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Preliminary Studies on the Expression and Purification of Functionally Active Recombinant Plasmepsin 9 from *Plasmodium falciparum*

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Author's contribution

Only author FMO designed the study, performed the work, analyzed the results and wrote the first draft of the manuscript. The author read and approved the final manuscript.

Research Article

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ABSTRACT

Aim: Plasmepsins, a group of homologous aspartic proteinases are attractive drug targets against malaria. Plasmepsin 9 (PM9) expressed in the blood stage of malaria life cycle with unknown function has been strongly considered as a potential target. However, recombinant expression of active PM9 for biochemical and structure-activity analysis of the enzyme has been very challenging. This paper presents preliminary report on the expression and purification of active recombinant plasmepsin 9 from *Plasmodium falciparum*.
Place and Duration of Study: Department of Biochemistry and Molecular Biology, College of Medicine, University of Florida, Gainesville, Florida between November 2011 and October, 2012.
Methodology: The plasmid of truncated proenzyme (pro*Pf*PM9) from *Plasmodium falciparum* was expressed in *Escherichia coli* Rosetta 2(DE3)pLysS competent cells. The protein was purified from inclusion bodies using combination of cation exchange and gel filtration chromatography. The expression and purification fractions were subjected to SDS-PAGE. The zymogen was activated to produce mature form of active recombinant P*f*PM9. Catalytic activity test of P*f*PM9 was determined using a chromogenic substrate, Lys-Pro-Ile-Glu-Phe-Nph*Arg-Leu (RS6). Substrate hydrolysis was examined from

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substrate cleavage scan.
Results: SDS-PAGE confirmed the expression and purification of truncated proenzyme (pro*Pf*PM9) with molecular weight of approximately 54 kD. Kinetic assays showed that the purified P*f*PM9 was active. The hydrolytic activity of P*f*PM9 on RS6 was confirmed by a shift in absorbance peak from 280 nm to 272 nm at the end of assay reactions. Inhibition studies on the mature *PfPM9* showed that the activity of the enzyme was inhibited by pepstatin A.
Conclusion: Protocols for successful expression and purification of active recombinant plasmepsin 9 in *E. coli* have been established in this preliminary report. These will aid production of sufficient yield of P*f*PM9 for detailed kinetic and structural characterization of the enzyme and development of future inhibitors against malaria.

Keywords: Expression; malaria; plasmepsin 9; Plasmodium falciparum; purification; recombinant.

1. INTRODUCTION

Malaria remains the most prevalent life threatening disease of global significance and a major cause of high infant mortality in over hundred endemic nations across the globe. There are several hundred million clinical cases of malaria annually resulting in about one million deaths, primarily, children [1,2]. Parasites of the genus *plasmodium* cause the disease by degrading human hemoglobin as a source of amino acids for their growth and maturation and the disease is transmitted by *Anopheles* mosquitoes [3]. Of the four human species, *P. falciparum* is by far the most deadly form and is responsible for the majority of malaria related deaths [4,5]. Malaria prevention and treatment currently relies on vector control and chemotherapy. The spread of parasite-drug resistance, particularly to inexpensive drugs, is thus a significant concern. Chloroquine resistance in the most pathogenic species, *P. falciparum*, is now almost universal and the declining efficacy of affordable antimalarial drugs, such as sulfadoxine-pyrimethamine is well documented [6]. Hence, novel anti-malarial drug targets are urgently needed to battle these variants.

Hemoglobin degradation is initiated by plasmepsins (PMs), a sub-group of 10 aspartyl proteases defined in the *P. falciparum* genome [4], which have been strongly considered as potential anti-malaria drug targets. The PM proteins were initially discovered by identifying enzymes from the hemoglobin digestion pathway [7]. Four PMs (PM1, PM2 and PM4 and HAP) are expressed in the erythrocytic stages of the lifecycle of *P. falciparum* [8-10] and are localized in the food vacuole [11]. PM5 is an integral membrane protein of the endoplasmic reticulum suggesting a role in protein processing within the parasite [12]. The functions of the remaining PMs 6–10 are unknown but the expression of PMs 5, 9 and 10 occurs in the blood stages of the malaria life cycle [9-11]. Plm5, Plm9 and Plm10 are expressed concurrently with PM1 to PM4 but are not transported to the digestive vaculole (DV). Recently, it has been reported that PM5 licenses *Pf* proteins for export into the host erythrocyte, therefore, it is essential for parasite viability [13]. The remaining PMs (PM6, PM7, PM8) are not expressed during the intraerythrocytic stage [11].

