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# Two-step chromatographic purification of recombinant *Plasmodium falciparum* circumsporozoite protein from *Escherichia coli*

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**Abstract**

The *Plasmodium falciparum* circumsporozoite (PfCS) protein (aa 19–405) has been cloned and expressed in *E. coli*. The protein was purified in a two-step process that was rapid and reproducible. *E. coli* cells were grown to a high density before induction for 1 h. Cells were disrupted by high pressure microfluidization and the total bacterial protein solubilized in 6 *M* Gu-HCl. The protein was refolded while bound to Ni–NTA agarose by exchange of 6 *M* Gu-HCl for 8 *M* urea and then slow removal of the urea. The eluted protein was further purified on Q Sepharose Fast Flow using conditions developed to remove *E. coli* proteins and reduce endotoxin (to 10 EU/50 μg). Yield was 20 mg of PfCS protein from 10 g of wet cell paste. The final protein product bound to HepG2 liver cells in culture and inhibited the invasion of those cells by sporozoites in an ISI assay greater than 80% over control cultures when used at 10 μg/ml. 

*Keywords:* *Plasmodium falciparum*; *Escherichia coli*; Recombinant protein expression; Purification; Circumsporozoite protein

## 1. Introduction

The circumsporozoite (CS) protein, the major surface protein on the infective sporozoite stage of *Plasmodium falciparum*, is a major vaccine candidate against malaria. It has been shown by Nussenzweig et al. to play a critical role in the invasion of sporozoites into hepatocytes [1]. The gene encodes a protein of 405 amino acids that contains an amino-terminal signal peptide and a carboxy-terminal anchor domain typical of a membrane protein. The PfCS protein contains a large central repeat domain composed of tetrapeptides of sequence NANP or NVDP. The *P. falciparum* gene was first cloned by Dame et al. [2] but its recombinant expression and purification was difficult and disappointing. Even at the time of its initial cloning using the lambda-gt11 system, its stable expression proved precarious. Four out of five genes analyzed from that expression screening were in reverse order to the gene's promoter and the fifth gene had an early nucleotide deletion and was out of reading frame. It was presumed, at the time, that the product of the gene was so toxic to the *E. coli* that only those clones with extremely low expression levels were able to grow and, thus, were selected by the anti-repeat domain specific monoclonal antibodies. Attempts to subclone the entire

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gene and express it in bacteria to achieve a full-length recombinant PfCS protein to be used for a malaria vaccine failed [3]. Consequently, the first recombinant malaria vaccine, FSV1, contained only 32 tetrapeptide repeat units expressed as a fusion protein with 32 amino acids derived from an open reading frame in the $Tet^r$ gene.

While the development of a malaria vaccine based on recombinant expression of various portions of the PfCS protein has proceeded [3–6], the analysis of the immune response to the vaccine has relied primarily on the recognition of synthetic peptides. These small sequences may not be able to reproduce the tertiary structure that is an inherent part of the full-length PfCS protein. Because the native PfCS molecule contains five cysteines and 54 prolines, amino acids that can greatly influence protein structure, the tertiary structure of the recombinant PfCS is likely to influence its presentation to the immune system. There is strong evidence to suggest disulfide bridge formation between some cysteines. Reduction and alkylation of the protein abolish binding to sulfatides [7] and mutagenesis of the cysteines to alanines greatly affects the binding of the PfCS protein to liver cells [8].

