Expression and enzymatic characterization of the soluble recombinant plasmepsin I from *Plasmodium falciparum*

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The plasmepsins are involved in the degradation of host cell hemoglobin during malaria infection. Plasmepsin I (PM I) initiates the degradative process, and has been suggested as an attractive target for the treatment of malaria. The production of active recombinant PM I, however, has been challenging. We report for the first time, the expression and partial characterization of soluble recombinant PM I from *Plasmodium falciparum* in which a truncated form of PM I (Lys77P–Leu329) (P indicates a propart residues) was fused to thioredoxin in the pET32b (+) vector, Trx-tPM I and expressed in *Escherichia coli* Rosetta-gami B (DE3)pLysS. The soluble fusion protein was purified from cell culture using a combination of Ni2+ affinity and gel filtration chromatography and was capable of autocatalytic activation at pH 4.0–5.5, which occurred at Leu116P–Ser117P, seven residues upstream of the native cleavage site (Gly123P–Asn1). The mature tPM I (mtPM I) was capable of hydrolyzing both human hemoglobin with a pH optimum of pH 2.8–4.0 and the synthetic fluorogenic peptide EDANS-CO-CH2-CH2-CO-ALERMFLSFP-Dap(DABCY)l-OH with a dual pH optima of pH 2.5–3.0 and pH 4.5–5.5. Using the synthetic substrate, mtPM I exhibited kinetic parameters comparable to native PM I.

Keywords: aspartic protease/malaria/plasmepsin I/recombinant expression

Introduction

Malaria afflicts up to several hundred million people resulting in the death of approximately 1–2 million (Greenwood et al., 2005). The disease is caused by parasites of the genus *Plasmodium*. During *P. falciparum* infection, the most lethal of the four parasites infecting humans, the parasite invades erythrocytes and degrades large amounts of host cell hemoglobin in food vacuole via aspartic, cysteine and metalloproteases (Kolakovich et al., 1997). The development of *P. falciparum* resistance to drugs such as chloroquine and sulfadoxine/pyrimethamine is a major challenge for malaria control; therefore, new treatment methods and alternative drugs are needed (Ridley, 2002). The plasmepsins produced by the *Plasmodium* parasite are aspartic proteases and have been recognized as an attractive target for the design of novel chemotherapeutic compounds for the control of malaria (Olliario and Goldberg, 1995; Silva et al., 1996; Asojo et al., 2002; Boss et al., 2003; Johansson et al., 2004; Ersmark et al., 2006).

There are 10 plasmepsins, type II membrane proteins, reported in the genome of *P. falciparum*, PM I, II and IV–X and histo-aspartic protease (HAP) (Coombs et al., 2001). Four of them, PM I, PM II, HAP and PM IV, lie in a cluster on chromosome 14, and share 50–79% amino acid sequence identity (Berry et al., 1999). These four plasmepsins have been localized in the food vacuole of the parasite, and have been shown to be involved in hemoglobin degradation (Coombs et al., 2001; Banerjee et al., 2002). PM I and II appear to initiate the degradative process by cleaving a conserved hinge region in the α-chain of native hemoglobin (Gluzman et al., 1994; HAP, PM IV and falcipain-2 and -3 subsequently act on the denatured or fragmented globin (Salas et al., 1995; Francis et al., 1996; Berry et al., 1999; Humphreys et al., 1999; Sijwali et al., 2001; Banerjee et al., 2002), PM V was shown to be localized in the endoplasmic reticulum and was suggested to have a role in intraerythrocytic biology that is distinct from PM I, PM II, HAP and PM IV (Klemba and Goldberg, 2005). 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 (Berry et al., 1999; Coombs et al., 2001). To date, much of our knowledge of recombinant expression, crystallographic analysis, kinetics and inhibitor development comes mainly from the successful expression of PM I and II and IV in *Escherichia coli* (Hill et al., 1994; Silva et al., 1996; Asojo et al., 2002; Li et al., 2004; Clemente et al., 2006; Gutierrez-de-Teran et al., 2006; Kim et al., 2006). Until recently, the recombinant expression of HAP has been unsuccessful due to insolvency of the enzyme (Berry et al., 1999; Banerjee et al., 2002); however, our group was the first to successfully express, and partially characterize, soluble recombinant HAP as a thioredoxin fusion protein from *E. coli* (Xiao et al., 2006). Expression of active recombinant PM I also has been a major challenge (Coombs et al., 2001; Ersmark et al., 2006). Luker et al. (1996) found that recombinant PM I did not resemble its native homolog kinetically and was unable to degrade hemoglobin due to its inability to autoactivate. Moon et al. (1997) obtained active plasmepsin I by introducing a Lys110P—Val (P indicates a propart residues) mutation into the propart of thezymogen, but found that the overall efficiency of refolding and autoactivation remained low. In a further study, Tyas et al. (1999) reported a 3–10-fold diminution in the *k*_cat values for substrate hydrolysis by this mutant, as compared with the native PM I. Although attempts to crystallize recombinant PM I have been made (Moon et al., 1997), no crystal structure has been reported to date. Furthermore, a large proportion of the inhibitors reported recently have not been
assessed regarding PM I inhibition due to the difficulties in obtaining active enzyme (Ersmark et al., 2006). In the present work, we report the successful expression and partial characterization of active recombinant PM I using the strategy to express HAP (Xiao et al., 2006).