For several years, the structure-based drug design of antimalarial compounds targeting *P. falciparum* DV plasmepsin inhibition has received much attention due to their potential biomedical use [14]. However, a recent study showed that a wide range of previously characterized aspartic protease inhibitors exert their antimalarial activities primarily on one or

more non-DV plasmepsins and secondarily on the DV plasmepsins [15]. This finding indicates the relevance in the intraerythrocytic stage of the non-DV plasmepsins as PM5 [13], PM9, and PM10 could be novel drug targets against malaria although their structures have not been solved yet. Up to date, there is scarcity of information on expression and purification of functionally active PM9 and PM10 which is an important prerequisite for proteins to be crystallized and structures determined through X-ray crystallography [16].

Heterologous expression of recombinant plasmodial proteins in *E. coli* is fraught with difficulties as reflected by the paucity of plasmodial protein structures in the Protein Data Bank (PDB, ~250), when compared to the approximately 50,000 protein structures available in the PDB [17]. A variety of factors contribute to make the heterologous expression of *P. falciparum* proteins challenging, some of which include higher molecular weight (> 56 kDa), greater protein disorder, more basic isoelectric point (pI), and lack of homology to *E. coli* proteins) [18]. The full sequence of proenzyme *Pf*PM9 is made up of 627 amino acids long with molecular weight of 74.18 kDa (UniProtKB/TrEMBL accession number: Q8ILG2) [19], which is responsible for the hurdles encountered in expressing it in the full sequence form.

Although, the production of active recombinant *Pf*PM9 has been very challenging, this manuscript reports for the first time, the expression of a truncated proenzyme (pro*Pf*PM9) from *P. falciparum* in *E. coli* and the activation of the zymogen to produce an active recombinant (*Pf*PM9).

2. MATERIALS AND METHODS

2.1 Bacterial Strains and Plasmids

E. coli strains used in this study were Escherichia coli Rosetta 2 (DE3) pLysS Competent Cells and pET3a plasmids were purchased from Novagen. Antibiotics were from Sigma and electrophoresis reagents were obtained from BioRad.

2.2 Expression of Pro *Pf*PM9

A truncated version of proenzyme *Pf*PM9 (pro*Pf*PM9) was provided as earlier done for *Pf*PM1 and *Pf*PM4 [23, 24]. The plasmids were transformed into *Escherichia coli* Rosetta 2 (DE3)pLysS competent cells (Novagen). Cultures of transformed *E.coli* strain Rosetta 2 (DE3)pLysS harbouring the recombinant pET3a plasmids were grown overnight at 37ºC in fresh Luria-Bertani (LB) medium overnight in a shaking incubator at 200 rpm. The culture was used to inoculate fresh 1 L LB medium (2% inoculation) containing ampicillin (50 µg/ml) and chloramphenicol (34 µg /ml) as selection antibiotics. This was incubated at 37ºC, 200 rpm until the OD_{600} reached 0.7 (exponential growth phase of cells). Expression was induced by adding 1 ml of 1M isopropyl β-D-1-thiogalactopyranoside (IPTG) to the 1 L LB expression culture (final concentration of 1 mM). This was incubated at 37ºC, 200 rpm for 3 hours. Cells were harvested by centrifugation at 4000 rpm for 20 minutes at 4ºC.

2.3 Inclusion Bodies Extraction and Preparation

Cell pellets were thawn on ice and suspended in ice cold buffer 1 (0.01 M Tris-HCl, pH 8.0 0.02 M $MgCl_2$, 0.005 M $CaCl_2$) at 4.2 ml/g wet cell pellet. Cells were lysed via French Pressure Cell at 1000 psi for 4 times keeping the lysed cells on ice. DNAse 1 was added to

