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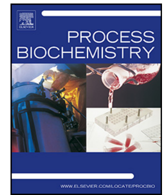

Novel and cost-effective refolding of a recombinant receptor binding domain of *Plasmodium falciparum* EBA-175

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ABSTRACT

Plasmodium falciparum erythrocyte binding antigen (EBA-175), which is a 175 kDa protein, binds with sialic acid residues on glycophorin A during invasion of human erythrocytes. Receptor-binding residues of EBA-175 lie in a conserved, amino-terminal, cysteine-rich region, region F2 of EBA-175 (PfF2), that is homologous to the binding domains of other erythrocyte binding proteins. Recombinant PfF2 was expressed in *Escherichia coli*, purified from inclusion bodies, renatured by rapid dilution in presence of proline and purified to homogeneity by ion-exchange chromatography. Recombinant PfF2 was refolded at higher concentrations with improved refolding efficiency and yield. The refolded protein was characterized using biochemical methods and shown to be pure, homogenous and functional as it binds human erythrocytes with specificity. Immunization of mice and rabbits with recombinant PfF2 formulated with Freund's and Montanide ISA720 adjuvants, elicited high and potent antibody responses. These observations support the development of a novel and cost-effective process for production of recombinant PfF2, a blood stage vaccine candidate for *P. falciparum* malaria.

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1. Introduction

Plasmodium falciparum is responsible for majority of the mortality associated with malaria [1–3]. All the clinical symptoms of malaria are associated with the blood-stage of *P. falciparum* infection [2–4]. The life cycle of blood-stage development involves erythrocyte invasion, parasite multiplication, red cell rupture, and egress of next generation merozoites and re-invasion of red cells [1,2,4]. Erythrocyte invasion requires specific receptor–ligand interactions [5,6]. The merozoites of *P. falciparum* interact with sialic acid residues on glycophorin A as receptors during erythrocyte invasion [2,7,8]. Binding to sialic acid–glycophorin A is mediated by a 175 kDa erythrocyte binding antigen (EBA-175) [9,10]. EBA-175 belongs to a family of erythrocyte binding proteins (EBPs) which bind with sialic acid residue on glycophorin A [1,7–10]. The erythrocyte binding domain of EBA-175 is region II (RII), which is a 616 amino acid fragment consists of two cysteine-rich regions F1 and F2 [1]. The receptor-binding domains of EBPs lie in conserved N-terminal cysteine-rich regions that are referred to as Duffy-binding-like (DBL) domains [1]. The N-terminal cysteine-rich region of EBA-175 contains a tandem duplication of DBL domains, F1 and F2, at the N-terminal end, which contain the receptor-binding sites for sialic acid residues [9]. PfF2 has been shown a potent candidate for vaccine against *P. falciparum* malaria [3,4,10,11]. Antibodies raised against PfF2, which contains receptor-binding residues, have been shown to inhibit erythrocyte invasion by *P. falciparum* [3,4,11–13]. A recombinant subunit vaccine candidate based on PfF2 has recently been tested for safety and immunogenicity in healthy adults [3].

Protein aggregation is a frequently observed phenomenon in protein folding pathways. Correct folding *in vitro* or *in vivo* competes with unproductive events such as misfolding or aggregation [14–16]. The prevention of aggregation is an important task during refolding and purification; therefore, protocols to control aggregation and promote refolding using chemical chaperones have been intensively studied [14–16]. Inclusion body formation is based on over-expression of recombinant proteins, which induces formation of inactive aggregates due to hydrophobic interactions. Osmolytes (polyols, sugars, polysaccharides, neutral polymers, amino acids (proline) and their derivatives) have been reported to prevent aggregation [14–16]. These osmolytes increase the viscosity of the

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refolding solution which affects protein refolding dynamics [17]. As a result the partially folded protein intermediates successfully finish their folding pathway, rather than being blocked by aggregation [15,16].

Recombinant PfF2 (∼43 kDa) accumulates in inclusion bodies in *Escherichia coli* and was refolded and purified followed by characterization as described earlier [10,11,18,19]. A recombinant subunit vaccine based on PfF2 has been tested recently in healthy adults and found that purified IgG from sera displayed significant growth inhibitory activity against *P. falciparum* CAMP strain [3]. However, the yield of purified PfF2 is very low after refolding. Therefore, in the present study we report the development of a novel and cost-effective refolding of recombinant PfF2 from inclusion bodies with improved yield and refolding efficiency. Recombinant PfF2 expressed in *E. coli* was refolded by rapid dilution in presence of proline under conditions that allow refolding at significantly higher protein concentrations (up to 1 mg/ml) than described earlier. Biochemical characterization confirmed that recombinant PfF2 was pure, homogenous and functionally active. Recombinant PfF2 formulated with Freund's and Montanide ISA720 adjuvants was highly immunogenic in laboratory animals supporting its clinical development as a malaria vaccine candidate. This paper describes a novel and cost-effective method for refolding of recombinant PfF2 that can be employed for development of vaccine against *P. falciparum* malaria.