Methods

Cloning of PM I and construction of expression plasmid

The method for cloning of plasmspin I (PM I) was similar to that used for HAP (Xiao et al., 2006). The PM I gene was isolated from the genomic DNA of *P. falciparum* strain 3D7 (MR4/American Type Culture Collection, Manassas, VA, USA) using polymerase chain reaction (PCR) with primers PMIFW (5’-AAGGATCCATGGCTTTATCAATTA AAC-3’) and PMIRV (5’-AATGGATCTCTTACATTTTTTG GCC-3’). The PCR products were cloned into vector pUC-19 to produce the plasmid construct pUC19–PM I. The sequence of the PM I gene was confirmed by sequencing with M13 primers using Dye Terminator Cycle Sequencing on an ABI PRISM Model 377 at the Guelph Molecular SuperCenter, University of Guelph. The transmembrane region of the propeptide was then deleted by amplification from pUC19-PM I using primer PMI-H227 (5’-CGGGATCCGAAACA TGTAATAATTGG-3’) and PMIRV to yield the truncated PM I (tPM I) (Lys77P–Leu329). The amplified fragment (tPM I) was digested with BamHI, and cloned into pET-32b (+) to give rise to PET32b–tPM I in which tPM I was fused to thioredoxin. The resulting construct plasmid encoded thioredoxin, followed by Tags including thrombin cleavage site, His-Tag, S-Tag and enterokinase cleavage site, and the truncated PM I (Lys77P–Leu329) (Fig. 1). The DNA sequence of the expression construct was confirmed by sequencing with T7 promoter and T7 terminator primers.

Expression of the fusion protein

*Escherichia coli* Rosetta-gami B (DE3)pLysS cells were transformed with the expression construct pET32b–tPM I.

**Fig. 1.** Schematic of PM I and the recombinant Trx-tPM I. The zymogen of PM I is a 51.4 kDa protein with its prosegment containing a transmembrane domain (line box), and processed to a mature form of 37 kDa (Gluzman et al., 1994). The cleavage site (indicated by a two-side arrow) and adjacent amino acids of the native protein (PM I) are shown. The fusion protein of the recombinant Trx-tPM consists of thioredoxin (Trx, 11.8 kDa), Tags (6 kDa) including thrombin cleavage site, His-Tag, S-Tag and enterokinase cleavage site, and truncated PM I with the removal of the first 76 amino residues (tPM I, K77P–L329, 42.5 kDa). The autocatalytic cleavage site (indicated by a two-side arrow) and adjacent amino acids of the recombinant tPM I are shown. The fusion protein, Trx-tPM I (simplified as F), the products produced during the autocatalysis, which are intermediate Trx-tPM I (I), zymogen of tPM I (Z), and the mature form of tPM I (M) and their sizes (kDa) are indicated with a dot-start arrow.

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 OD600 reached 1.0 at 37°C, and then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3–4 h at 30°C as suggested by Xiao et al. (2006). Cells were collected by centrifugation (2500 × g for 15 min).

Purification of the fusion protein

Cell pellets were resuspended in BugBusterTM 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 with anti-thioredoxin primary antibody (Invitrogen, Burlington, ON, Canada). The solubile fraction obtained above was applied on a HisSelect™ Cartridge (6.4 ml) (Sigma-Aldrich, Oakville, ON, Canada); the column was first washed with 50 mM sodium phosphate/0.3 M NaCl/10 mM imidazole pH 7.5 washing buffer and then with 10% elution buffer (50 mM sodium phosphate/0.3 M NaCl/250 mM imidazole pH 7.5) and finally eluted with 100% elution buffer. The sample was further purified by gel filtration with a Superose™ 12 10/300 GL (GE Healthcare, Uppsala, Sweden) column equilibrated with 50 mM sodium phosphate/150 mM NaCl pH 7.5 to obtain the fusion Trx-tPM I protein. Purification was monitored using SDS–PAGE and western blot with anti-PM I antibody (MR4/American Type Culture Collection, Manassas, VA, USA).