lysed cell suspension to a final concentration of 80 units/ml. The suspension was gently swirled and left at room temperature for 20 minutes. 10 ml of lysate was laid on 10 ml of 27% (w/v) sucrose solution in 30 ml Corex tubes and centrifuged at 12000g, 4ºC for 45 minutes. An aliquot of supernatant was saved for SDS-PAGE. Each pellet obtained was suspended in 10 ml of buffer 2 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 2 mM β-mercaptoethanol (BME), 100 mM NaCl). The suspension was again laid on 10 ml of 27% (w/v) sucrose solution in 30 ml Corex tubes and centrifuged at 12000g, 4ºC for 45 minutes. An aliquot of supernatant was saved for SDS-PAGE. Each pellet obtained was suspended in 10 ml buffer 3 (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 2.5 mM BME, 0.5% Triton X-100). Suspension was centrifuged at 12 000g, 4ºC for 15 minutes. An aliquot of supernatant was stored for SDS-PAGE. Pellet was suspended in buffer 4 (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 2.5 mM BME). Suspension was transferred into pre-weighed centrifuge tubes and spun at 12000g, 4ºC for 15 minutes. An aliquot of supernatant was again stored for SDS-PAGE. The weight of the wet inclusion bodies (IB) pellet was determined. IB pellet was suspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) to a final concentration of 100 mg/ml and stored at –80ºC until ready for use.

2.4 Refolding and Purification of pro*Pf*PM9

8 M urea solution was freshly prepared. For every 10 ml of urea solution, 1 gm of Amberlite ion exchange resin was added (10 ml urea solution/g Amberlite resin). This was stirred for 60 minutes at room temperature to deionize urea. The resin was removed by filtering the urea solution containing the resin. Sodium phosphate was added to the filtered urea solution to give final concentration of 50 mM and NaCl was added to a final concentration of 500 mM. The buffer was made up to final volume with distilled water and pH adjusted to 7.5. This was filtered and referred to as IB solubilization buffer. IB stored in TE buffer at -80ºC was thawn on ice and centrifuged at 12000 g, 4ºC for 15 minutes. Solubilization buffer (Urea-phosphate buffer pH 7.5) was added to IB pellet at 1 ml 8M urea / 68 mg IB. Denaturing/solubilization of IB was carried out at room temperature for 2 hours. This allowed complete extraction of pro*Pf*PM9. The solution was centrifuged at 13000g, 4ºC for 30 minutes. Supernatant was obtained and an aliquot of supernatant was kept for SDS-PAGE. Protein concentration was determined by Bradford method [25].The supernatant was dialyzed in 20 mM Tris-HCl, pH 8.0 at 4ºC (X 100 volume of protein solution) with stirring and three changes of buffer. The first buffer change was after 4 hours. The buffer was exchanged for fresh 20 mM Tris-HCl, pH 8.0 and left to dialyze overnight. The buffer was changed the next morning making the third change of buffer and left to dialyze finally for 4 hours. The resulting dialysate contained refolded pro*Pf*PM9 and was centrifuged at 13000g, 4ºC for 30 minutes. An aliquot was stored for SDS-PAGE, protein concentration determination and PfPM9 activity assay. For the purification to be carried out on a cation exchanger, the dialysate in Tris-HCl buffer was diluted 10X with cold 20 mM Tris-HCl, pH 8.0 buffer before further dialysis in cold 50 mM MES, pH 6.5 buffer at 4ºC (X 20 volume of protein solution) with two buffer changes, each for 3 hours. The resulting dialysate was centrifuged at 13000g, 4ºC for 30 minutes. An aliquot was stored for SDS-PAGE, protein concentration determination and enzyme activity assay as described earlier. The dialysate in 50 mM MES, pH 6.5 buffer containing the refolded was stored at 4ºC.

2.5 Purification of pro*Pf*PM9 on Cation Exchange Chromatography using HiTrap SP Column

The HiTrap SP column was first washed with 25 ml of start buffer (50 mM MES buffer pH 6.5) at 3 ml/min. The column was again washed with 25 ml of elution buffer (50 mM MES buffer pH 6.5, 1 M NaCl). Final equilibration was done by washing the column with 100 ml of start buffer (50 mM MES buffer pH 6.5). The refolded protein in 50 mM MES pH 6.5 buffer was loaded onto the SP column. The flow through was collected and tested for presence of protein. Purified protein was eluted with a salt gradient up to 1 M NaCl on Amersham Pharmacia FPLC. Fractions collected were tested for protein at OD_{280} and chromatogram of OD_{280} was plotted against eluted fraction volume to identify peak fractions. The NaCl gradient (0-100%) was also plotted on the chromatogram to determine the NaCl gradient-protein elution profile. Aliquots from fractions containing protein were run on SDS-PAGE and catalytic assay were performed on the fractions. Peak fractions showing P*f*PM9 activity were found to be eluted with the 450 mM NaCl gradient. Purified fractions containing active PfPM9 were pooled and subjected to gel filtration on Superdex 75. Purified fractions were stored at 4ºC.