2. Materials and methods

2.1. Expression of recombinant receptor binding domain of P. falciparum EBA-175 (PfF2) using fed-batch fermentation

Media composition for fed-batch cultivation was same as described earlier [18]. Aliquots (1 ml) from frozen cell banks of *E. coli* BLR(DE3) pLysS pET28a(+) synPfF2 were used to inoculate 50 ml primary seed culture and grown at 37 °C for 8-10 h. Recombinant strain was grown in 10 L defined medium in the reactor vessel of a 20 L bioreactor (Applikon, The Netherlands) equipped with analytical devices including mass flow controller, feed control system for automated feeding and software for data sampling and analysis (Bio-expert from Applikon, The Netherlands) in fed-batch mode. All fermentation conditions were maintained as described earlier [18] and glucose feeding was carried out to allow the volumetric cell mass concentration to increase exponentially based on equations described by Korz et al. [20]. The glucose feed with a specific growth rate of 0.12 h^{-1}, was continued during the growth phase. During the induction phase specific growth rate was decreased to 0.1 h^{-1}. Culture was induced with 1 mM IPTG and harvested 4 h post-induction. The cells were harvested by centrifugation. The cell pellet was stored at −80 °C until use.

2.2. Refolding and purification of recombinant PfF2

Cells harvested from 10 L culture were lysed by Dynomill (WAB, switzerland) in the 5 L lysis buffer (Tris.Cl 10 mM, NaCl 50 mM, EDTA 5 mM, Benzamidine.HCl 3.92 g, DTT 7.77 g, lysozyme 100 mg, pH 8.0). Inclusion bodies (IBs) were collected by centrifugation at 12,000 × *g* for 45 min [11]. The resultant IBs were dissolved in 8 M guanidine-hydrochloride. The whole denatured protein was purified by metal affinity chromatography using streamline-100 column (GE healthcare USA) [bed volume 300 ml, 4 cm] charged with Ni^{2+} as described earlier [11]. Protein thus obtained after immobilized metal affinity chromatography was used in the development of a novel and cost-effective refolding process for improved efficiency. Each refolding was performed with 30 mg protein diluted in 250 ml refolding buffer. Different refolding buffers were tested to identify conditions for improved refolding efficiency. The following conditions were used in the refolding buffer:

1. Control refolding buffer (50 mM citrate-phosphate buffer, pH 5.8, 2 mM cystine base, 0.67 mM cystamine dihydrochloride, 1 M urea and 0.5 M arginine, 0.034% Triton X-100 and 0.54% β-cyclodextrin).
2. Test buffer 1 (50 mM citrate-phosphate buffer, pH 5.8, 2 mM cystine base, 0.67 mM cystamine dihydrochloride, 1 M urea and 0.5 M proline)
3. Test buffer 2 (50 mM citrate-phosphate buffer, pH 5.8, 2 mM cystine base, 0.67 mM cystamine dihydrochloride, 1 M urea, 0.5 M proline, 0.034% Triton X-100 and 0.54% β-cyclodextrin)
4. Test buffer 3 (50 mM citrate-phosphate buffer, pH 5.8, 2 mM cystine base, 0.67 mM cystamine dihydrochloride, 1 M urea and 0.5 M arginine),
5. Test buffer 4 (50 mM citrate-phosphate buffer, pH 5.8, 2 mM cystine base, 0.67 mM cystamine dihydrochloride, 1 M urea, 0.5 M arginine, 0.5 M proline,0.034% Triton X-100 and 0.54% β-cyclodextrin).

Solution of β-cyclodextrin was added after 10 min addition of the protein wherever it is used. The protein was refolded by rapid dilution in the refolding buffer for 24 h at 10 °C, dialyzed for 48 h in dialysis buffer (50 mM citrate-phosphate buffer, pH 5.8, 1 M urea). During dialysis the buffer was exchanged three times. Refolded PfF2 was loaded on a SP-Sepharose column (XK 16/20, GE Healthcare, USA) equilibrated with 50 mM citrate-phosphate buffer (pH 5.8) [10,13]. The bound protein was eluted with a segmented gradient of NaCl (100, 300, 750 and 1000 mM NaCl) using AKTA purifier (GE Healthcare, USA). Fractions containing refolded PfF2 were pooled and concentrated using Amicon ultra 10 kDa membranes (Millipore, USA). Based on comparison test buffer 1 was selected to refold different amount of PfF2 (120–1000 mg per litre refolding buffer) followed by dialysis and purification as described above.

2.3. Scale up of refolding conditions

Based on comparison of refolding yields under test buffer 1 condition, recombinant PfF2 (600 mg) was refolded in 1000 ml of optimized refolding buffer (50 mM citrate-phosphate buffer, pH 5.8, 2 mM cystine base, 0.67 mM cystamine dihydrochloride, 1 M urea and 0.5 M proline) for 24 h at 10 °C. The refolded protein was dialyzed for 48 h with three buffer exchanges and further purified as described above.

2.4. Characterization of refolded and purified PfF2

2.4.1. Electrophoresis mobility shift

Refolded PfF2 (10 μg) was reduced with SDS-PAGE buffer containing β-mercaptoethanol and separated by SDS-PAGE. Refolded PfF2 was also dissolved in non-reducing SDS-PAGE buffer lacking β-mercaptoethanol or other reducing agents. The samples were treated for 10 min at 100 °C. Reduced and non-reduced PfF2 were separated by SDS-PAGE and detected by Coomasie Brilliant Blue R-250 staining.

2.4.2. Analysis of refolded, purified PfF2 by reverse phase chromatography

Refolded PfF2 was loaded on a C8 column. The gradient used for elution was developed using Buffer A [0.05% trifluoroacetic acid (TFA) in water] and Buffer B (0.05% TFA in 100% acetonitrile). The column was initially equilibrated with 95% Buffer A and 5% Buffer B, reached a composition of 90% Buffer A and 10% Buffer B in 10 min

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