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High-level expression in *Escherichia coli*, purification and kinetic characterization of *Plasmodium falciparum* M1-aminopeptidase

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ABSTRACT

Plasmodium falciparum neutral metallo-aminopeptidase (PfAM1), a member of the M1 family of metallo proteases, is a promising target for malaria, a devastating human parasitic disease. We report the high-level expression of PfAM1 in *Escherichia coli* BL21. An optimized gene, with a codon adaptation index and an average G/C content higher than the native gene, was synthesized and cloned in the pTrcHis2B vector. Optimal expression was achieved by induction with 1 mM IPTG at 37 °C for 18 h. This allowed obtaining 100 mg of recombinant PfAM1 (rPfAM1) per L of culture medium; 19% of the *E. coli* soluble protein mass was from rPFAM1. rPfAM1, fused to an amino-terminal 6×His tag, was purified in a single step by immobilized metal ion affinity chromatography. The protein showed only limited signs of proteolytic degradation, and this step increased purity 27-fold. The kinetic characteristics of rPfAM1, such as a neutral optimal pH, a preference for substrates with basic or hydrophobic amino acids at the P1 position, an inhibition profile typical of metallo-aminopeptidases, and inhibition from Zn^{2+} excess, were similar to those of the native PfAM1. We have thus optimized an expression system that should be useful for identifying new PfAM1 inhibitors.

Introduction

Aminopeptidases (APs)[1] are proteolytic enzymes that hydrolyze peptide bonds at the amino terminus of polypeptide chains. Metallo-APs use one or two active site metal ions for catalysis [1]. A neutral metallo-AP, belonging to the M1 family of metallo-proteases [2], is a molecular target for malaria [3], a human parasitic disease caused by several species of protozoan unicellular parasites belonging to the genus *Plasmodium,* mainly *Plasmodium falciparum* [4]. This enzyme, named PfAM1, has biological functions that are crucial for *P. falciparum* intra-erythrocytic stages inside the human host. PfAM1 is involved in the degradation of erythrocytic hemoglobin [1,5–11], trophozoite maturation [7,12,13], and reinvasion [7,9,11,13–15] and appears to be essential for the parasite during the erythrocytic stage [1,8,10,16–20]. Furthermore, inhibitors of PfAM1 block the *in vitro* growth of the parasite at micromolar concentrations [7,10,16,18–22], and one of them reduces infection in a murine malaria model [19]. The identification of potent and selective PfAM1 inhibitors is therefore a major target for malaria treatment.

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[1] *Abbreviations used:* AP(s), aminopeptidase(s); PfAM1, M1-aminopeptidase from *P. falciparum*; p96, PfAM1 96-kDa form; p68, PfAM1 68-kDa form; ePepN, M1-aminopeptidase from *E. coli*; rPfAM1, recombinant PfAM1; IMAC, immobilized metal ion affinity chromatography; DNA, deoxyribonucleic acid; G, guanine; C, cytosine; PCR, polymerase chain reaction; LB, Luria-Bertani; $OD_{600(280)nm}$, optical density at 600 or 280 nm; IPTG, isopropyl-β-D-1-thiogalactopyranoside; SDS–PAGE, sodium dodecyl-sulfate polyacrylamide gel electrophoresis; EA, enzymatic activity; Leu, leucine; *p*NA, *p*-nitroanilide; C1, culture of *E. coli* BL21 non-transformed and induced; C2, culture of *E. coli* BL21 transformed with the pTrcHis2B vector and induced; C3, culture of *E. coli* BL21 transformed with the pTrcHis2B-rPfAM1 construct and non-induced; BSA, bovine serum albumin; LC–MS/MS, liquid chromatography coupled to tandem mass spectrometry; UNAM, Autonomous National University of Mexico; His, histidine; DMSO, dimethyl sulfoxide; U, unit of EA; MES, 2-(N-morpholino)ethanesulfonic acid; Arg, arginine; Lys, lysine; Ala, alanine; Val, valine; Ile, isoleucine; Gly, glycine; Pro, proline; bp, base pairs; CAI, Codon Adaptation Index; spEA, specific EA; AMC, 7-amido-4-methylcoumarin; Glu, glutamic acid; βNA, β-naphtylamide; dNTPs, deoxy-nucleoside-triphosphates; PMSF, phenyl-methylsulfonyl-fluoride.

