Our findings are consistent with this prediction. Furthermore, we clarify that the prosegment of PMV has no discernible contribution to the enzyme's correct folding unlike the purported folding role in BACE [49], and therefore its function, if any exists, remains unknown. A functional requirement for zymogen processing to mature PMV in its PEXEL-cleaving role in malarial protein export *in vivo* thus appears to be unlikely. Most importantly, the above findings suggest that both zymogen and mature forms of PMV should be further characterized structurally and treated as potentially independent targets for *in silico* antimalarial drug development.

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Abbreviations:

DABCYL	4-((4-(dimethylamino)phenyl)azo)benzoic Acid
EDANS	5-((2-Aminoethyl)amino)naphthalene-1-sulfonic acid
KNI	Kinostatin
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHES	N-Cyclohexyl-2-aminoethanesulfonic acid
MES	2-(N-morpholino)ethanesulfonic acid

References

- [1]. World Health Organization. World malaria report Switzerland: WHO Press; 2008 pp. vii.
- [2]. White NJ, Pukrittayakamee S, Hien TT, Faiz MA, Mokuolu OA, Dondorp AM. Malaria. *Lancet* 2014; 383:723–35. [PubMed: 23953767]
- [3]. Mackintosh CL, Beeson JG, Marsh K. Clinical features and pathogenesis of severe malaria. *Trends Parasitol* 2004;20:597–603. [PubMed: 15522670]
- [4]. Ersmark K, Samuelsson B, Hallberg A. Plasmepsins as potential targets for new antimalarial therapy. *Med Res Rev* 2006;26:626–66. [PubMed: 16838300]
- [5]. Coombs GH, Goldberg DE, Klemba M, Berry C, Kay J, Mottram JC. Aspartic protease of *Plasmodium falciparum* and other parasitic protozoa as drug targets. *Trends Parasitol* 2001;17:532–7. [PubMed: 11872398]
- [6]. Banerjee R, Liu J, Beaty W, Pelosof L, Klemba M, Goldberg DE. Four plasmepsins are active in the *Plasmodium falciparum* food vacuole, including a protease with an active-site histidine. *PNAS* 2002;99:990–5. [PubMed: 11782538]
- [7]. Klemba M, Goldberg DE. Characterization of plasmepsin V, a membrane-bound aspartic protease homolog in the endoplasmic reticulum of *Plasmodium falciparum*. *Mol Biochem Parasitol* 2005;143:183–91. [PubMed: 16024107]
- [8]. Russo I, Babbitt S, Muralidharan V, Butler T, Oksman A, Goldberg DE. Plasmepsin V licenses *Plasmodium* proteins for export into the host erythrocyte. *Nature* 2010;463:632–6. [PubMed: 20130644]
