Research Note

Expression of *Plasmodium falciparum* plasmepsin I *in Escherichia coli* and *Pichia pastoris*

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The widespread occurrence of drug resistant malaria parasite, particularly the most virulent species, *Plasmodium falciparum* has resulted in a desperate need to develop new drugs to combat the disease. Currently, studies are being focused on the identification of novel antimalarial compounds acting against molecular targets essential for the parasite viability. One promising new antimalarial drug target is the aspartic proteinase plasmepsin I which is involved with haemoglobin degradation (Berry, 1997). In this study, our aim is to produce biologically active recombinant plasmepsin I for screening of inhibitory compounds using *in vitro* molecular assays.

Expression of the malaria protease plasmepsin I was carried out using both bacterial and yeast systems. The EasySelect *Pichia* expression kit (Invitrogen) was used to prepare recombinant *Pichia pastoris* clones expressing the proplasmepsin I from *P. falciparum* as an intracellular protein. Using genomic DNA from *P. falciparum*, the gene fragment encoding the last 48 amino acids of the propart region and the entire mature plasmepsin I (Francis *et al.*, 1994) was amplified by PCR using a forward primer with an additional *Kpn* I restriction site (5'-ACAGGTACCA ATACCTCAAAACATG TAATAATTG-3') and a reverse primer with an additional *Not* I restriction site (5'-TTAGCGGCCG CCAATTTTTTTTTGGCAAGGGCG-3'). The 1157 bp PCR product obtained was ligated with the pCR2.1-TOPO vector (Invitrogen). The cloned PCR product was excised out of the vector by consecutively digested with *Not* I, treated with DNA polymerase I Klenow fragment and digested with *Kpn* I before insertion into the *Pichia* expression vector, pPICZ B which had been digested with *Xba* I, treated with DNA polymerase I Klenow fragment and digested with *Kpn* I. The PMI.pPICZ B plasmid generated was linearised with *Sac* I and transformed into the competent cells of Pichia strains X-33 (wild type) and SMD1168 (protease-deficient) by electroporation according to the manual for the expression kit.

For small-scale protein expression studies, 8 to 12 recombinant *Pichia* clones from each of the strains were grown overnight at 30°C in 5 ml of buffered glycerol-complex medium (BMGY). Cells were harvested by low-speed centrifugation, resuspended in 5 ml of buffered methanol-complex medium (BMMY), and cultured for 3 days. Methanol was added to 1% every 24 hours. Cell lysates were analysed for the presence of recombinant protein on Coomassie brilliant blue stained-SDS-PAGE gels (12.5%) as well as by immunoblotting using monoclonal antibody directed against the vector *myc* epitope fused to the C-terminal of proplasmepsin I, and peroxidase-conjugated goat anti-mouse IgG.

For protein expression in *E. coli*, the proplasmepsin I gene fragment along with the *myc* tag was subcloned from PMI.pPICZ B into the bacterial expression vector, pRSET B (Invitrogen). The PMI.pPICZ B recombinant plasmids were first digested with Sal I, treated with mung bean nuclease and digested with *Kpn* I before ligation with pRSET B which had been digested with *Eco*R I, treated with DNA polymerase I Klenow fragment and digested with *Kpn* I to give PMI.pRSET B.

Culture of *E. coli* strain BL21(DE3)pLysS harbouring the PMI.pRSET B plasmid was grown at 37°C in LB media to an A_{600} of 0.5. At this point, IPTG was added to 0.5 mM and cultured for an additional 3 hours. Cells were harvested by low-speed centrifugation and the recombinant proplasmepsin I was purified using the ProBond resin (Invitrogen) under denaturing conditions according to the manufacturer's instructions. For protein refolding, the purified protein sample was dialysed against buffer containing 10 mM Tris, pH 8.0 and 0.1% Triton X-100 overnight at 4°C. To convert the proenzyme to its mature form, the refolded protein sample was incubated in 100 mM sodium acetate pH 4.4 overnight at 37°C.

