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Construction, Overexpression, and Preliminary Purification of a *Plasmodium falciparum* Plasmepsin 6 Prosegment Chimera

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ABSTRACT

Malaria is a parasitic infection that affects 300-500 million people annually. The *Plasmodia* parasites are increasingly resistant to current drugs, and plasmepsins are essential hemoglobinases that are putative new targets of *Plasmodium falciparum*. The structures of the plasmepsins reveal prosegment inactivation of the enzyme by conferring a structural change in the protein and an alteration in the active site cleft. The enzyme self-activates at acidic pH by removing the prosegment, leaving the mature enzyme in the proper conformation.

This project aims to study plasmepsin 6 (PM6), a recently discovered enzyme with amino acid sequence similarity to plasmepsin 2 (PM2) and plasmepsin 1 (PM1). Prior attempts to overexpress PM6 with both a full length and truncated prosegment have failed. It is hypothesized that if a protein shows poor levels of overexpression, a different prosegment from a homologous protein may be used to mediate expression and refolding of the protein.

A chimeric protein comprised of the prosegment of PM2 joined to the mature PM6 sequence (pPM2gPM6) was created through overlap PCR. Overexpression and refolding conditions were optimized, and purification is currently underway. Preliminary assays indicate that pPM2gPM6 self-activates at acidic pH and activity was observed by hydrolysis of aspartic proteinase substrates.

INTRODUCTION

Malaria is a parasitic infection that results in over 1.5 million deaths annually, and 300-500 million people are currently infected with malaria worldwide (1). The parasite spends one part of its life cycle in female *Anopheles* mosquitoes and the other part in humans (2). As the mosquito is feeding, sporozoites in the saliva are transferred to the blood of the host. They travel through the blood and initially infect liver cells, in which they develop into trophozoites. These trophozoites develop into merozoites, which erupt from the liver cells and travel into the blood stream to invade erythrocytes. During this intraerythrocytic stage they develop into trophozoites, and they degrade massive amounts of human hemoglobin as a nutrient source, which causes the major presentation of disease in the human host (2,3). It is during this stage, from the acidic digestive

vacuole, that the first two plasmepsins were initially isolated (4).

One of the drugs used to treat malarial infections with minimal side effects is chloroquine. Chloroquine-resistant *Plasmodium falciparum* strains emerged in Africa in 1978, and strains that are resistant to many of the current therapeutics are widespread (5). This increasing resistance has made the development of new drugs a necessity. One of the possibilities for new drug targets includes a class of enzymes called plasmepsins. Plasmepsins are *Plasmodia* aspartic proteases that function in hemoglobin degradation during the intraerythrocytic stage of the life cycle (6). Hemoglobin is an essential nutrient source, and various aspartic protease inhibitors have been shown to kill intraerythrocytic cultures of *P. falciparum* (7,8,9).

Aspartic proteases exist both in an immature zymogen form, with the prosegment blocking the active site of the enzyme, and an active form, in which the prosegment has been autocatalytically cleaved (10). Ten plasmepsins have been identified to date, only a few of which have been characterized and studied (11). Attempts to express full-length plasmepsin 6 have failed in the past, and a new method was needed to study this enzyme. Prosegment chimeras are advantageous, because a different prosegment can be used to help mediate the expression and refolding of the protein, but at a low pH, the prosegment gets cleaved off, leaving only the desired enzyme behind.

MATERIALS AND METHODS

Background Primers were designed that were specific for the prosegment of PM2 (pPM2) and the gene segment of PM6 (gPM6). Primer 2 had an overlapping region that was complementary to gPM6, and primer 3 had a region that was complementary to pPM2. Primers 1 and 4 had BamHI cleavage sites incorporated into them. The pPM2 and gPM6 fragments were amplified through PCR and run on an agarose gel. They were excised from the gel and gel purified. The fragments were allowed to extend at 72 ºC for 10 minutes with Taq polymerase before the primers (1 and 4) were added and amplified through overlap PCR (see **Fig. 1**). This PCR product was run on a gel, gel purified, and ligated into a pCR.2.1 TOPO cloning vector (see **Fig. 2**). The vector was transformed into TOP10F' One Shot *E. coli* cells, which were plated on LB/ampicillin plates. Colonies were plucked, cultures were grown overnight, and plasmid DNA was isolated from them with a QIAprep Spin Miniprep kit. The clones were screened with a BamHI restriction digest, and those that appeared correct were sent for verification through sequence analysis.

