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Expression and Characterization of Catalytic Domain of *Plasmodium falciparum* Subtilisin-like Protease 3

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Abstract

PfSUB3 is the third subtilisin-like protease annotated in *Plasmodium* genome database “PlasmoDB”. The other two members, PfSUB1 and PfSUB2 have been implicated in merozoite egress and invasion in asexual blood stages. In this study, we recombinantly expressed a region of PfSUB3 spanning from Asn³³⁴ to Glu⁷⁶⁹ (PfSUB3c) which encompassed the predicted catalytic domain with all the active site residues and predicted mature region spanning from Thr⁵¹⁶ to Glu⁷⁶⁹ (PfSUB3m) in *E. coli*. PfSUB3m showed PMSF-sensitive proteolytic activity in *in vitro* assays. Replacement of active site serine with alanine in PfSUB3m resulted in inactive protein. We found that PfSUB3c and PfSUB3m undergo truncation to produce a 25-kDa species which was sufficient for proteolytic activity. Quantitative real-time PCR, immunofluorescence assay and Western blot analyses revealed that PfSUB3 is expressed at late asexual blood stages. Serine protease activity of PfSUB3 and its expression in the late stages of erythrocytic schizogony are indicative of some possible role of the protease in merozoite egress and/or invasion processes.

Keywords

Plasmodium falciparum; serine protease; subtilisin-like protease; erythrocytic schizogony

Short Communication

Malaria is a global burden for human health and causes nearly 500 million cases and 1-2 million deaths every year [1]. Among human malaria parasites, *Plasmodium falciparum* causes the most severe form of malaria and accounts for most of the malaria-related deaths. Drug resistance in malaria parasites and toxicity associated with available antimalarials necessitate the discovery of novel drug targets and chemotherapeutics against the disease [2]. Proteases are attractive targets for drug development against malaria as they are involved in many parasite-specific processes including hemoglobin digestion, merozoite egress and invasion of fresh RBCs during blood stages [2-5]. *P. falciparum* subtilisin-like protease 3 (PfSUB3) is one of three subtilisin-like proteases of *P. falciparum* annotated in

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Plasmodium genome database “PlasmoDB” [6-8]; the other two members PfSUB1 and PfSUB2 have been well characterized and known to have roles in egress and invasion.

Both PfSUB1 and PfSUB2 are synthesized as preproteases and undergo posttranslational maturation. PfSUB1 is a 77.6-kDa protein which when expressed in insect cells undergoes truncation to yield a 54-kDa catalytically active form [9]. PfSUB2 is a micronemal, 154.8-kDa type I integral membrane protein [10] and also undergoes posttranslational proteolytic processing [11, 12]. PfSUB1 is a multifunctional protease, secreted in ‘exonemes’ an apical organelle with the morphology of dense granules in the parasite [13] and has roles in merozoite egress [13, 14] and invasion [15] in blood stages. A recent work has suggested a variety of novel substrates of PfSUB1, hence validating its potential as a promising drug target [16]. PfSUB2 has not been expressed in recombinant proteolytically active form, but it has been shown to carry out the secondary proteolytic processing of merozoite surface protein 1 (MSP1) and apical membrane antigen 1 (AMA1) which are adhesions involved in merozoite invasion of RBCs [17, 18]. PfSUB3 is encoded by a 2307-bp single exon open reading frame and possesses subtilase domain at its C-terminus [7]. Microarray studies have suggested that PfSUB3 is expressed at blood stages but its expression is not restricted to blood stages with significant amount of expression in sporozoites and gametocytes [19]. In this study, we have characterized PfSUB3 with respect to its proteolytic activity and localization in asexual erythrocytic stages of *P. falciparum*.

Locations of catalytic domains of PfSUB3 and its *Plasmodium* homologs were determined by Pfam server (<http://www.pfam.sanger.ac.uk>). Sequence alignment of catalytic domain of PfSUB3 with the homologs by bl2seq program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) revealed significant sequence conservation (Table S1). Alignment of catalytic domains by ClustalW (<http://www.ebi.ac.uk/clustalw/>) and positions of predicted catalytic residues is shown in figure S1. Besides, we predicted the mature region in PfSUB3 by CPHmodels-3.0 server (<http://www.cbs.dtu.dk/services/CPHmodels/>) which was found to span from Asp⁵¹⁹ to Phe⁷⁵⁸ by taking *Bacillus* Ak. 1 protease (PDB ID: 1DBI) as template which had 29% identity with PfSUB3. Based on these analyses, the regions spanning from Asn³³⁴ to Glu⁷⁶⁹ (referred as catalytic domain or PfSUB3c) and Thr⁵¹⁶ to Glu⁷⁶⁹ (referred as mature protein or PfSUB3m) were selected for expression in *E. coli*.