Eukaryotic proteins are frequently not folded correctly when synthesized in prokaryotes, resulting in an insoluble, non-native form of recombinant protein which is usually stored in inclusion bodies [9]. Published protocols for purification of inclusion bodies often include solubilization by detergents or by strong chaotropic salts. Some refolding strategies have included dialysis and stepwise or continuous removal of the denaturant while the target protein is bound to the resin. Although the refolding of globular proteins from inclusion bodies is used more often today, only a few membrane proteins have been refolded. Rogl et al. [10] reported refolding of two different membrane proteins using chaotropic salts and detergents. Frankel et al. [11] diluted protein with renaturation buffer containing oxidized glutathione and 0.5 *M* L-arginine HCl and refolding was allowed to proceed at 4°C for 44–60 h followed by 24 h of dialysis. Gupta et al. [12] tried to purify a protein immobilized on Ni–NTA resin under denaturating conditions using gradual removal of the denaturant followed by several dialysis steps. Another group, Holzinger et al. [13] accomplished solubilization of the target protein bound to a Ni–NTA column in a single step by omitting the denaturant from the last wash and elution buffers. Takacs et al. [14] successfully expressed and purified a fragment of the PfCS protein both as a fusion with a ''merozoite protein'' and as a 6×-His-tagged molecule. However, that procedure did not work to purify the full length the PfCS molecule produced here. Therefore, we report the development of a process that yields a significant amount of soluble, stable full-length PfCS protein. The purification protocol presented in this study allows for a gradual renaturation of the PfCS protein in an effort to refold it into a more native structure while at the same time achieving a purity acceptable for a human-use vaccine. The function of the CSP molecule on the sporozoite is to enable it to bind to receptors on the liver cells, therefore, in order to test the biological function of the recombinant CSP we allowed the protein to bind to heptocytes in culture and tested its ability to interfere with the invasion of sporozoites in an inhibition of invasion assay (ISI).

## 2. Experimental

### 2.1. Chemicals, solutions and buffers

Guanidine hydrochloride (Gu-HCl), urea and glycerol (Fisher Biotech, Fair Lawn, NJ, USA); imidazole, β-mercaptoethanol (β-ME), EGTA and $NiCl_2$ (Sigma Chemical Co., St. Louis, MO, USA); magnesium chloride, monobasic sodium phosphate (monohydrate) and 10 *M* NaOH solution (J.T. Baker, Phillipsburg, NJ, USA); 0.5 *M* EDTA solution (disodium) and 10×phosphate buffered saline (PBS) solution (Digene, Beltsville, MD, USA); goat anti-rabbit AP conjugate and goat anti-mouse AP conjugate (Promega, Madison, WI, USA); benzonase (EM Industries, Merck KGaA, Darmstadt, Germany); NOVEX buffer system (Invitrogen Corporation/Novex, Carlsbad, CA, USA); rabbit anti *E. coli* (Dako Corporation, Carpinteria, CA, USA); mouse anti-poly-His-tag 6×His monoclonal antibody (Clontech Laboratories, Palo Alto, CA, USA); anti-PfCS C-term peptide $K_{282}$–$S_{383}$ (generous gift from G. Corradin of the University of Lausanne, Switzer-

land); NBT/BCIP ready-to-use tablets (Roche, Indianapolis, IN, USA); dibasic sodium phosphate (heptahydrate) (Spectrum Chemical Mfg. Corp, Gardena, CA, USA); Ni–NTA Superflow resin (Qiagene, Valencia, CA, USA); 2 *M* HCl solution (VWR, West Chester, PA, USA); Q Sepharose Fast Flow (Pharmacia LKB, Biotechnology AB, Uppsala, Sweden); Bio-Rad DC protein assay kit (Melville, NY, USA); Limulus amebocyte lysate (LAL) chromogenic endpoint assay kit (Associates of Cape Cod, Falmouth, MA, USA).

All solutions and buffers were made with deionized water (18 mΩ) and where appropriate the pH was adjusted with either 2 *M* HCl or 2 *M* NaOH. Water, solutions and buffers were prepared fresh for each run and filtered through sterile 0.45-μm filters (Nalgene, Rochester, NY, USA).

L1: (Lysis buffer): 20 m*M* phosphate buffer (1.06 m*M* $NaH_2PO_4 \cdot H_2O$, 18.94 m*M* $Na_2HPO_4 \cdot 7H_2O$, 100 m*M* NaCl, 2 m*M* EGTA, 1 m*M* imidazole, 2 m*M* $MgCl_2$) pH 8.0.

N1: (Ni–NTA stripping solution): 0.1 *M* HCl.

N2: (Ni–NTA recharging solution): 0.25 *M* $NiCl_2$.

N3: (Ni–NTA loading buffer): 8 *M* urea, 10 m*M* phosphate buffer (0.53 m*M* $NaH_2PO_4 \cdot H_2O$, 9.47 m*M* $Na_2HPO_4 \cdot 7H_2O$), 20 m*M* NaCl, 1 m*M* imidazole, pH 8.0.

N4: (Ni–NTA refolding buffer): 10 m*M* phosphate buffer (as above), 20 m*M* NaCl, 1 m*M* imidazole, pH 8.0.