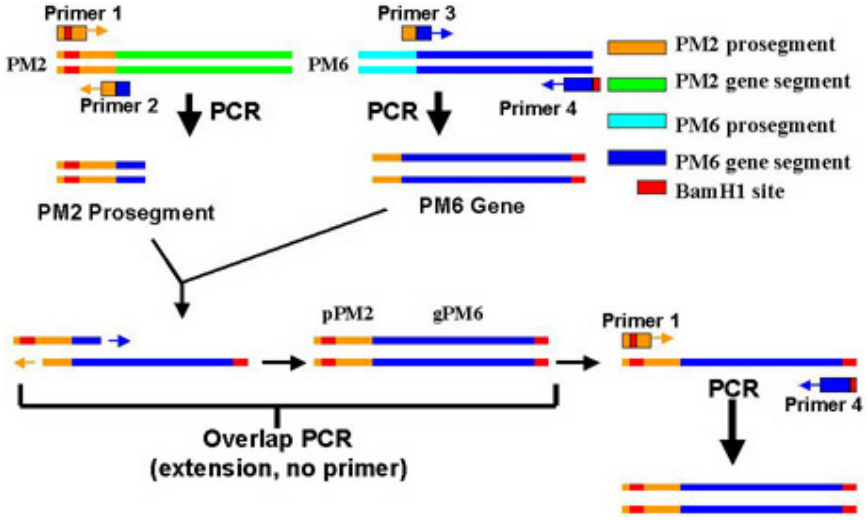

Figure 1. pPM2gPMG Prosegment Chimera Construction

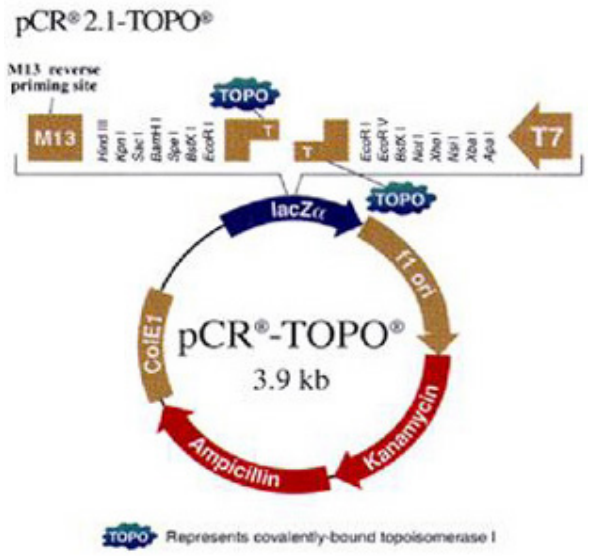

Figure 2. TOPOs.1 Cloning Vector

The TOPO vector plus insert and a pet3a vector were both digested with BamHI and run on a gel. The inserted gene and the digested pet3a vector were excised from the gel, gel purified, and the insert was ligated into the pet3a expression vector (see **Fig. 3**), and subsequently transformed into TOP10F' One Shot cells. Clones were screened for directional insertion through a restriction digest with XbaI, and clones were verified through sequence analysis. Plasmid DNA was isolated from a positive clone, and a transformation was done with the DNA and BL21(DE3)pLysS supercompetent E. coli cells. Colonies were plucked from the plates and grown overnight in LB media with ampicillin. Plasmid DNA was isolated and the clones were subsequently digested with XbaI and run on a gel to screen for positives. Possible positive clones were verified through sequence analysis, and cell stocks were made by adding 15% glycerol and storing at −20ºC.