PfSUB3m and PfSUB3c were expressed in *E. coli* with N- and C-terminal hexahistidine tags respectively and their identities were confirmed by mass spectrometry and sequencing of tryptic peptides (Table S2, S3 and S4 and Fig. S2). PfSUB3m, expressed in *E. coli* BL21(DE3) cells was purified by Ni-NTA chromatography after refolding on the column which migrated at its expected position on SDS-PAGE gel (Fig. 1A). Soon after purification, PfSUB3m started to undergo cleavage at the N-terminal to produce a truncated polypeptide that migrated at ~25 kDa on SDS-PAGE gel (termed as PfSUB3mt) (Fig. 1B). Ni-NTA eluate containing both the polypeptides, when probed with anti-hexahistidine antibody recognized intact PfSUB3m but not the truncated polypeptide, suggesting that the truncation took place near the N-terminus of PfSUB3m (Fig. S4A). When attempted to purify the protein by gel-permeation chromatography, PfSUB3m underwent complete degradation during purification in buffers of pH 7.5 and 8.0 and large amounts of smaller fragments were seen late in the chromatogram (not shown). However, at pH 8.5, we were able to purify the protein but it was present in the truncated 25-kDa form (Fig. S3). In the chromatogram, large amount of late eluting degradation products were also seen which were supposed to be generated by the degradation of the protein (not shown). We generated an active site mutant of PfSUB3m, in which the active site serine-701 was replaced with alanine by site-directed mutagenesis. The mutant (referred as “PfSUB3m-S701A”) was also expressed in *E. coli* BL21(DE3) cells and purified after refolding as described for PfSUB3m. Unlike PfSUB3m, the mutant was found to be stable and seen as a single

polypeptide after purification (Fig. 1C). PfSUB3c, when expressed in *E. coli* KRX cells with C-terminal hexahistidine tag underwent truncation to produce a smaller fragment of ~25 kDa which was detectable with anti-hexahistidine antibody in induced cells (Fig. S4B). The expressed protein accumulated both in soluble and insoluble fractions. Upon purification from insoluble fraction after column refolding, a C-terminal 25-kDa fragment of PfSUB3c (PfSUB3ct) was co-purified along with the full-length PfSUB3c (Fig. 1D). Both the polypeptides were found to be PfSUB3 fragments as confirmed by sequencing of tryptic peptides by tandem mass spectrometry (Table S3). Upon purification under soluble conditions, the C-terminal 25-kDa fragment of PfSUB3 was predominantly eluted from Ni-NTA column (Fig. 1E) whose identity was confirmed by sequencing of tryptic peptides by tandem mass spectrometry (Table S4). This may be due to the autocatalytic truncation after activation of the protease after refolding which could be a probable maturation event.