- [9]. Boddey JA, Hodder AN, Günther S, Gilson PR, Patsiouras H, Kapp EA, Pearce JA, de Koning-Ward TF, Simpson RJ, Crabb BS, Cowman AF. An aspartyl protease directs malaria effector proteins to the host cell. *Nature* 2010;463:627–31. [PubMed: 20130643]
- [10]. Meyers MJ, Goldberg DE. Recent advances in plasmepsin medicinal chemistry and implications for future antimalarial drug discovery efforts. *Curr Top Med Chem* 2012;12:445–55. [PubMed: 22242846]
- [11]. Hiller NL, Bhattacharjee S, van Ooij C, Liolios K, Harrison T, Lopez-Estraño C, Haldar K. A host-targeting signal in virulence proteins reveals a secretome in malarial infection. *Science* 2004;306:1934–7. [PubMed: 15591203]
- [12]. Marti M, Good RT, Rug M, Knuepfer E, Cowman AF. Targeting malaria virulence and remodeling proteins to the host erythrocyte. *Science* 2004;306:1930–3. [PubMed: 15591202]
- [13]. Boddey JA, Carvalho TG, Hodder AN, Sargeant TJ, Sleebs BE, Marapana D, Lopaticki S, Nebl T, Cowman AF. Role of plasmepsin V in export of diverse protein families from the *Plasmodium falciparum* exportome. *Traffic* 2013; 14:532–50. [PubMed: 23387285]
- [14]. Tarr SJ, Cryar A, Thalassinou K, Haldar K, Osborne AR. The C-terminal portion of the cleaved HT motif is necessary and sufficient to mediate export of proteins from the malaria parasite into its host cell. *Mol Microbiol* 2013; 87: 835–50. [PubMed: 23279267]
- [15]. Sleebs BE, Lopaticki S, Marapana DS, O'Neill MT, Rajasekaran P, Gazdik M, Günther S, Whitehead LW, Lowes KN, Barford L, Hviid L, Shaw PJ, Hodder AN, Smith BJ, Cowman AF, Boddey JA. “Inhibition of Plasmepsin V activity demonstrates its essential role in protein export, PfEMP1 display, and survival of malaria parasites.” *PLoS Biol* 2014;12:e1001897. [PubMed: 24983235]
- [16]. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–5. [PubMed: 5432063]
- [17]. Xiao H, Sinkovits AF, Bryksa BC, Ogawa M, Yada RY. Recombinant expression and partial characterization of an active soluble histidine-aspartic protease from *Plasmodium falciparum*. *Protein Exp Purif* 2006;49:88–94.
- [18]. Cheng Y, Prusoff WH. Relationship between the inhibition constant (KI) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* 1973;22:3099–108. [PubMed: 4202581]
- [19]. Xiao H, Tanaka T, Ogawa M, Yada RY. Expression and enzymatic characterization of the soluble recombinant plasmepsin I from *Plasmodium falciparum*. *Protein Eng Des Sel* 2007;20:625–33. [PubMed: 18073224]