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

In order to determine the pH optimum for activation of PM I, 2 μg of purified thioredoxin-tPM I (Trx-tPM I) was incubated in 100 mM sodium acetate at various pH values (pH 3.5–6.5) at 37°C for 4 h. For the time course activation, 2 μg of Trx-tPM I was incubated in 100 mM sodium acetate, pH 4.5, for 15 min, 30 min, 1 h, 2 h and 4 h. To examine the effect of protease inhibitors on the autocatalytic processing of PM I, 2 μg of Trx-tPM I was incubated with or without the aspartic protease inhibitor pepstatin A (10 μM), cysteine protease inhibitor N-acetyl-Leu-Leu-norleucinal (PMSF, 1 mM) and serine protease inhibitors (100 μM) in 100 mM sodium acetate, pH 4.5, at 37°C for 4 h (Kim et al., 2006). The processing of PM I was detected by SDS–PAGE.

The activated sample at pH 4.5 was further purified by washing in an Ultracel YM50 Centricon (Millipore, Billerica, MA, USA) to remove the cleaved prosegment and thioredoxin to obtain mature tPM I (mtPM I). mtPM I protein was subjected to SDS–PAGE and electroblotted to a polyvinylidene difluoride (PVDF) membrane for N-terminal sequence analysis by the Advanced Protein Technology Center at the Hospital for Sick Children (Toronto, ON, Canada).

Protein concentration determinations

Enzyme and protein concentrations were determined in triplicate by the DC protein assay (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard (Lowry et al., 1951).
SDS–PAGE and Western blotting
SDS–PAGE was performed according to the method of Laemmli (1970) in a Mini-Protean II electrophoresis cell (Bio-Rad, Hercules, CA, USA). Protein samples were mixed with sample buffer in the presence of β-mercaptoethanol and SDS, heated for 5 min at 100°C, and loaded onto a 15% polyacrylamide gel with SDS. Gels were run at a constant voltage (200 V) for 1 h. The separated proteins were either stained with Coomassie blue or transferred onto PVDF membrane (Bio-Rad, Hercules, CA, USA) for western blotting according to the method of Towbin et al. (1979) using either anti-thioredoxin antibody or anti-PM I antibody. Electroblotting was carried out for 1 h at a constant voltage (100 V, 4°C) on a Mini Transblot electrophoretic transfer cell (Bio-Rad, Hercules, CA, USA).

Native PAGE
Native PAGE was carried out similar to SDS–PAGE except that the sample buffer contained neither SDS nor β-mercaptoethanol (samples were not heated), and the 10% polyacrylamide gel and electrophoresis solutions were prepared without SDS. Gels were run at a constant voltage (80 V) for 2.5 h. The gels were developed with GelCode Blue Stain Reagent (Pierce, Rockford, IL, USA).

Hemoglobin zymography
The hemoglobin zymography gel was prepared similar to native PAGE except 0.8 mg of human hemoglobin (dissolved in 100 mM acetic acid-HCl, pH 2.0) was co-polymerized in 5 ml of 10% polyacrylamide. After electrophoresis, the gels were incubated in 100 mM sodium acetate buffer (pH 2.8 or 5.0) at 37°C for 2.5 h, and then developed with GelCode Blue Stain Reagent.

pH optimum determination
The pH optimum of mtPM I was determined using the internally quenched fluorescent synthetic peptide substrate EDANS-CO-CH₂-CH₂-CO-Ala-Leu-Glu-Arg-Met-Phe-Leu-Ser-Phe-Pro-Dap-(DABCYL)-OH (2837b) with a cleavage site of Phe-Leu (AnaSpec Inc., San Jose, CA, USA), previously described by Istvan and Goldberg (2005), at various pH values between 2.0 and 6.5 in 100 mM sodium acetate buffers (pH 2.0–3.0 acetic acid solutions were adjusted with hydrochloric acid). 3 nM mtPM I (37.8 kDa) and 1.5 μM substrate were used for the assays performed at 25°C. The assays were carried out using a Victor 2 1420 Multilabel Counter (Perkin Elmer, Woodbridge, ON, Canada) with excitation at 335 nm and emission at 500 nm (Istvan and Goldberg, 2005).

Molecular modeling
The sequence of mature PM I was submitted to the Geno3D server at the Institute for the Biology and Chemistry of Proteins, University of Lyon, France (Combet et al., 2002) and a predicted molecular model was created. This model was then energy-minimized using the NAMD molecular modeling program (Kale et al., 1999). The calculation used topology force field data provided with the package, and was carried out in a water-filled box. The parameters were adjusted to a 15-Å cut-off distance and for 10 000 runs.

Hemoglobin degradation
In order to determine the hemoglobin degradation by PM I, 5 μg of human hemoglobin (Sigma-Aldrich, Oakville, ON, Canada) were incubated with 10 nM mtPM I in 100 mM sodium acetate at various pH values (pH 2.0–6.5, pH 2.0–3.0 acetic acid solutions were adjusted with hydrochloric acid) for 6 h. The effect of protease inhibitors on the hemoglobin degradation by PM I was assessed by incubating 5 μg of hemoglobin with 10 nM mtPM I in 100 mM sodium acetate (pH 2.8) in the presence or absence of pepstatin A (10 μM) or ALLN (15 μM) for 3 and 6 h at 37°C. The reaction was terminated by heating at 95°C in sample buffer containing 1.2% SDS. Hemoglobin degradation was detected by SDS–PAGE (Xiao et al., 2006).