Freshly purified PfSUB3m along with a fraction of its truncated product “PfSUB3mt” underwent autolysis when incubated at 37°C for prolonged time (16 hours) as evident by the loss of intensities of the protein bands on silver-stained SDS-PAGE gel which was abrogated by serine protease inhibitor, PMSF (Fig. 2A). Similarly, truncated form of PfSUB3m purified by size exclusion chromatography (PfSUB3mt) when incubated at 37°C also showed autolysis in a time-dependent manner as evident by the loss of the intensities of bands of PfSUB3mt and its degradation product on silver stained SDS-PAGE gel (Fig. 2B). This activity was inhibited by PMSF in a dose-dependent manner as evident by the intensities of the bands of the protein and its stabilized degradation product (Fig. 2C). The active site mutant, PfSUB3m-S701A was found to be inactive as it was stable upon incubation at different time intervals up to 12 hours at 37°C (Fig. 2D) confirming that the autocatalytic activity was specific to the protease and not due to some contaminating *E. coli* protease. Proteolytic activity of PfSUB3m (with a fraction of PfSUB3mt) was further assessed on general protease substrates, azocasein and azoalbumin [20]. Dose-dependent activity was seen on both the substrates as determined by the absorbance at 440 nm as a measure of the released azo dye upon hydrolysis of peptide bonds. PfSUB3m-S701A, on the other hand showed no activity in these assays (Fig. 2E and 2F). Besides, the 25-kDa polypeptide generated by the truncation of PfSUB3c (PfSUB3ct) purified from soluble fraction also showed dose-dependent activity on azocasein which was abrogated by PMSF (Fig. 2G). Although the C-terminal 25 kDa region of PfSUB3c was active on azocasein, the full-length PfSUB3c purified after column refolding was inactive which may be due to improper folding of PfSUB3c. There is also the probability that PfSUB3c is a precursor form whereas the 25-kDa C-terminal region is the mature proteolytically active form. These results demonstrated that PfSUB3 serine protease activity resides in its C-terminal region spanning from Thr⁵¹⁶ to Glu⁷⁶⁹ and serine-701 is the active site serine. Proteolytic activity of PfSUB3 on two different general protease substrates, azocasein and azoalbumin was indicative of broad substrate range of the protease, like its closely related subtilase, PfSUB1 which is known to be a multifunctional enzyme that could cleave a variety of parasite proteins [16]. Location of the active serine was determined by alignment with known bacterial and eukaryotic subtilisins. The signature sequence SSGTSFSASV (active site serine is shown in bold) was in agreement with the active site consensus region as found in many bacterial and eukaryotic subtilisins [6]. In case of subtilases, the active site residues are arranged in the order of Asp, His and Ser which constitute the triad at the active site. The active site residues for PfSUB3 described were Asp³⁷², His⁵²³ and Ser⁷⁰¹ and hence the active protease should have all these residues. However, in contrast to this, our findings have demonstrated that the region of the protein lacking the predicted aspartate (25-kDa C-terminal region) was still proteolytically active. Although the position of the active site serine was confirmed by our mutagenesis study, positions of active site aspartate could not be confirmed. However, in PfSUB3 a few unusual features were evident in predicted triad residues. The distance between aspartate and histidine was unusually long (Thr³⁷³ to Gly⁵²²)

when compared to other subtilases from bacterial and eukaryotic origin [6]. Similarly, the presence of phenylalanine in the signature sequence of active site histidine (shown in bold) **HGHGTFIAG** was also an unusual feature. With the present available information, although the positions of the catalytic triad aspartate and histidine remain unclear; our findings suggest that region of the protease from Thr⁵¹⁶ to Glu⁷⁶⁹ possesses proteolytic activity.

Expression of PfSUB3 at different asexual erythrocytic stages was determined by quantitative real-time PCR, immunofluorescence assay and Western blotting. Transcript abundance of PfSUB3 gene was determined at ring, trophozoite and late schizont stages by quantitative real-time PCR relative to the transcripts of 18S rRNA at these stages. Highest level of expression was found at schizont stage with the ratio of transcripts at schizont, trophozoite and ring stages as 1:0.724:0.0934. In immunofluorescence assays, different asexual blood stages were probed with anti-PfSUB3 antibody. Cy3-labelled anti-mouse IgG was used as secondary antibody and nuclei were counterstained with DAPI. PfSUB3 was detected in schizont stage parasites but not in ring and trophozoite stage parasites suggesting some role in late asexual blood stages. No signal was seen with preimmune antibody (Fig. 3A). To check the release of the protease in the culture medium, supernatants of *P. falciparum* culture at late schizonts and mature trophozoite stages were probed with anti-PfSUB3 antibody which detected major bands corresponding to the mature protein and its probable degradation product in the supernatant of schizont stage parasite but no signal was detected in that of trophozoite stage parasites (Fig. 3B). These results suggest that PfSUB3 is mainly expressed at late asexual blood stages and undergoes proteolytic processing. Similarly, PfSUB1, which is known to have roles in egress is secreted in the parasitophorous vacuole and released in the culture supernatant following egress in processed form [13]. Detection of PfSUB3 in schizonts and culture supernatant of schizont stage parasites may be taken to suggest its possible role in egress and/or invasion processes, however its essentiality for parasite survival and biological substrates are not known. To functionally characterize PfSUB3, search for its probable biological substrate(s) is the focus of study in our laboratory. In this study we have demonstrated that PfSUB3, the third *P. falciparum* subtilisin-like protease possesses serine protease activity in its C-terminal region spanning from Thr⁵¹⁶ to Glu⁷⁶⁹. During asexual blood stages it is maximally expressed at schizont stage and shed in culture medium after schizont rupture. These results suggest some probable role of the protease in the late stages of erythrocytic schizogony, which would require further investigations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