- [20]. Xiao H, Dee D, Yada RY. The native conformation of plasmepsin II is kinetically trapped at neutral pH. *Arch Biochem Biophys* 2011;513: 102–109 [PubMed: 21767524]
- [21]. Clark EDB. Refolding of recombinant proteins. *Curr Opin Biotechnol* 1998;9:157–163. [PubMed: 9664053]
- [22]. Pan JC, Wang JS, Cheng Y, Yu Z, Rao XM, Zhou HM. The role of detergent in refolding of GdnHCl-denatured arginine kinase from shrimp *Penaeus chinensis*: the solubilization of aggregate and refolding in detergent solutions. *Biochem Cell Biol* 2005;83:140–6. [PubMed: 15864323]
- [23]. Tsumoto K, Umetsu M, Kumagai I, Ejima D, Philo JS, Arakawa T. Role of Arginine in Protein Refolding, Solubilization, and Purification. *Biotechnol Prog* 2004;20:1301–8 [PubMed: 15458311]
- [24]. Arakawa T, Ejima D, Tsumoto K, Obeyama N, Tanaka Y, Kita Y, Timasheff SN. Suppression of protein interactions by arginine: A proposed mechanism of the arginine effects. *Biophysical Chemistry* 2007;127:1–8 [PubMed: 17257734]
- [25]. Abdel-Rahman HM, Kimura T, Hidaka K, Kiso A, Nezami A, Freire E, Hayashi Y, Kiso Y. Design of inhibitors against HIV, HTLV-I, and *Plasmodium falciparum* aspartic proteases. *Biol Chem* 2004; 385: 1035–9. [PubMed: 15576323]
- [26]. Nezami A, Kimura T, Hidaka K, Kiso A, Liu J, Kiso Y, Goldberg DE, Freire E. High-affinity inhibition of a family of *Plasmodium falciparum* proteases by a designed adaptive inhibitor. *Biochemistry* 2003;42:8459–64. [PubMed: 12859191]
- [27]. Hidaka K, Kimura T, Tsuchiya Y, Kamiya M, Ruben AJ, EFreire E, Hayashi Y, Kiso Y. Additional interaction of allophenylnorstatine-containing tripeptidomimetics with malarial aspartic protease plasmepsin II. *Bioorg Med Chem Lett* 2007; 17: 3048–52. [PubMed: 17400453]
- [28]. Miura T, Hidaka K, Uemura T, Kashimoto K, Hori Y, Kawasaki Y, Ruben AJ, Freire E, Kimura T, Kiso Y. Improvement of both plasmepsin inhibitory activity and antimalarial activity by 2-aminoethylamino substitution. *Bioorg Med Chem Lett* 2010; 20:4836–39. [PubMed: 20634066]
- [29]. Gruninger-Leitch F, Schlatter D, Kung E, Nelbock P, Dobeli H. Substrate and inhibitor profile of BACE (beta-secretase) and comparison with other mammalian aspartic proteases. *J Biol Chem* 2002;277:4687–93. [PubMed: 11741910]
- [30]. Liu K, Shi H, Xiao H, Chong A, Bi Z, Chang Y, Tan K, Yada R, Yao S. Functional Profiling, Identification and Inhibition of Plasmepsins in Intraerythrocytic Malaria Parasites. *Angewandte Chemie* 2009;48:8293–7. [PubMed: 19784986]
- [31]. Karlström AR, Levine RL. Copper inhibits the protease from human immunodeficiency virus 1 by both cysteine-dependent and cysteine-independent mechanisms. *Proc Natl Acad Sci USA* 1991;88:5552–6. [PubMed: 2062837]
- [32]. Marva E, Cohen A, Saltman P, Chevion M, Golenser J. Deleterious synergistic effects of ascorbate and copper on the development of *Plasmodium falciparum*: an in vitro study in normal and in G6PD-deficient erythrocytes. *Int J Parasitol* 1989;19:779–85. [PubMed: 2687184]
- [33]. Mohapatra SC, Tiwari HK, Singla M, Rathi B, Sharma A, Mahiya K, Kumar M, Sinha S, Chauhan SS. Antimalarial evaluation of copper(II) nanohybrid solids: inhibition of plasmepsin II, a hemoglobin-degrading malarial aspartic protease from *Plasmodium falciparum*. *J Biol Inorg Chem* 2010;15:373–85. [PubMed: 19946719]
- [34]. Horimoto Y, Dee DR, Yada RY. Multifunctional aspartic peptidase prosegments. *N Biotechnol* 2009;25:318–24. [PubMed: 19491047]
- [35]. Sohl JL, Jaswal SS, Agard DA. Unfolded conformations of alpha-lytic protease are more stable than its native state. *Nature* 1998;395: 817–9. [PubMed: 9796818]
- [36]. Cunningham EL, Jaswal SS, Sohl JL, Agard DA. Kinetic stability as a mechanism for protease longevity. *Proc Natl Acad Sci USA* 1999;96:11008–14. [PubMed: 10500115]
- [37]. Truhlar SME, Cunningham EL, Agard DA. The folding landscape of *Streptomyces griseus* protease B reveals the energetic costs and benefits associated with evolving kinetic stability. *Protein Sci* 2004;13:381–90. [PubMed: 14718653]
- [38]. Zhu X, Ohta Y, Jordan F, Inouye M. Pro-sequence of subtilisin can guide the refolding of denatured subtilisin in an intermolecular process. *Nature* 1989;339:483–4. [PubMed: 2657436]