N5: (Ni–NTA elution buffer): 10 m*M* phosphate buffer (as above), 20 m*M* NaCl, 50 m*M* imidazole, pH 8.0.

Q1: (Q Sepharose sanitizing solution): 0.5 *M* NaOH.

Q2: (Q Sepharose 10×loading buffer): 100 m*M* sodium phosphate buffer (5.3 m*M* $NaH_2PO_4 \cdot H_2O$, 94.7 m*M* $Na_2HPO_4 \cdot 7H_2O$), 200 m*M* NaCl, pH 8.0.

Q3: (Q Sepharose concentrated salt solution): 1 *M* NaCl

WB1: (WB blocking buffer): 1×PBS, 0.1% Tween-20, 5% powdered milk.

WB2: (WB wash buffer): 1×PBS, 0.1% Tween-20.

### 2.2. Cloning and cell expression

Because the use of penicillin-like antibiotics is prohibited in the production of human used recombinant protein vaccine production, the plasmid pET-32a (Invitrogen, San Diego, CA, USA) was modified by the insertion of a cassette encoding the gene for kanamycin resistance (Amersham Pharmacia Biotech, Piscataway, NJ, USA) into the single Tth111 site to yield pET-32aK. The gene for thioredoxin was removed from pET–32aK by Nde I digestion and religation to yield pET-32aK-trx. The PfCS gene (bp 51–1218 including the terminal stop codon) from the 3D7 clone of *P. falciparum* was cloned into the Bam HI–Sal I site of the multiple cloning site (MCS) of pET-32aK-trx. The PfCS gene was obtained from another laboratory clone, CS-FL pMV261 used to make the *P. falciparum* (3D7) PfCS gene insert for NYVAC-Pf7 [5], by restriction digestion, ligation and transformation into DH5-α *E. coli* cells. The inserted gene and flanking regions were sequenced after cloning for verification. The resulting PfCS-FL–pET-32aK-trx plasmid translation product was: MHHHHHHSSGLVPRGSGMKETAAAKFEAQHMNSPNLGTNNNNKAMANIGS-$CSP_{L19\text{-}N405}$. The recombinant protein contained 438 amino acids, had a calculated molecular mass of 47 kDa and had a p*I*-value of 5.48. The 6×-His upstream region also contained the sites for cleavage by thrombin and enterokinase as well as the S-tag site for binding S-protein (Invitrogen). For expression of the PfCS protein, the plasmid was transformed into BLR (DE3) cells. Using a Bioflo 3000 10 l fermenter (New Brunswick, Edison, NJ, USA) cells were grown at 37°C in SB medium (12 g/l tryptone, 24 g/l yeast extract, 6.3 ml/l glycerol, 12.5 g/l $K_2HPO_4$, 3.8 g/l $KH_2PO_4$; pH 7.2) with kanamycin to an optical density at 600 nm ($OD_{600}$) of 6.0. The PfCS protein production was induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 0.2 m*M* and cell growth continued for 1 h. At this point the $OD_{600}$ was 10 and cell growth had ceased.

The cells were harvested by centrifugation, the

supernatant discarded and the cell paste stored at −70°C. It was found that storage of cell paste in thin layers on wax paper wrapped in aluminum foil greatly facilitated the removal of small quantities of paste for process development.

### *2.3. Extraction, solubilization, and denaturation of the inclusion bodies*

For extraction, 100 g of frozen paste was re-suspended in a final volume of 500 ml of lysis buffer (L1) and stirred on ice for 30 min. Ten minutes prior to cracking, 120 μl of benzonase (to a final concentration of 60 units/ml) and 200 μl of β-ME (to a final concentration of 2 m*M*) were added. The cells were cracked using a microfluidizer (Model 1109, Microfluidic Corp., Newton, MA, USA) until the solution became translucent. The chamber of the microfluidizer was cooled during the process and the starting and recipient bottles were kept on ice throughout the procedure. After microfluidization, 200 ml of glycerol was added to the cell lysate. The solution was then added to a beaker containing 573 g of solid Gu-HCl to achieve a final concentration of 6 *M* Gu-HCl in a total volume of approximately 1 l. The suspension was stirred for 30 min on ice, then the cell lysate was centrifuged for 1 h at 22 000 *g* at 4°C. The pellet was discarded and the supernatant was aliquoted and stored at −70°C for further process development.