2.6 Gel Filtration Chromatography of pro*Pf*PM9 on Superdex™ 75

The Superdex™ 75 column was first calibrated using standard proteins with known molecular weights before carrying out gel filtration on the partially purified proP*f*PM9. The pooled fraction from cation exchange chromatography was concentrated to a smaller volume (≤ 2 ml) before loading on Superdex 75 column with elution buffer, 50 mM MES pH 6.5, 450 mM NaCl. Fractions collected were tested for protein at OD_{280} and chromatogram of OD_{280} was plotted against fraction number to identify peak fractions. SDS-PAGE was run on peak fractions and catalytic assay was also performed to determine the activity of the pure pro*Pf*PM9. Protein peak fractions with active *Pf*PM9 were pooled together and the total volume was recorded. Protein concentration was determined by Bradford method [25].

2.7 Activation and Assay of *Pf*PM9

The Pro*Pf*PM9 was activated by incubating it at 37ºC for 20 minutes in 50 mM sodium acetate buffer, pH 4.5. The pre-incubation time allows zymogen conversion to the mature form which shows catalytic activity. Plasmepsin 9 kinetic assay was done using the chromogenic substrate, Lys-Pro-Ile-Glu-Phe-Nph*Arg-Leu (RS6) where * represents cleavage site. Catalytic activity was confirmed by monitoring the decrease in average absorbance from 280 to 272 nm [26] using a Cary 50 Bio UV-visible spectrophotometer (Varian). Stock peptide solutions were made in 10% formic acid and 90% distilled water. Reactions were carried out in 50 mM sodium acetate buffer, pH 4.5 at 37ºC. The total reaction volume was 250 µl. The reaction mixture had a 50 µM substrate solution. Reaction was initiated when the mature *Pf*PM9 was added to the substrate solution. The substrate cleavage reaction was monitored in a Cary50 UV/Visible spectrophotometer by the decrease in average absorbance from 302–410 nm.

2.8 Inhibition Assay on Activity of *PfPM9*

Inhibition assay on the activity of *PfPM9* was done with 5 µM of pepstatin A, which is the general tight-binding aspartic protease inhibitor. The reaction was carried out in 50 mM sodium

acetate buffer, pH 4.5 using mature *PfPM9* and 50 µM chromogenic substrate, Lys-Pro-Ile-Glu-Phe-Nph*Arg-Leu (RS6). Control reaction was without pepstatin A.

3. RESULTS AND DISCUSSION

The plasmid vector used in cloning of truncated pro*Pf*PM9 gene was pET3a which contained a T7-tagged sequence. The expressed truncated pro*Pf*PM9 protein contains a T7 tag on its amino-terminal end (Scheme 1). The expressed zymogen (Fig. 1a) accumulated in the form of inclusion bodies in *E. coli* (Fig. 1b). A total of 1.47 g of purified inclusion bodies was obtained from 4 L of expression culture (data not shown). The inclusion bodies were solubilized in 8 M Urea-phosphate buffer pH 7.5 and the supernatant obtained contained the unfolded pro*Pf*PM9 as confirmed by SDS-PAGE (Fig. 1b). This was subjected to the refolding procedure described under experimental section. It was observed that refolding pro*Pf*PM9 by dialysis in 20 mM Tris-HCl pH 8.0 yielded 94% of soluble protein. The soluble dialysate was the refolded pro*Pf*PM9 as revealed by the SDS-PAGE ((Fig. 1c). The pI of pro*Pf*PM9 is 9.15 (Scheme 1) so, the initial purification of the refolded protein was by cation exchange chromatography. The refolded pro*Pf*PM9 was dialysed in 50 mM MES buffer pH 6.5. Dialysis of the refolded pro*Pf*PM9 directly in MES buffer pH 6.5 resulted in 83 % precipitates of the refolded protein (data not shown). This could be due to the very high concentration of protein in the Tris-HCl buffer (21 mg/ml). The refolding protocol was optimized by 10X dilution of refolded protein solution in 20 mM Tris-HCl buffer, pH 8.0 before dialysis in 50 mM MES buffer pH 6.5 (Table 1).