- [39]. Eder J, Rheinneck M, Fersht AR (1993) Folding of subtilisin BPN': role of the pro-sequence. *J Mol Biol* 1993;233: 293–304. [PubMed: 8377204]
- [40]. Bryan PN. Prodomains and protein folding catalysis. *Chem Rev* 2002;102:4805–16. [PubMed: 12475207]
- [41]. Dee DR, Yada R Y. The prosegment catalyzes pepsin folding to a kinetically trapped native state. *Biochemistry* 2010;49:365–71. [PubMed: 20000477]
- [42]. Mach L, Mort JS, Glossl J. Maturation of human procathepsin B. Proenzyme activation and proteolytic processing of the precursor to the mature proteinase, in vitro, are primarily unimolecular processes. *J Biol Chem* 1994;269: 13030–5. [PubMed: 8175723]
- [43]. Carmona E, Dufour E, Plouffe C, Takebe S, Mason P, Mort JS and M'enard R Potency and selectivity of the cathepsin L propeptide as an inhibitor of cysteine proteases. *Biochemistry* 1996;35:8149–57. [PubMed: 8679567]
- [44]. Taylor MAJ, Baker KC, Briggs GS, Connerton IF, Cummings NJ, Pratt KA, Revell D F, Freedman RD, Goodenough PW. Expression of the pro-regions of papain and papaya proteinase IV in *Escherichia coli* and their inhibition of mature cysteine proteinases. *Protein Eng* 1995;8:59–62. [PubMed: 7770454]
- [45]. Ohta Y, Hojo H, Aimoto S, Kobayashi T, Zhu X, Jordan F, Inouye M. Pro-peptide as an intermolecular chaperone: renaturation of denatured subtilisin E with a synthetic propeptide. *Mol Microbiol* 1991;5:1507–10. [PubMed: 1686294]
- [46]. Dee DR, Filonowicz S, Horimoto Y, Yada RY. Recombinant prosegment peptide acts as a folding catalyst and inhibitor of native pepsin. *Biochim Biophys Acta* 2009; 1794:1795–801. [PubMed: 19715777]
- [47]. Basak A, Lazure C. Synthetic peptides derived from the prosegments of proprotein convertase 1/3 and furin are potent inhibitors of both enzymes. *Biochem J* 2003; 373:231–9. [PubMed: 12662153]
- [48]. Cumin F, Evin G, Fehrentz JA, Seyer R, Castro B, Menard J, Corvol P. Inhibition of human renin by synthetic peptides derived from its prosegment. *J Biol Chem* 1985;260:9154–7 [PubMed: 3894354]
- [49]. Shi XP, Chen E, Yin KC, Na S, Garsky VM, Lai MT, Li YM, Platchek M, Register RB, Sardana MK, Tang MJ, Thiebeau J, Wood T, Shafer JA, Gardell SJ. The pro domain of beta-secretase does not confer strict zymogen-like properties but does assist proper folding of the protease domain. *J Biol Chem* 2001;276:10366–73. [PubMed: 11266439]
- [50]. Kennedy ME, Wang WY, Song LX, Lee J, Zhang LL, Wong G, Wang LY & Parker E. Measuring human beta-secretase (BACE1) activity using homogeneous time-resolved fluorescence. *Anal Biochem* 2003;319:49–55. [PubMed: 12842106]
- [51]. Gorfe AA, Caflisch A. Functional plasticity in the substrate binding site of beta-secretase. *Structure* 2005;13:1487–98. [PubMed: 16216580]
- [52]. Lenz T, Sealey JE, Lappe RW, Carilli C, Oshiro GT, Baxter JD, Laragh JH. Infusion of recombinant human prorenin into rhesus monkeys. Effect on hemodynamics, renin-angiotensin-aldosterone axis and plasma testosterone. *Am J Hypertens* 1990;3:257–61. [PubMed: 2189442]
- [53]. Lenz T, Sealey JE, Maack T, James GD, Heinrikson RL, Marion D, Laragh JH. Half-life, hemodynamic, renal, and hormonal effects of prorenin in cynomolgus monkeys. *Am J Physiol* 1991;260:R804–10. [PubMed: 2012251]