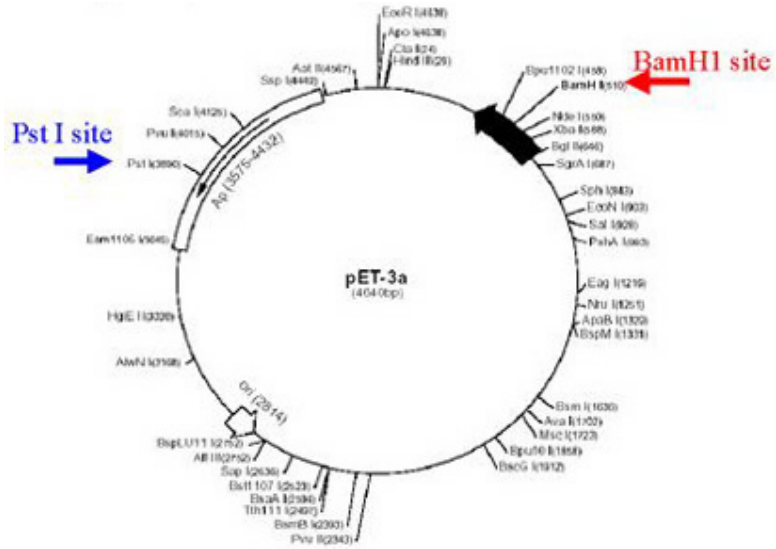

Figure 3. pet3a Expression Vector

A 200mL culture of a positive clone was grown overnight with 200µL ampicillin, and was used to inoculate 4L of fresh LB media. Once the cells had reached an OD at 600nm of 0.5-0.8 AU, 0.4mM IPTG was added to induce overexpression of the plasmid protein. Samples and OD readings were taken at t=0, t=1, t=2, and t=3 hours. The OD readings were used to normalize the amount of protein loaded on the SDS-PAGE gel. After three hours, the cells were spun down and the cell pellet was weighed and re-suspended in TE buffer. The suspension was lysed through French Pressure Cell lysis and inclusion bodies, which are insoluble protein aggregates, were purified by centrifugation with several different buffers. The resulting pellet was weighed after the final step, and was re-suspended in TE buffer at 50mg/mL.

Inclusion bodies were solubilized at 1mg/mL in 8M urea, 0.05M CAPS, 0.005M EDTA, and 0.2M BME. The solubilized protein was put in dialysis tubing and was dialyzed against 50mM Tris pH 11.0 at room temperature for four hours. Then the buffer was changed and allowed to sit overnight at 4»C. The buffer was changed three more times with 50mM Tris pH 8.0, 20mM Tris pH 8.0, and 20mM Tris pH 8.0, respectively, at twelve-hour intervals. For the anion exchange column, the last three buffers were pH 9.5 instead of pH 8.0. Refolded protein in a 20mM Tris pH 8.0 buffer was loaded onto a Superdex 75 gel filtration column, and fractions were tested for activity and run on an SDS-PAGE gel. Protein in a pH 9.5 buffer was loaded onto a Sepharose High Performance anion exchange column, and the fractions that eluted were run on an SDS-PAGE gel and tested for activity. For the activity assays, the enzyme was incubated at 37ºC for 10 minutes in 0.5M sodium formate buffer pH 4.5, and then the substrate RS6P2V was added. The average absorbance change from 284-324 nm was monitored with a spectrophotometer. A decrease in absorbance was indicative of substrate cleavage.