M A S M T G G Q Q M G R G S N H M N K I K D E K Y K Q E Y E E E K E I Y D N T N T S
Q E K N E T N N E Q N L N I N L I N N D K V T L P L Q Q L E D S Q Y V G Y I Q I G T P P
Q T I R P I F D T G S T N I W I V S T K C K D E T C L K V H R Y N H K L S S S F K Y Y E
P H T N L D I M F G T G I I Q G V I G V E T F K I G P F E I K N Q S F G L V K R E K A S
D N K S N V F E R I N F E G I V G L A F P E M L S T G K S T L Y E N L M S S Y K L Q H
N E F S I Y I G K D S K Y S A L I F G G V D K N F F E G D I Y M F P V V K E Y Y W E I H
F D G L Y I D H Q K F C C G V N S I V Y D L K K K D Q E N N K L F F T R K Y F R K N K
F K T H L R K Y L L K K I K H Q K K Q K H S N H K K K K L N K K K N Y L I F D S G T S
F N S V P K D E I E Y F F R V V P S K K C D D S N I D Q V V S S Y P N L T Y V I N K M P
F T L T P S Q Y L V R K N D M C K P A F M E I E V S S E Y G H A Y I L G N A T F M R Y
Y Y T V Y R R G N N N N S S Y V G I A K A V H T E

Scheme 1. Schematic representation of the amino acid sequence of translated N and C-terminal pro*Pf*PM9 Truncation

(Red = T7 tag from pET3a vector, Green = beginning and end of truncated proPfPM9) with theoretical pI/Mw: 9.15 / 53606.