PMSF	phenylmethylsulfonyl fluoride
Ni-NTA	nickel-nitrilotriacetic acid

MALDI-MS matrix-assisted laser desorption ionization
MS mass spectrometry

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Figure 1. Purification of PfSUB3m and PfSUB3c

(A) Coomassie blue-stained SDS-PAGE gel showing the Ni-NTA purified PfSUB3m. (B) Coomassie blue-stained SDS-PAGE gel showing PfSUB3m and its truncated product (PfSUB3mt). (C) Coomassie blue-stained SDS-PAGE gel showing the Ni-NTA purified PfSUB3m-S701A. (D) Coomassie blue-stained SDS-PAGE gel showing the Ni-NTA purified PfSUB3c and its truncated product (PfSUB3ct) after column refolding (E) Coomassie-stained SDS-PAGE gel showing the Ni-NTA purified PfSUB3c under native conditions. A major 25-kDa truncated product of PfSUB3c (PfSUB3ct) along with some degradation products was seen.

Figure 2. Serine protease activity of PfSUB3

(A) Autolytic activity of PfSUB3m. Equal amount of protein was incubated at 37°C for 16 hours with and without PMSF and analyzed by SDS-PAGE and silver staining. Autolysis was inhibited in presence of PMSF. (B) Autolytic activity of PfSUB3mt. Time-dependent autolysis of PfSUB3mt was seen upon incubation of equal amount of protein at 37°C for different time intervals when analyzed by SDS-PAGE and silver staining. Lanes 1, 2, 3, 4 and 5 µg protein incubated for 1 hour, 3 hours, 5 hours, 7 hours and 18 hours respectively. Positions of PfSUB3mt and its degradation product are marked by asterisks. (C) Inhibition of PfSUB3mt autolysis by PMSF. Equal amount of protein was incubated at 37°C for 3 hours with and without PMSF and analyzed by SDS-PAGE and silver staining. Lanes 1, 2,

3, 4, 5 and 6 correspond to protein incubated without PMSF and with 1 mM, 2 mM, 3 mM, 4 mM and 5 mM PMSF respectively. Dose-dependent inhibition of autolysis was seen with increasing concentrations of PMSF. Positions of PfSUB3mt and its degradation product are marked by asterisks. (D) Stability checking of PfSUB3m-S701A. PfSUB3m-S701A was found to be stable towards autolysis upon incubation at 37°C for different time intervals up to 12 hours when analyzed by SDS-PAGE and silver staining along with zero hour control. (E) Proteolytic activity of PfSUB3 on chromogenic substrate, azocasein. Dose-dependent proteolytic activity of PfSUB3m was seen. No activity was seen in case of PfSUB3m-S701A. (F) Proteolytic activity of PfSUB3 on chromogenic substrate, azoalbumin. Dose-dependent proteolytic activity of PfSUB3m was seen. No activity was seen in case of PfSUB3m-S701A. (G) Proteolytic activity of PfSUB3ct on azocasein. Dose-dependent activity was seen with increasing amount of enzyme which was completely abrogated by 1 mM PMSF.

Figure 3. Localization of PfSUB3 at asexual blood stages

(A) Localization of PfSUB3 in the parasite by immunofluorescence assay. Immunofluorescent staining of ring, trophozoite and schizont stage parasites with anti-PfSUB3 antibody. Cy3-labeled anti-mouse IgG was used as secondary antibody and nuclei were counterstained with DAPI. Cy3 fluorescence signals were seen in schizont stage parasites. No signal was seen in ring and trophozoite stage parasites. Similar immunofluorescent staining of schizonts with preimmune antibody did not show any fluorescent signals. (B) Localization of PfSUB3 in culture supernatant (CS) by Western blotting with anti-PfSUB3 antibody. Signals corresponding to various sizes were seen with major bands corresponding to the size of the predicted mature protein and its probable degradation product (marked by asterisks) in the CS of schizont stage parasite. No signal was seen in CS of trophozoite stage parasites.