Enzyme kinetic assays and inhibition studies
Kinetic parameters were determined using substrate 2837b as described above. The reaction was carried out at 25°C in 100 mM sodium acetate pH 2.8 and 5.0 using 3 nM mtPM I and 0.1 μM–4.0 μM substrate. The initial reaction rates were determined by calculating the slope of the linear portion of the curve (fluorescence/min). The observed fluorescence units were converted to micromoles per minute 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 (0.1, 1.0, 2.0 and 5.0 μM) (Xiao et al., 2006). Non-linear regression of the Michaelis-Menten model was used to derive $K_m$ and $k_{cat}$ which were calculated from $k_{cat} = V_{max} [E]$. The concentration of active enzyme [E] used in each assay was determined by active site titration with pepstatin A. Inhibition assays of mtPM I were done with pepstatin A at various concentrations (0.16–40 nM) and ALLN (5–160 μM). The reaction was carried out in 100 mM sodium acetate pH 2.8 and 5.0 using 3 nM mtPM I and 1.5 μM peptide substrate. $K_i$ values for pepstatin A and ALLN were determined with GraphPad Prism (Version 3.03) using the equation of Cheng and Prusoff (1973).

Results and discussion
Soluble expression of recombinant plasmepsin I in E. coli
Similar to the protocol used to express HAP (Xiao et al., 2006), a dual-approach was used for PM I where the truncated PM I (K77P–L329) combined with the deletion of the membrane-binding domain was fused with the highly soluble protein, thioredoxin (Trx) using pET-32b(+) vector and expressed in E. coli Rosetta-gami B (DE3)pLysS. As observed for recombinant HAP (Xiao et al., 2006), a small proportion of total tPM I was soluble in the cell lysate supernatant as illustrated in SDS–PAGE (Fig. 2A, lane 1) and the western blot with anti-thioredoxin antibody (Fig. 2B, lane 1). Eight percent of the total Trx-tPM I fusion protein was expressed in the soluble form as determined by densitometric analysis of a Coomassie stained SDS–PAGE gel using Syngene GeneToolsTM software (data not shown). In contrast, about 13% of the total fusion protein was expressed in a soluble form for Trx-hAP (Xiao et al., 2006) indicating that PM I was more difficult to express in vitro than HAP.

Purification of Trx-tPM I from the cell lysate supernatant was accomplished by FPLC using a combination of Ni²⁺
affinity and size exclusion chromatography (Fig. 2C and D).

The supernatant was initially applied on a HisSelect™ column on an AKTA FPLC system (GE Healthcare, Uppsala, Sweden). The elution was further purified by gel filtration with a Superose™ 12 10/300 GL column to obtain the fusion Trx-tPM I protein (Fig. 2C and D). The purification steps are shown from SDS–PAGE (Fig. 2C) and western blot using anti-PM I antibody (Fig. 2D) and summarized in Table I. This purification procedure produced 62 mg of Trx-tPM I fusion protein from 1 l of cell culture, which could be activated by acidification to the active mature form (Fig. 3).

Autocatalytic processing of PM I

The recombinant Trx-tPM I fusion protein was incubated in 100 mM buffer over the pH range 3.5–6.5 at 37°C for 4 h. The fusion protein (Trx-tPM I) underwent activation between pH 4.0 and 5.5 with a pH optimum of 4.5 (Fig. 3A, lane 4), as evidenced by the band shifting from 60.3 kDa (F) to 37.8 kDa (M), which is similar to the reported size of mature form of PM I (Banerjee et al., 2003). The pH optimum of activation was similar to that reported for non-recombinant native PM I, pH 4.0–5.5 (Banerjee et al., 2003). The time course study showed that hydrolysis for 2 h at 37°C at pH4.5 was sufficient to convert fusion protein to the mature form (Fig. 3B). There were intermediate products, intermediate Trx-tPM I (identified as I) and tPM I (identified as Z) produced during the activation (Fig. 3). N-terminal sequencing showed that a cleavage between Met and Asp in the region of the Tags (Fig. 1) yielded an intermediate Trx-tPM I (I) with a molecular mass of 44.5 kDa, and a cleavage between Phe83P and Ser84P which resulted in tPM I (Z) with a

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)a</th>
<th>Amount of Trx-tPM I (mg)b</th>
<th>Fold enrichment</th>
<th>Percentage yield (%)</th>
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<td>Supernatant</td>
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<td>2.56</td>
<td>1.00</td>
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<tr>
<td>Post-Ni2⁺ affinity</td>
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<td>0.179</td>
<td>19.4</td>
<td>7</td>
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<tr>
<td>Post gel filtration</td>
<td>0.075</td>
<td>0.062</td>
<td>82.8</td>
<td>2.4</td>
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aProtein concentrations were determined in triplicate by the DC protein Assay (BIO-RAD, Hercules CA, USA); bThe amount of the Trx-tPM I fusion protein was determined by densitometric analysis using Syngene GeneTools™ software from coomassie stained SDS–PAGE gel.