### *2.4. Chromatographic procedure*

All experiments were performed at room temperature (RT). All chromatographic purification steps were performed using a Waters HPLC 600E system with Waters 486 UV tunable detector or a Waters FPLC 650E system with Waters 441 UV absorbance detector (Waters Corporation, Milford, MA, USA). The PfCS protein was purified in a two-step procedure: Ni–NTA affinity chromatography and anion-exchange chromatography on Q Sepharose Fast Flow resin.

#### *2.4.1. Immobilized metal affinity chromatography on Ni–NTA superflow resin*

A Waters AP-2 glass column was packed with Ni–NTA Superflow resin to a bed volume of 28 ml. Due to lot-to-lot variation with commercial Ni–NTA resin we found it more consistent to strip and recharge the resin before each run. The resin was recharged as follows: packed resin was washed with five column volumes (CVs) of water and then was stripped with two CVs of solution N1. The resin was further washed with five CVs of water or until the pH of the eluate reached pH 4–5. The resin was then recharged with solution N2 at a flow-rate of 2 ml/min. The resin was washed again with five CVs of water and equilibrated with ten CVs of buffer N3.

Frozen denatured cell lysate (100 ml of lysate from 10 g wet paste) was thawed and loaded (2 ml/min) onto the Ni–NTA column through the 600E manifold using a 60-ml syringe. After loading, non-specific and unbound proteins were removed by washing the resin with buffer N3 until the $OD_{280}$ reached a background level. During this wash step the protein denaturant, 6 *M* Gu-HCl, was exchanged for 8 *M* urea. The refolding–renaturation of the PfCS protein was then performed while bound on the Ni–NTA column using two linear gradients. The first gradient reduced the urea concentration from 8 to 4 *M* in 30 min. This was followed with the second gradient that further reduced the urea concentration from 4 to 0 *M* in 3 h. This was followed by elution of a major protein peak with buffer N5. Collection of the protein started when the $OD_{280}$ went above the initial baseline of 0.01 and finished when a new baseline was reached, higher than the starting baseline due to the presence of the imidazole in the elution buffer. The protein sample eluted from the Ni–NTA column could be stored at 4°C after adding EDTA to a final 1 m*M* concentration.

#### *2.4.2. Anion-exchange chromatography on Q Sepharose fast flow resin*

A Waters AP-2 glass column was packed with Q Sepharose Fast Flow resin to 22-ml bed volume. The resin was sanitized before each run with five CVs of solution Q1 followed by ten CVs of water, or until the pH was lowered to pH 7. Then the Q Sepharose resin was equilibrated with ten CVs of 1×Q2 (isocratic proportion: 10% Q2, 90% water). The protein sample eluted from the Ni–NTA column was diluted with an equal volume of 1×Q2, lowering the imidazole concentration and the ionic strength, allowing it to be loaded directly onto the anion-

exchange resin. This diluted sample was applied to the Q Sepharose column through the Waters FPLC 650E manifold using a syringe at the flow-rate of 2 ml/min. The column was washed with five CVs of 1×Q2 or until the $OD_{280}$ reached a background level. The purified PfCS protein was eluted (Fig. 1A) at the beginning of the isocratic proportion: 10% Q2, 15% Q3, 75% water.

### 2.5. Analytical procedures

#### 2.5.1. Electrophoresis

To monitor the purification during the chromatographic steps, protein was analyzed by SDS–PAGE using the Nu PAGE Electrophoresis System (Invitrogen/Novex) with precast 4–12% Bis–Tris gels according to the manufacturer's guidelines. Protein samples were diluted 3:1 in NuPAGE 4×sample buffer, 1:10 (v/v) of 0.1 *M* DTT was added and the sample heated to 80°C for 5 min. Electrophoresis was performed at 150 V for 1 h. Visualization of the proteins were done by Coomassie Brilliant Blue R 250 staining; multi-colored molecular mass markers (Invitrogen/Novex) were employed on all gels.