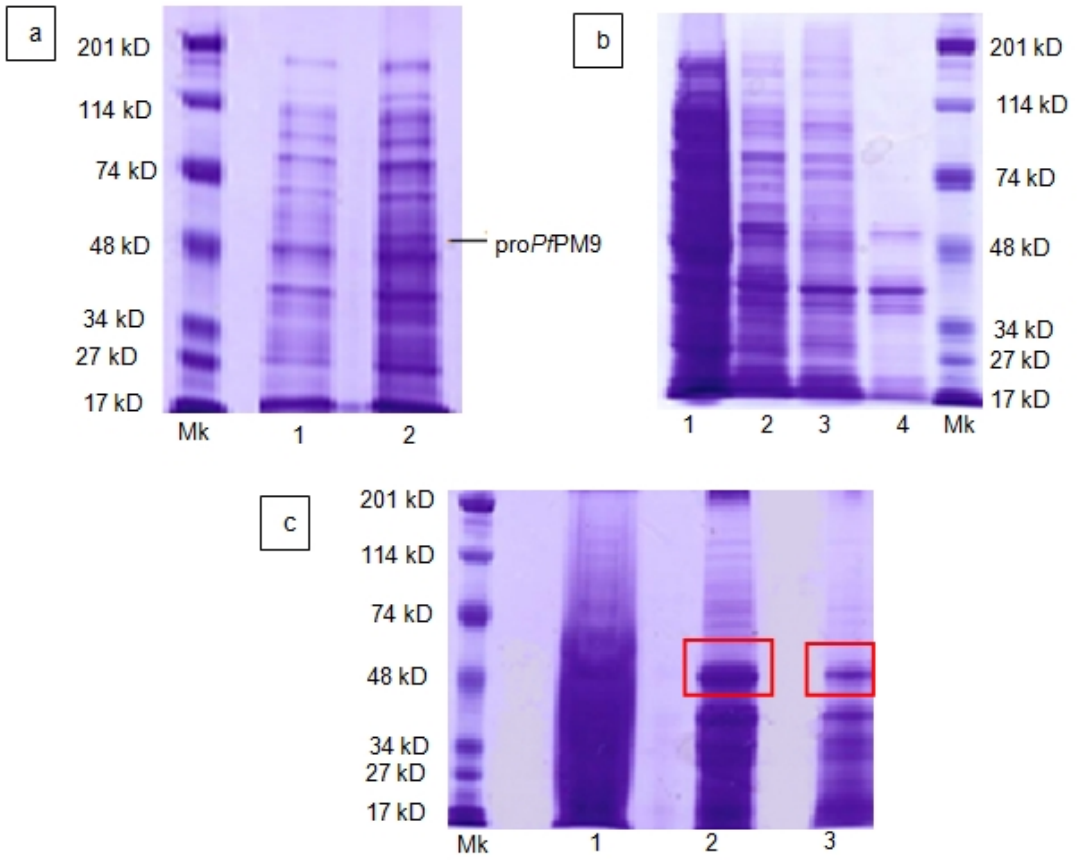

Fig. 1. **Expression of pro*Pf*PM9 from *E.coli* Rosetta 2(DE3)pLysS. on 10% SDS-PAGE**
a). showing non-induced expression culture (1) and IPTG induced expression culture (2). (b) Inclusion bodies preparation showing soluble fractions of lysate (1-4). (c) Solubilized inclusion bodies in Urea-phosphate buffer pH 7.5 (1), refolded proPfPM9 in 20 mM Tris-HCl pH 8.0 buffer (2) and 10X dilution of refolded proPfPM9 dialysed in 50 mM MES, pH 6.5 buffer (3). Bands of proPfPM9 highlighted in red boxes.

An average of 0.64 mg of purified activatable *Pf*PM9 zymogen was obtained from 4 L of cell culture (Table 1). The yield appears low but very encouraging when compared with first reported yield of pro*Pf*PM1 [20], which was 0.9 mg of purified activatable *Pf*PM1 zymogen from 14 L of cell culture. As earlier reported for processing of proplasmepsins 1, 2 and 4 which required acidic conditions in the range of 4.0 – 5.0 to become catalytically active [21-23], the refolded pro*Pf*PM9 was also activated to the mature enzyme following incubation at pH 4.5. Spectrophotometric assay procedure described under experimental section using chromogenic substrate, RS6 (Lys-Pro-Ile-Glu-Phe-Nph*Arg-Leu) was followed. The kinetic assay showed that the refolded PfPM9 was active (Figs. 2a and 2b). Confirmation of active PfPM9 was made from substrate cleavage scan at the end of the assay reaction which showed hydrolysis of RS6 by shift of absorbance peak from 280 nm to 272 nm (Figs. 2c and 2d).