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

Fig. 2. Expression and purification of Trx-tPM I fusion protein from E.coli Rosetta-gami B (DE3)pLysS. (A) SDS–PAGE and (B) corresponding anti-thioredoxin western blot of expression cultures induced with IPTG. Lane 1, soluble fraction (25 μl, 1.2 μg/μl); lane 2, insoluble fraction (25 μl). (C) SDS–PAGE and (D) corresponding anti-PM I western blot for Trx-tPM I purification steps. Lane 1, lysate supernatant (30 μg); lane 2, post-Ni2⁺ affinity chromatography (15 μg); lane 3, post-size exclusion chromatography (5 μg). Lane M1, Page Rager™ Prestained Protein Ladder (Fermentas).

Fig. 3. SDS–PAGE on the processing of soluble recombinant tPM I. (A) Effects of pH on the processing of PM I. The recombinant fusion Trx-tPM I (2 μg) was incubated in 100 mM sodium acetate of various pH for 4 h at 37°C. Lane 1 (without incubation), lane 2 (pH 3.5), lane 3 (pH 4.0), lane 4 (pH 4.5), lane 5 (pH 5.0), lane 6 (pH 5.5), lane 7 (pH 6.0), lane 8 (pH 6.5). (B) Time course of the autoactivation of tPM I. The recombinant fusion Trx-tPM I (2 μg) was incubated in 100 mM sodium acetate (pH 4.5) at 37°C for 0 min (lane 1), 15 min (lane 2), 30 min (lane 3), 1 h (lane 4), 2 h (lane 5), and 4 h (lane 6). (C) Effects of protease inhibitors on the processing of PM I. The recombinant fusion Trx-tPM I (2 μg) was incubated with or without protease inhibitors in 100 mM sodium acetate, pH 4.5 for 3 h at 37°C. Lane 1 (without incubation), lane 2 (without inhibitor), lane 3 (pepsstatin A, 10 μM), lane 4 (ALLN, 15 μM), lane 5 (PMSF, 1 mM), lane 6 (leupeptin, 100 μM), lane 7 (DMSO, control). F, I, Z and M indicate the fusion protein, intermediate fusion protein, zymogen and mature form of tPM I, respectively, as shown in figure 1.
molecular mass of 41.8 kDa (Figs 1 and 3). It would appear that the activation process proceeded in three steps: from fusion protein (Trx-PM I) to intermediate Trx-tPM I (I), from intermediate Trx-tPM I (I) to truncated zymogen tPM I (Z) and finally from tPM I (Z) to mature PM I (M) (Figs 1 and 3).

After incubation at pH 4.5 for 4 h, the resultant protein was washed in an Ultracel YM50 Centricon to remove thioredoxin and the cleaved prosegment to obtain the mature enzyme (mtPM I) (Fig. 4A). N-terminal sequencing of the purified mtPM I (Fig. 4A) confirmed the identity of mtPM I and showed that autocatalytic cleavage occurred between amino acids Leu116P and Thr117P, seven residues upstream of the native cleavage site at Gly123P–Asn1 identified by Gluzman et al. (1994) (Fig. 1). Moon et al. (1997), in a study which mutated Lys110P to Val110P at the propart of the zymogen, reported that there were two autocatalytic cleavage sites for an activation site mutant of the PM I; the first one was 12 residues upstream of the native cleavage site and the second one is the same as reported in the present study. Tyas et al. (1999), who also mutated Lys110P→Val, reported only the first cleavage site.

In order to determine which protease inhibitors could prevent the autocatalytic processing of PM I, the Trx-tPM I fusion protein was incubated with various protease inhibitors in 100 mM sodium acetate (pH 4.5) for 4 h at 37°C. The results showed that the processing of PM I was completely inhibited by aspartic protease inhibitor pepstatin A (Fig. 3C, lane 3). The cysteine protease inhibitor ALLN showed some inhibition (Fig. 3C, lane 4), while the serine protease inhibitors PMSF and leupeptin were not inhibitory (Fig. 3C, lanes 5 and 6). It has been reported that in vivo, pepstatin A does not inhibit proplasmepsin processing, whereas ALLN completely inhibits proplasmepsin processing (Francis et al., 1997).