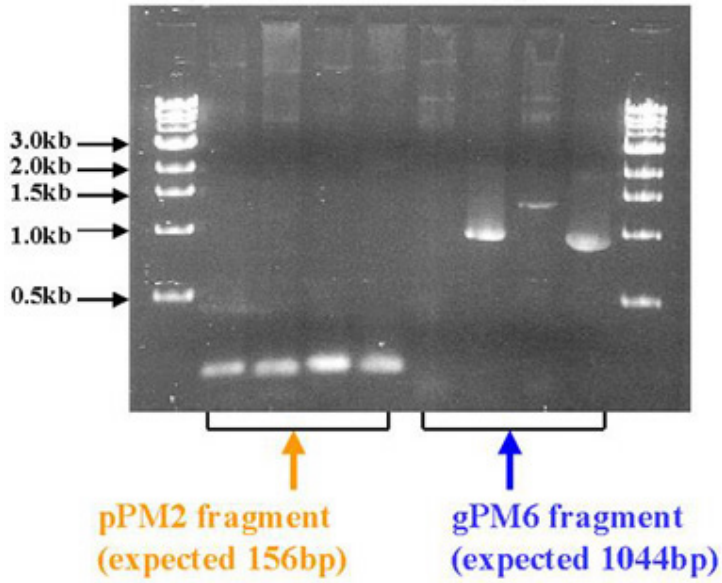

Figure 4. PCR Amplification pPM2 and gPM6

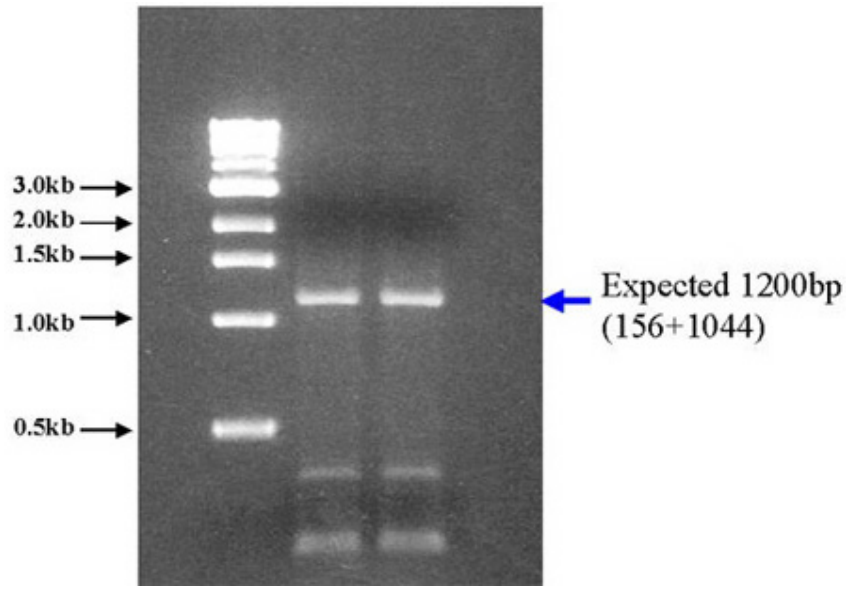

Figure 5. Overlap PCR Amplification of pPM2gPM6

RESULTS

The construction of the pPM2gPM6 chimera through overlap PCR was successful, as shown in **Fig. 4** and **5**, and so was the overexpression and refolding, as show in **Fig. 6** and **7**, respectively. Gel filtration (**Fig. 8**) and anoin exchange (**Fig. 9**) both helped in purification of the protein, but additional purification is still necessary. Preliminary activity of the post-dialysate (refolded protein) was seen,with 100µL of PD giving a negative change in absorbance of 132x10-6 Absorbance Units/sec from 284-324 nm for the initial rate.

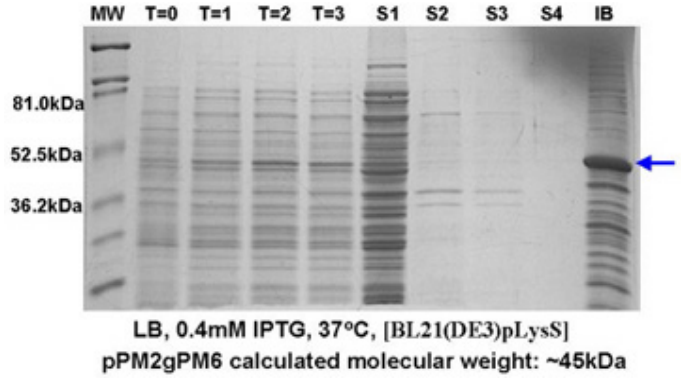

Figure 6. Expression of pPM2gPM6 in *E.coli* and Inclusion Body Purification

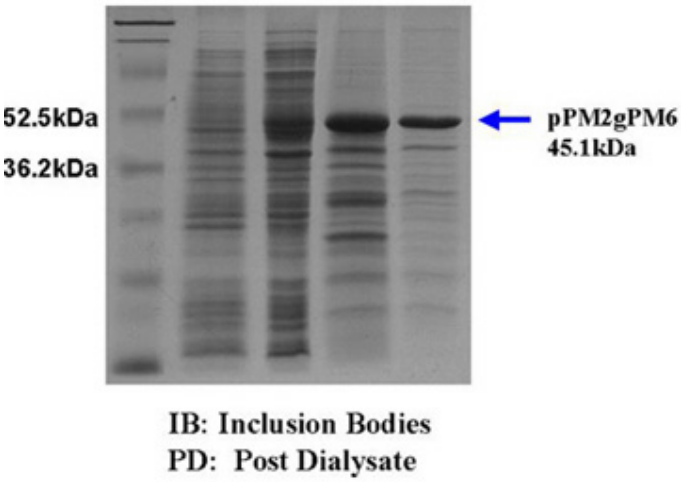

Figure 7. Post-Dialysate (Refolded Protein)