Figure 1.

Purification and processing of Trx-proPMV. (A) Purification of Trx-proPMV using size-exclusion chromatography at 1 mL/min with 1 mL fractions collected throughout. (B) Non-reducing 15% SDS-PAGE analysis of size exclusion chromatography fractions; Lane "M": protein marker, lanes 1–9: fractions 1–9 (i.e., the first 9 minutes of the chromatography run from (A) above; "AP": aggregation protein, "MP": monomeric protein. (C) Reducing 15% SDS-PAGE analysis of fractions from size exclusion chromatography as in (B) above; "F": Trx-proPMV fusion protein. (D) SDS-PAGE band shifting indicated that Trx-proPMV was

processed when incubated for 12 h at various pH values at 37°C; “CK”: fusion protein negative control; “F”: fusion protein Trx-proPMV; “Z”: zymogenic proPMV, and “M”: mature PMV.

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Figure 2.

Partial characterization of PMV. (A) SDS-PAGE gel for 1 μg of purified PMV under reducing (lane 1) and non-reducing (lane 2) conditions. Lane “M”; protein molecular weight marker. (B) Enzyme activity of PMV as a function of pH. Enzyme activity was determined as described in Materials and Methods, and percent activity is expressed relative to the highest observed activity. Values are reported as the mean ± SD from triplicate determinations. (C) Enzyme stability of PMV as a function of pH. Samples at the indicated pH values were incubated at 37°C for 1 h prior to activity determination at pH 6.0 and 37°C. Percent activity is expressed relative to control sample at 4°C. Values are reported as the mean ± SD from triplicate determinations. (D) Kinetic analysis of PMV for internally quenched fluorescent HRPII peptide substrate. Kinetic assays were completed over the substrate range of 0.5–12.0 μM using 12 nM PMV in 50 mM MES pH 6.0 with 0.005% Tween 20 at 37°C.

Figure 3.

Verification of PMV substrate specificity. **(A)** Untreated substrate HRPII. **(B)** PMV-treated HRPII. **(C)** Untreated control substrate differing from HRPII only by an L→A mutation; **(D)** PMV-treated control substrate. All samples were incubated for 20 h at 37 °C. Mass spectrometry analyses were carried out by the Advanced Analysis Centre, University of Guelph, Guelph, ON, Canada using electrospray ionization on an Agilent LC-UHD Q-Tof (Agilent Technologies).

Figure 4.

Effects of pepstatin A, KNI-10742, KNI-10743, Cu²⁺ and Hg²⁺ on the activity of PMV. 10 nM PMV in 50 mM MES pH 6.0 with 0.005% Tween-20 was incubated at various inhibitor/cation concentrations for 5 min at 37°C after which 5 μM HRP II was added. Relative activity is expressed as a percentage of the activity of control and values are reported as the mean ± SD from triplicate determinations.

Figure 5.

Effects of PMV prosegment on refolding. Five μL of 1 mg/mL PMV in 8 M urea, 50 mM Tris-HCl pH 8.0 was added to 95 μL of various pH buffers or refolding buffer (50 mM Tris-HCl pH 9.0 containing 0.25 M arginine, 0.5 mM oxidized glutathione and 1.25 mM reduced glutathione) either with (filled bar) or without (unfilled bar) 20 μM prosegment and kept at 4°C for 18 h. Refolding with prosegment and without prosegment, reported as the mean $\pm$ SD from triplicate determinations, were not significantly different ($P > 0.05$).

Figure 6.

Effects of PMV prosegment on the PMV activity. PMV (10 nM) in the assay buffer (50 mM MES, pH 6.0, 0.005% Tween-20) was incubated at various concentrations of the PMV prosegment for 5 min at 37 °C, and then 5 μM substrate was added. Hydrolysis of the substrate was measured as the release of fluorescence as described in the Method section. The relative activity is expressed as a percentage of the activity of mPMV sample without PMV prosegment. Each data point represents the mean and SD of three determinations.

Figure 7.