The presence of aspartic proteinase activity in the acidified protein sample was determined using the haemoglobin-based chromogenic peptide substrate Leu-Glu-Arg-Ile-Phe*Nph-Ser-Phe. The assay was performed by mixing different amounts of acidified protein sample with the substrate at a final concentration of 50 µg/ml in 100 mM sodium acetate buffer, pH 4.4 at 37°C and

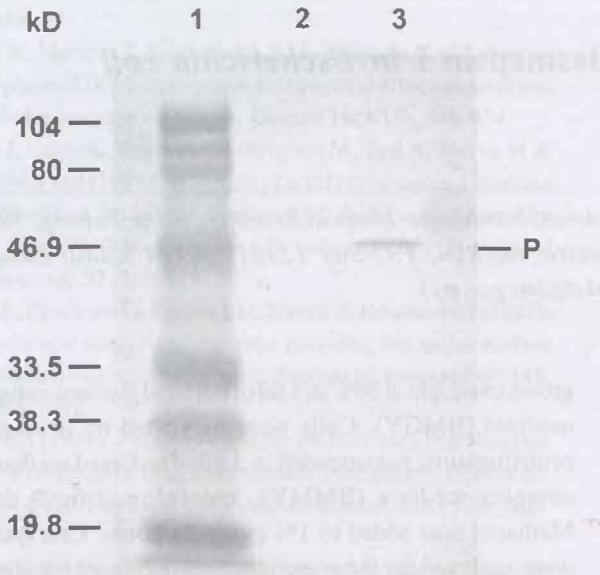

Figure 1. Expression of proplasmepsin I (P) in *E. coli*. Cell lysates from induced bacterial cultures were separated by SDS-PAGE and analysed by immunoblotting. Lane 1: protein marker; Lane 2: lysate from *E. coli* transformed with parent plasmid; Lane3: lysate from recombinant clone.

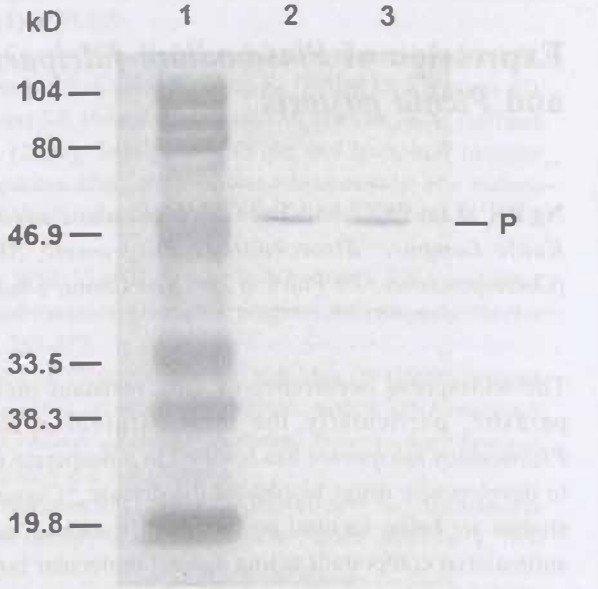

Figure 2. Recombinant proplasmepsin I (P) does not undergo autoactivation. Purified recombinant proplasmepsin I was refolded by dialysis, incubated at pH 4.4 and analysed by immunoblotting. Lane 1: protein marker; Lanes 2 & 3: recombinant proplasmepsin I before and after acidification.

decrease in absorbance at 300 nm was monitored for 5 min using the Lambda Bio 20 spectrophotometer (Perkin Elmer). A second sample containing the standard aspartic proteinase inhibitor, isovaleryl-pepstatin (Sigma) at a final concentration of 1 μM was included in each assay. A control assay was run using another malaria aspartic proteinase, the recombinant plasmepsin II (Hill *et al.*, 1994).

The proplasmepsin I gene product with the expected size of 53 kDa was not detected in all recombinant clones of wild type and protease-deficient *Pichia* strains. However, following sub cloning of the gene fragment from the yeast vector into the bacterial vector, the recombinant protein was successfully expressed in *E. coli* although the expression levels was not high (Fig. 1). The negative results observed in *Pichia* could be attributed to one or more factors. The protein expression levels may be too low to be detected by immunoblotting. In this case, it may be desirable to screen for recombinant yeast clones harbouring multicopy proplasmepsin I gene. The recombinant protein may have been degraded by host proteases other than the protease A absent in the strain SMD1168. The proplasmepsin I gene may contain a sequence which promotes premature transcriptional termination in *Pichia* thus resulting in a truncated protein without the *myc* tag which will not be detected by the anti-*myc* antibody. Premature transcriptional termination has been observed in expression of HIV-1 envelope protein in *Pichia* (Scorer *et al.*, 1993).

The size of the purified recombinant proplasmepsin I refolded by dialysis did not reduce to 37 kDa after acidification (Fig. 2). In addition, no enzyme activity was ever detected in the acidified protein samples (data not shown). At present, we are employing alternative folding protocols to determine whether the failure of the recombinant proplasmepsin I to autoactivate was due to misfolding.

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