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PfAM1 is a monomeric enzyme of 1,085 amino acid residues and 126 kDa (primary protein product), with an amino-terminal extension of 194 residues that is not conserved among the members of the M1 family [20]. In *P. falciparum* erythrocytic stages, two active and soluble 96- and 68-kDa PfAM1 processed forms (p96 and p68) have been detected, both lacking the amino-terminal extension [2,9,11]. The enzyme has 4 domains [20], a general fold very similar to that of bacterial neutral metallo-APs [23–25], and 35% identity to the full-length sequence of *Escherichia coli* M1-AP (ePepN) [20]. PfAM1 localizes in the food vacuole and nucleus of *P. falciparum* [10,26]. This AP is Zn^{2+}-dependent, has an optimal pH of 7.0–7.4 [9,20], and has a substrate specificity typical of M1-APs: preference for basic and hydrophobic (including aromatic and branched) residues at the P1 position (*i.e.*, the amino acid amino-terminal to the hydrolyzed peptide bond) [9].

Because of the difficulty with purifying quantities of native enzyme sufficient for kinetic studies and the search for inhibitors [26], recombinant PfAM1 (rPfAM1) expression is a priority. McGowan *et al.* [20] were the first to report recombinant expression of PfAM1. In *E. coli* BL21, they expressed a synthetic gene that was initially optimized for the *Pichia pastoris* system. This gene encodes a truncated PfAM1 form of approximately 100 kDa that lacks the first 194 residues. In this construct, the first residue of rPfAM1 correlates with the start of the ePepN orthologue, and a six-histidine tag is inserted at the carboxyl terminus for purification purposes. The protein is active and soluble, with molecular and kinetic characteristics similar to those of the native enzyme [20]. On the other hand, Azimzadeh *et al.* [11] obtained recombinant p68 (residues 191–802) in *E. coli* BL21(DE3). This protein, expressed in an insoluble form, was purified by immobilized metal ion affinity chromatography (IMAC) in 6 M urea. A third recombinant variant of PfAM1 lacking the first 191 residues and containing an histidine tag at its amino terminus was amplified from parasite genomic DNA and was expressed in a soluble and active form in the *E. coli* BL21(DE3) Rosetta 2 optimized strain [26]. However, comprehensive data on heterologous rPfAM1 expression are lacking. This work describes, in detail, a strategy that allowed high-level expression of rPfAM1. The purified rPfAM1 had kinetic characteristics similar to those of the native enzyme.

Materials and methods

Codon optimization, synthesis of the rpfam1 gene and cloning in the pTrcHis2B vector

The services of the company GeneArt AG (Germany) were contracted. The *rpfam1* gene (protein sequence available in UniProtKB/TrEMBL: **Q8IEK1**) was optimized for its expression in bacteria, using the software GeneOptimizer®. The optimization consisted of changing the codons of the native gene to codons with the highest usage in bacteria, increasing the G/C content and avoiding the formation of sequences that can affect transcription and translation. Synthesis was performed by PCR assembly of synthetic oligonucleotides and PCR products. The sequences of the native and synthetic rPfAM1 genes were analyzed and compared using the web site GeneScript (http://www.genscript.com/cgi-bin/tools/rare_codon_analysis). Cloning was performed in the bacterial expression vector pTrcHis2B (Invitrogen, USA) under the control of the strong, inducible and hybrid *trc* promoter [27], between *Pst* I and *Kpn* I restriction sites. This vector contains the *lacI*^{q} gene to regulate the expression of the heterologous gene. The resultant construct (pTrcHis2B-rPfAM1) was confirmed by DNA sequencing. *E. coli* DH5α (*supE*44 Δ*lac*U169 (ϕ80 *lacZ* ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*-1) competent cells were transformed with the pTrcHis2B-rPfAM1 construct. Four colonies were arbitrarily selected, and plasmid DNA was amplified and purified from liquid cultures using a mini-prep kit (Roche, Switzerland). The quality of the amplified plasmids was confirmed by agarose gel electrophoresis.