Characterization of recombinant proPMV. (A) SDS-PAGE gel showing 5 µg purified proPMV under non-reducing (lane 1) and reducing (lane 2) conditions. "M"; molecular weight marker. (B) Enzymatic activity of proPMV as a function of pH. Percent activity is expressed relative to the highest observed activity and values are reported as the mean $\pm$ SD from triplicate determinations. (C) Inhibition by protease inhibitors and cations. Ten nM proPMV was incubated for 5 min at 37°C with inhibitor/cations in 50 mM MES pH 6.0 containing 0.005% Tween-20 followed by activity measurement expressed relative to control. Values are reported as the mean $\pm$ SD from triplicate determinations. (D) The linear progress curve for the hydrolysis of peptide substrate HRPII. The assay was conducted with 12 nM enzyme tPMV and 5 µM substrate in 50 mM MES pH 6.0 with 0.005% Tween 20 at 37°C.

Table 1:

Effects of protease inhibitors and various cations on PMV activity

<u>Inhibitor \ cation</u>	<u>Concentration (μM)</u>	<u>Relative Activity (%) $\pm$ SD</u>
Pepstatin A	10	60 $\pm$ 5
KNI764	10	73 $\pm$ 2
KNI10006	10	77 $\pm$ 1
KNI10333	10	81 $\pm$ 3
KNI10343	10	68 $\pm$ 1
KNI10395	10	77 $\pm$ 0.1
KNI10742	20	65 $\pm$ 5
KNI10743	20	60 $\pm$ 3
G16	10	80 $\pm$ 5
Saquinavir	12.5	94 $\pm$ 3
PMSF	1000	102 $\pm$ 6
Leupeptin	25	112 $\pm$ 3
ALLN	25	110 $\pm$ 1
EDTA	10000	128 $\pm$ 1
Cu ²⁺	25	1.0 $\pm$ 0.5
Hg ²⁺	25	0.8 $\pm$ 0.6
Zn ²⁺	25	36 $\pm$ 3
K ⁺	25	98 $\pm$ 1
Ca ²⁺	25	94 $\pm$ 3
Mn ²⁺	25	93 $\pm$ 4
Ni ²⁺	25	39 $\pm$ 3
Co ²⁺	25	81 $\pm$ 5
Fe ²⁺	25	41 $\pm$ 2
Fe ³⁺	25	14 $\pm$ 1
Mg ²⁺	25	94 $\pm$ 3
Na ⁺	25	95 $\pm$ 2

10 nM of PMV enzyme was incubated with various protease inhibitors and cations in 50 mM MES, pH 6.0 / 0.005% Tween-20 for 5 min at 37 °C then assayed for activity by addition of substrate HRPII. Percent activity is expressed relative to the activity without addition of protease inhibitors. Values are reported as the mean $\pm$ SD from triplicate determinations.

Table 2:

Effects of EDTA and DTT on PMV inhibition by copper and mercury

First addition	Second addition	Third addition	Relative Activity (% $\pm$ SD)
PMV	---	substrate	100 $\pm$ 1
PMV + Cu ²⁺	---	substrate	1.2 $\pm$ 0.4
PMV + Cu ²⁺	EDTA	substrate	86 $\pm$ 4
PMV + Cu ²⁺	DTT	substrate	19 $\pm$ 1
PMV + Hg ²⁺	---	substrate	1.6 $\pm$ 0.4
PMV + Hg ²⁺	EDTA	substrate	76 $\pm$ 0.2
PMV + Hg ²⁺	DTT	substrate	138 $\pm$ 1
PMV	EDTA	substrate	100 $\pm$ 5
PMV	DTT	substrate	109 $\pm$ 4
Substrate + Cu ²⁺	EDTA	PMV	99 $\pm$ 3
Substrate + Hg ²⁺	EDTA	PMV	79 $\pm$ 1

10 nM PMV and 5 μ M HRPII, 25 μ M CuSO₄, 25 μ M HgCl₂, 1 mM EDTA and 10 mM DTT were added in the order indicated. The first and second additions were both incubated in 50 mM MES pH 6.0 containing 0.005% Tween-20 for 5 min at 37°C prior to the third addition. Percent activity is expressed relative to control PMV and values are reported as the mean $\pm$ SD from triplicate determinations.