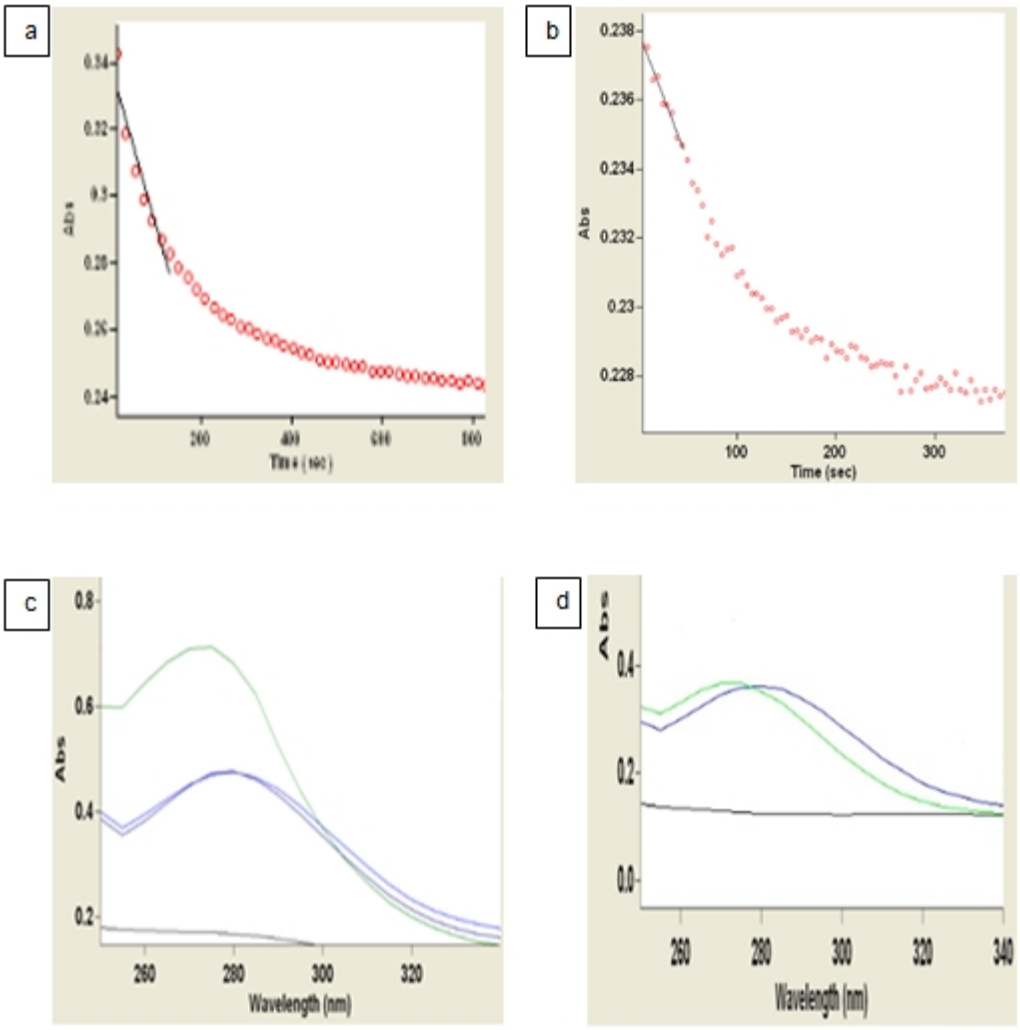

Fig. 2. **Kinetic assays on *PfPM9*** (*a). Velocity of hydrolysis of RS6 by refolded PfPM9 in 20 mM Tris-HCl pH 8 (4.7 *10^{-4} AU/sec). (b) Velocity of hydrolysis of RS6 by 10X diluted PfPM9 in 50 mM MES pH 6.5 (0.74 *10^{-4} AU/sec). (c.) Substrate cleavage by PfPM9 showing a shift in absorbance peak from 280 nm to 272 nm (in green). (d).Substrate cleavage by positive control, cathepsin D (in green)*

The refolded pro*Pf*PM9 was purified from the soluble dialysate by FPLC using cation exchange (Figs. 3a and b) and gel filtration chromatography (Figs. 3d and e) as described under experimental section. The SDS – PAGE of peak fractions from both purification techniques (Figs. 3c and f) confirm bands of pure pro*Pf*PM9 (approx. 54 kD).

Inhibition studies on the mature *PfPM9* showed that the activity of the enzyme was inhibited by pepstatin A (Figures 4a and b). *PfPM9* lost 75% activity in the presence of 5 µM pepstatin A (Figure 4b) relative to control. Similar reports were made on plasmepsins 1, 2 and 4 [2, 15, 23]. This further confirms that the purified protein is an active plasmepsin 9.