Expression of rPfAM1 in E. coli

rPfAM1 was expressed in *E. coli* BL21 (*hsdS gal* (λc*Its*857 *ind*1 *Sam*7 *nin*5 *lac*UV5-T7 gene *1*)). To check pTrcHis2B-rPfAM1 functionality, a small-scale proof of concept experiment was performed. For this, 1-mL aliquots of Luria–Bertani (LB) broth (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl) supplemented with 200 μg/mL ampicillin were inoculated with colonies of transformed cells and incubated overnight at 37 °C. Then, 50-μL aliquots were inoculated in 5-mL aliquots of ampicillin-supplemented LB broth. The 5-mL cultures were incubated at 37 °C until an $OD_{600\ nm}$ of 0.4–0.8 was reached, at which point expression was induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG, Sigma, USA) over 4 h. The following negative controls were used: (i) the non-transformed strain, induced in LB broth; (ii) the strain transformed with the pTrcHis2B vector, induced in ampicillin-supplemented LB broth; and (iii) the strain transformed with the construct pTrcHis2B-rPfAM1, in ampicillin-supplemented LB broth. Expression of rPfAM1 was evaluated by SDS–PAGE and AP enzymatic activity (EA), with Leu-*p*-nitroanilide (Leu-*p*NA) as a substrate. To keep the positive clones, aliquots from 1-mL cultures were mixed with glycerol and stored at −70 °C.

To select the induction method, 5 mL of medium was inoculated with an rPfAM1-producer bacterial clone stored in glycerol and incubated overnight at 37 °C. Thereafter, 100 mL of medium were inoculated with 1 mL of clone-containing medium, and induction was performed for 18 h at 37 °C. Cultures used as negative controls included (i) the non-transformed strain, induced (C1); (ii) the strain transformed with the pTrcHis2B vector, induced (C2); and (iii) the strain transformed with pTrcHis2B-rPfAM1, non-induced (C3). Induction with IPTG was performed in ampicillin-supplemented LB broth with 0.1 mM IPTG at the start of the culture or with 1 mM IPTG during the exponential phase ($OD_{600\ nm}$ = 0.4–0.8). Negative control C1 was prepared with LB broth, whereas C2 and C3 controls were prepared with ampicillin-supplemented LB broth. For auto-induction with 0.2% lactose, MDG non-inducing medium (25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4, 2 mM $MgSO_4$, 100 μM $FeCl_3$, 0.5% glucose, 0.25% aspartate; [28]) supplemented with 200 μg/mL ampicillin was used for preparation of the 5-mL culture. The ZYMB-5052 auto-induction medium (1% N-Z-amine, 0.5% yeast extract, 1% NaCl, 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4, 2 mM $MgSO_4$, 10 μM $FeCl_3$, 1 μM $ZnSO_4$, 0.5% glycerol, 0.05% glucose, 0.2% lactose; [28]) supplemented with 200 μg/mL ampicillin was used for the 100-mL culture. Negative control C1 was prepared with ZYMB-5052 broth. Control C2 was prepared with ampicillin-supplemented ZYMB-5052 medium, and control C3 was prepared with ampicillin-supplemented ZYMB-5260 broth (ZYMB-5052 without lactose and with 0.26% glucose). The final $OD_{600\ nm}$ was measured, and expression was evaluated as previously mentioned.

rPfAM1 purification

Bacterial biomass was collected by centrifugation at 8,000*g* for 10 min at 4 °C. Cells were resuspended in cold 50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, to obtain cellular suspensions at a concentration of 10 units of $OD_{600\ nm}$. These suspensions were subjected to 2 ultrasonic pulses (Branson Sonicator, USA) of 15 s at 40% output, with a 30-s pause on ice between pulses. The extracts were centrifuged at 12,000*g* for 20 min at 4 °C, and the pellets and supernatants were separated and stored at −20 °C.