Fig. 4. Purified mature tPM I and its pH profile against synthetic peptide and hemoglobin substrate. (A) SDS–PAGE of purified mtPM I (1 μg). Lane M2, Protein Molecular Weight Marker (Fermentas). (B) pH dependence of mtPM I activity against synthetic peptide substrate. The mtPM I activity in 100 mM sodium acetate, pH 2.0–6.5, was determined using 3 nM mtPM I after activation at 37°C for 1 h and for 4 h and 1.5 μM peptide substrate 2837b. Percent activity is expressed relative to the highest observed activity at pH 5.0 for 1 h-activated mtPM I, averaged from triplicate determinations. (C) Effects of pH on the 4 h-activated-mtPM I degradation of human hemoglobin shown on SDS–PAGE gel. Five micrograms of hemoglobin were incubated with 10 nM of 4 h-activated-mtPM I in 100 mM sodium acetate of various pH indicated for 6 h at 37°C. Lane 1 (without mtPM I), lane 2 (pH 2.0), lane 3 (pH 2.8), lane 4 (pH 3.0), lane 5 (pH 3.5), lane 6 (pH 4.0), lane 7 (pH 4.5), lane 8 (pH 4.7), lane 9 (pH 5.0), lane 10 (pH 5.2), lane 11 (pH 5.5), lane 12 (pH 6.0) and lane 13 (pH 6.5). (D) Effects of pH on the 1 h-activated-mtPM I degradation of human hemoglobin shown on SDS–PAGE gel. Five microgram of hemoglobin were incubated with 10 nM of 1 h-activated-mtPM I in 100 mM sodium acetate of various pH indicated for 3 h at 37°C. Lane 1 (without mtPM I), lane 2 (pH 2.8), lane 3 (pH 3.0), lane 4 (pH 3.5), lane 5 (pH 4.0), lane 6 (pH 4.5), lane 7 (pH 4.7), lane 8 (pH 5.0), lane 9 (pH 5.2), lane 10 (pH 5.5), lane 11 (pH 6.0) and lane 12 (pH 6.5).
confirmed through further experiments. The activation of PM suggested that the monomer is more active at pH 5.0 while and a decrease at pH 2.8 (Fig. 5B). These observations lane 2, which coincided with an increase in activity at pH 5.0, increase in the amount of monomers as shown in Fig. 5A, reduced the amount of aggregates with a corresponding ment of mtPM I with 0.2% CHAPS, zwitterionic surfactant, that mtPM I contained both oligomeric (aggregates) and peak near the void volume and a peak eluting later suggesting exclusion chromatography data where there was both a broad native PAGE (Fig. 5A, lane 1), which was supported by size mtPM I consisted of two forms when it was analyzed using native-, SDS–PAGE and zymography. It was shown that the recombinant mtPM I had a different pH optimum for the native PM I, i.e. pH 4.5–5.0 (Goldberg et al., 1991) and to that of the food vacuole (~pH 5.0) (Yayon et al., 1984); however, it became less prominent when the time of autoactivation at 37°C was greater than 4 h (Fig. 4B). Hydrolysis of human hemoglobin with 4 h activated mtPM I confirmed that mtPM I effectively degraded the native hemo- globin in 6 h and the degradation was faster at low pH range (pH 2.8–4.0) (Fig. 4C). Interestingly, the 1 h-activated mtPM I also had maximal activity at pH 2.8–4.0 in the degradation of hemoglobin (Fig. 4D), although it showed high activity at pH 4.5–5.5 on the synthetic peptide substrate (Fig. 4B). This indicated that the recombinant mtPM I had a different pH optima of activity on the peptide and protein substrate.

The pH dependency of mtPM I was further examined with native-, SDS–PAGE and zymography. It was shown that mtPM I consisted of two forms when it was analyzed using native PAGE (Fig. 5A, lane 1), which was supported by size exclusion chromatography data where there was both a broad peak near the void volume and a peak eluting later suggesting that mtPM I contained both oligomeric (aggregates) and monomeric forms, respectively (data not shown). The treatment of mtPM I with 0.2% CHAPS, zwitterionic surfactant, reduced the amount of aggregates with a corresponding increase in the amount of monomers as shown in Fig. 5A, lane 2, which coincided with an increase in activity at pH 5.0 and a decrease at pH 2.8 (Fig. 5B). These observations suggested that the monomer is more active at pH 5.0 while the aggregates are more active at pH 2.8. This suggestion was confirmed through further experiments. The activation of PM I at 4°C (A1) and 37°C (A2) gave similar amounts of the mature form shown in the SDS–PAGE gel (Fig. 6A). However, A1 sample contained a higher amount of monomers in contrast to A2 sample as indicated in the Native PAGE (Fig. 6B). Correspondingly, the activity of A1 sample at pH 5.0 was more pronounced than that of A2 sample at pH 5.0 (Fig. 6E). Native PAGE also indicated that the higher activity of A1 at pH 2.8 as compared to A2 at pH 2.8 paralleled the higher amount of aggregates in A1 than that in A2 (Fig. 6B and E). In addition, A1, which had more monomers, showed a higher activity at pH 5.0 than at pH 2.8, while A2, which consisted of more aggregates, showed a higher activity at pH 2.8 than at pH 5.0 (Fig. 6B and E). The activity of the aggregate form of mtPM I was further illustrated using hemoglobin zymography, in which the aggregates in both A1 and A2 could degrade hemoglobin and the degradation was faster at pH 2.8 than at pH 5.0 (Fig. 6C). These results clearly indicated that the monomers and aggregates of PM I had a different pH optimum, with monomers at pH 4.5–5.5 and the aggregates at pH 2.5–3.0, and thus the observed dual pH optima described above resulted from the existence of two states of the mature enzymes (i.e., monomeric form and aggregate form). Different oligomeric states with different pH optimum have been reported for various enzymes (Campillo et al., 1981; Wong et al., 1987; Klyachko et al., 2005). For example, Campillo et al. (1981) found that the tetrameric α-galactosidase have a pH optimum of about pH 7.0, whereas the monomeric form display a pH optimum of 5.6.