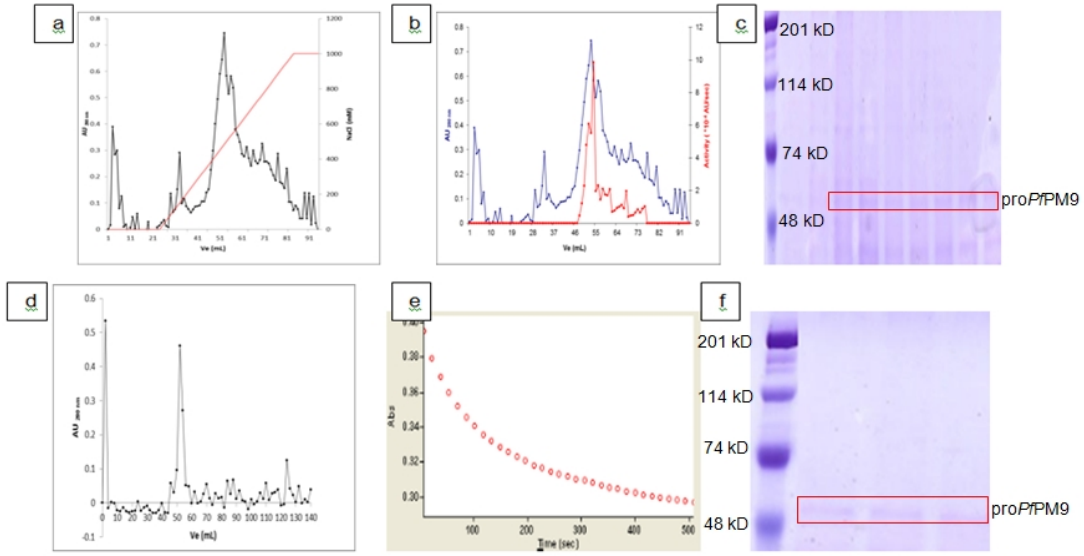

Fig. 3. Purification and activity of P*f*PM9

*(a) Chromatogram of proPfPM9 on cation-exchange chromatography using HiTrap SP column. (b).Chromatogram of proPfPM9 vs activity of eluted fractions. (c). SDS – PAGE of peak fractions from cation exchange chromatography (d). Chromatogram of proPfPM9 on Superdex™ 75 gel filtration chromatography. (e). Velocity of hydrolysis of RS6 by unconcentrated pooled PfPM9 peak fraction from gel filtration chromatography (5.9 *10^{-4} AU/sec). (f). SDS – PAGE of peak fractions from gel filtration chromatography.*

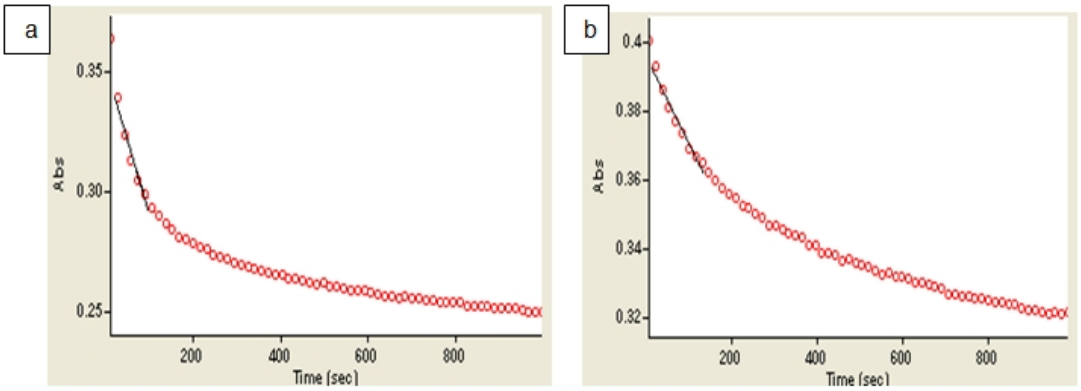

Fig. 4. Inhibition assays on *Pf*PM9

*(a). Velocity of hydrolysis of RS6 by purified PfPM9 without pepstatin A (**control**) (7.6 *10^{-4} AU/sec). (b) Velocity of hydrolysis of RS6 by purified PfPM9 in the presence of 5 µM pepstatin A (1.9 *10^{-4} AU/sec). The relative activity (%) was calculated relative to the activity obtained in the control reaction which was taken as 100%.*

Table 1. Purification table of recombinant pro*Pf*PM9 from 4 L expression

Step	Volume (mL)	Total protein (mg)	Total activity (*10^{-4} AU/sec)	Specific activity (units/mg)	Purification fold	Yield (%)
Refolding (Dialysis in 20 mM Tris-HCl pH 8)	20	462.6	11844	25.6	1	100
Dialysis in 50 mM MES pH 6.5	202	385.5	8282	21.48	1	70
SP-Cation Exchange chromatography	8	1.57	633.1	403.2	16	5.3
Superdex 75 Gel filtration chromatography	6	0.64	412.2	644.06	25	3.5

4. CONCLUSION

Functionally active recombinant pro*Pf*PM9 has been successfully expressed and purified. This preliminary report provides good background to further optimize protocols for improved yield of pro*Pf*PM9 for detailed kinetic and structural characterization of the enzyme.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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