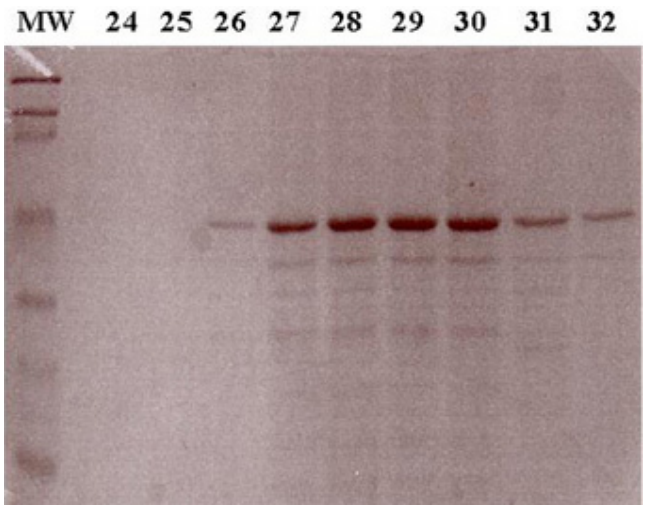

Figure 8. Superdex 75 GF Column Fractions

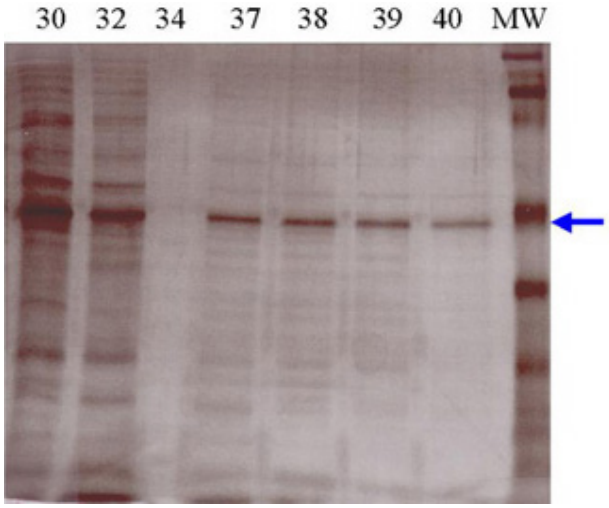

Figure 9. Sephorex Anion Exchange Column Fractions

DISCUSSION

The amplification of the segments was successful, as was the overlap PCR to join them. Overexpression and refolding of pPM2gPM6 was successful, and in all of the publications to date, attempts to express PM6 have failed. Preliminary activity was seen with the post-dialysate and the post-gel filtration column protein samples, but the pPM2gPM6 enzyme that was used for the activity assays was in either a pH 8.0 or a pH 9.5 Tris buffer. When the sodium formate pH 4.5 buffer was added to drop the pH and induce activation of the enzyme, it induced precipitation of the protein. This precipitation could be seen as opacity in the cuvette, and caused significant scattering of light, which interfered with the assay.

A new refolding method is currently underway, which uses rapid dilution and then the addition of oxidized glutathione instead of dialysis to promote refolding of the protein. The rapid dilution method is advantageous, because the pH of the buffer can be adjusted from 9.5 to 4.5 without causing the protein to precipitate out of solution (this was determined by experimentation). This protein could be loaded directly onto a cation exchange column and eluted with a pH 4.5 sodium formate buffer along with increasing concentrations of NaCl to purify it, allowing the activity assays to be done without having to cross over the isoelectric point. This will hopefully help to eliminate the precipitation during the activity assay and yield better results.

Post-anion exchange column activity has not yet been obtained, but this was due to several reasons. The amount of protein that was loaded on the column was approximately 10.8 mg, much of which was *E. coli* proteins. Only a fraction of that was pPM2gPM6, some of which may have failed to correctly refold. In addition to the small amount of enzyme that could have even been present in the 1.5mL fractions, pPM2gPM6 eluted at around 600 mM NaCl, and the fractions were not desalted before they were tested for activity because the method used to desalt the fractions lost so much protein. If the experiment were up-scaled, fractions could be pooled and a different method for desalting could be used that would avoid so much protein loss. Additionally, a strong anion exchange column was used, and perhaps with a weak anion exchange resin, the protein would still bind,

but would elute with less salt.

ACKNOWLEDGEMENTS

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