Further analysis also revealed that the monomers and aggregates of PM I had different substrate specificities on synthetic peptide and human hemoglobin. As shown in Fig. 6D, both monomer-dominant A1 and aggregate-dominant A2 samples showed higher activities to human hemoglobin and BSA at pH 2.8 than at pH 5.0. If the monomeric form hydrolyzes hemoglobin efficiently, then the monomer-domi- nant A1 should degrade hemoglobin faster at pH 5.0 than at pH 2.8, since it exhibited higher activities towards the synthetic peptide 2837b at pH 5.0 compared to pH 2.8 (Fig. 6E, A1). However, the opposite was observed, i.e. the monomer-dominant A1 degraded hemoglobin faster at pH 2.8 than at pH 5.0. Therefore, it was suggested that the monomers and aggregates of PM I hydrolyze synthetic peptide and human hemoglobin differently. The aggregates were able to hydrolyze both proteins and peptides with similar efficiency while the monomers were more efficient at hydrolyzing the syn- thetic peptides as compared to proteins. It was reported that all plasmepsin structures determined, so far, are tightly associated homodimers (Asojo et al., 2003). The observation
that aggregates and monomers hydrolyze proteins and peptides with different efficiencies may have biological relevance in vivo.

The dual pH optima and the possible mechanism of soluble aggregates for mtPM I in hemoglobin degradation were further investigated by molecular modeling. The molecular modeling of PM I gave similar structural models to PM II and other aspartic proteases (Fig. 7) (Asojo et al., 2003). The examination of the structure models of PM-I and other aspartic protease models revealed that the active site

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**Fig. 6.** Substrate specificities of mature tPM I. Thirty micrograms of fusion protein (trx-tPM I) in 100 μl of 100 mM sodium acetate (pH 4.5) was incubated at 4 °C (A1) overnight or 37 °C (A2) overnight. (A) SDS–PAGE of A1 and A2. Twenty microliters of the samples were loaded into the 15% acrylamide gel. Lane M2, Protein Molecular Weight Marker (Fermentas). (B) Native PAGE of A1 and A2. Twenty microliter of the samples was loaded into the 10% acrylamide gel. MF and AF indicate monomeric form and aggregate form of mtPM I, respectively. (C) Hemoglobin zymogram. Twenty microliters of A1 and A2 samples were loaded into the 10% acrylamide gel with hemoglobin. After electrophoresis, the gels were incubated at pH 2.8 or pH 5.0. (D) SDS–PAGE gel showing the A1 and A2 mtPM I degradation of human hemoglobin and bovine serum albumin (BSA). Ten micrograms of hemoglobin or BSA were incubated with 1 μl of A1 or A2 in 25 μl of 100 mM sodium acetate (pH 2.8 or pH 5.0) at 37 °C for 2.5 h. CK indicates human hemoglobin without addition of enzyme. (E) Activity assay of A1 and A2 mtPM I (0.5 μl) against synthetic peptide 2837b at pH 2.8 and pH 5.0. Percent activity is expressed relative to the highest observed activity of A1 at pH 5.0, averaged from triplicate determinations.

**Fig. 7.** Superposition of a calculated PM-I model on PM-II (PDB: 1IL3). Yellow ribbon is PM-II crystal model, and blue ribbon is PM-I.
clefts have little difference in the residues that can be affected by pH changes around pH 4.0, i.e. Asp or Glu. There is an Asp residue at position 303 near the active centre, Asp34 and Asp214. However, Asp303 was conserved among the most aspartic proteases that exhibit single pH optima (Fruton, 1971). In other words, Asp303 is not likely to be the cause of the double pH optima observed in this PM I. According to Asojo et al. (2003), PM II has a unique and close relation between two monomers in the crystals that showed an unusual P2 space group. They suggested that PM II has the ability to form a dimeric structure in the solution unlike other aspartic proteases, which suggests that PM I can form a dimeric structure similar to PM II since a BLAST search showed that PM I shares 80% amino acid sequence homology with other plasmepsins (PM II, PM IV, HAP) while only 52% or less with the most of other aspartic proteases (such as rennin, cathepsin D). The model of PM I was, therefore, examined to see if dimer formation can be affected by pH of the environment. The assumed interface of two monomers had two pairs of salt bridges in PM I (Fig. 8) whereas PM II (PDB: 1IL3) had one pair. Asp107–Lys238 existed in both enzymes and this Lys238 is on the flexible loop that protrudes into the active site cleft of the other monomer. This flexible loop would have the similar local structures between PM I and PM II. The other pair, Lys53–Asp279 was observed only in PM I. Between pH 2.0 and 4.0, which is below the pK\text{a} of free Asp (pH 3.9), Asp279 is likely protonated and loses the ability to form the salt bridge. As a result, the mode of interaction changes from what exists in the monomer–monomer interaction in the pH 4.0–6.0 range leaving Lys53 charged without pairing to a neutralizing residue, i.e. inability to form a salt bridge with another Asp or Glu which are abundant on the surface of PM I. We speculate that this situation will initiate the aggregation of PM I in the pH 2.0–4.0 range. The formation of salt bridges among aggregated monomers is consistent with the observation in the present study where CHAPS treatment reduced the amount of aggregated monomers (Fig. 5A). This formation of aggregates would involve the change of environments of active site clefts in the aggregated PM I and result in the pH optima shift from pH 5.0 to pH 2.8.

The degradation of hemoglobin by mtPM I could be inhibited by pepstatin A, but not by ALLN (Fig. 9). Kim et al. (2006) reported that hemoglobin degradation by PM II or IV was completely inhibited by 10 \mu M pepstatin A, and PM II-mediated hemoglobin degradation was partially inhibited by 15 \mu M ALLN, while the same concentration of ALLN completely blocked hemoglobin degradation by PM IV. The above results indicate that homologous plasmepsins have different affinities for ALLN.

**Kinetic analysis of mature PM I**

Kinetic parameters of mtPM I for cleavage of the Phe33–Leu34 peptide bond in \(\alpha\)-globin at the optimum pH of 2.8 and 5.0 were determined using the quenched fluorescent peptide substrate 2837b previously used for the kinetic characterization of PM II (Istvan and Goldberg, 2005) and HAP (Xiao et al., 2006). The amount of active mtPM I was titrated with pepstatin A, and the kinetic data (Table II) were determined using the quenched fluorescent peptide substrate 2837b previously used for the kinetic characterization of PM II (Istvan and Goldberg, 2005) and HAP (Xiao et al., 2006).

<table>
<thead>
<tr>
<th>Condition</th>
<th>(K_m) (\mu M)</th>
<th>(k_{cat}) (s(^{-1}))</th>
<th>(k_{cat}/K_m) (\mu M(^{-1})s(^{-1}))</th>
<th>Pepstatin (K_i) (pM)</th>
<th>ALLN (K_i) (\mu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mtPM I pH2.8</td>
<td>0.87 ± 0.27</td>
<td>1.7</td>
<td>1.95</td>
<td>11.7</td>
<td>2.42</td>
</tr>
<tr>
<td>mtPM I pH5.0</td>
<td>2.02 ± 0.1</td>
<td>1.24</td>
<td>0.61</td>
<td>69.9</td>
<td>6.82</td>
</tr>
<tr>
<td>Native PM I*</td>
<td>0.49 ± 0.12</td>
<td>2.3</td>
<td>4.7</td>
<td>1.2</td>
<td>—</td>
</tr>
</tbody>
</table>

*From Luker et al. (1996).*
Expression of soluble recombinant plasmepsin I

compared with data reported for native PM I from Luker et al. (1996), who also used the similar quenched fluorescent peptide substrate. mtPM I had a 1.3-fold higher $K_m$ and 0.3-fold lower $k_{cat}$ at pH 5.0 than at pH 2.8, which was 69% less effective in the hydrolysis of the peptide substrate at pH 5.0 than at pH 2.8. mtPM I at pH 2.8 had a $K_m$ of 0.87 μM and a $k_{cat}$ of 1.7 s⁻¹ that were comparable to the kinetic parameters observed for native PM I at pH 5.0 (Table II) (Luker et al., 1996). mtPM I bound pepstatin A was similar to native PM I. However, as compared to pepstatin A, mtPM I had a very low affinity for ALLN, which may explain why the degradation of native hemoglobin was not inhibited by ALLN (15 μM) (Fig. 9).

In conclusion, the above results showed that the properties of recombinant PM I were generally comparable to native PM I (Table II, Luker et al., 1996). The finding that the aggregates of the recombinant PM I was active is significant with a relatively easy purification and produces sufficient amounts of protein, will undoubtedly allow for comprehensive structure-function studies of PM I to be undertaken which may lead to the potential development of specific inhibitors.

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References

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