#### 2.5.2. Western blotting

Proteins were transferred to 0.2-μm nitrocellulose membranes (Invitrogene Corporation/Novex) at 30 V for 1 h. The membranes were blocked with WB1 for 1 h at RT. After three 10-min washes with WB2 each membrane was incubated for 1 h at RT with a solution of specific primary antibodies in WB2 (see figure legends). The membranes were washed as above and then reacted with the appropriate secondary antibody in WB2 for 1 h at RT. The blots were washed as above and immunoreactive bands were revealed by using NBT/BCIP ready-to-use tablets as detailed by the manufacturer.

#### 2.5.3. Protein concentration

Protein concentration was estimated using the Lowry method [15] according to the Bio-Rad DC protein assay instruction manual except for micro-scale experiments for optimizing the Q Sepharose binding where the method of Bradford [16] was used. A Beckman DU 600 spectrophotometer was used and bovine serum albumin (BSA) was used as the standard. Protein concentration results correlated well with UV detection and calculations using a molar extinction coefficient of 23 500 from the deduced amino acid sequence to achieve a calculated $OD_{280}=1$ for a 2.0-mg/ml solution of PfCS protein [17]. Imidazole has a significant $OD_{280}$ absorbance so reference blanks were prepared with the same imidazole content as the analyzed samples.

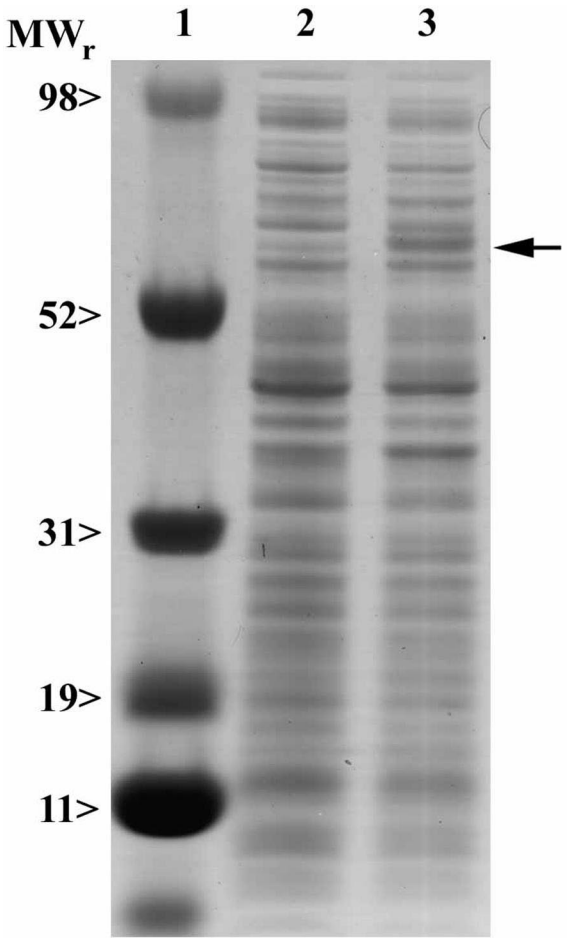


Fig. 1. Induction of bacterial expression of recombinant PfCS protein. Coomassie blue-stained 4–12% SDS–PAGE of total bacterial lysate before induction (lane 2) and 1 h after induction (lane 3). Relative molecular mass markers are in lane 1 and their molecular masses ($\times 10^{-3}$) are indicated on the left. The arrow indicates PfCS expression in lane 3.

#### 2.5.4. Endotoxin test

Endotoxin content was estimated using the chromogenic LAL endpoint assay [18]. Dilutions of

all protein samples and standards for the LAL test were prepared in pyrogen-free vials. Positive control solutions prepared for the standard curves ranged from 1 endotoxin unit (EU)/ml to 0.06 EU/ml in two-fold serial dilutions (total of a five-point standard curve). A 96-well plate heater (Associates of Cape Cod) was used for incubation at 37°C for 20 min. The plates were read at 405 nm on a $V_{max}$ kinetic microplate reader (Molecular Devices, Sunnyvale, CA, USA).

### *2.6. Inhibition of sporozoite invasion*

The inhibition of sporozoite invasion (ISI) assay was preformed by a previously described method [19]. Briefly, HepG2 A16 cells were grown on Labtek slides. NF54 sporozoites were obtained from *An. stephensi* mosquitoes. Aliquots of 50-μl of diluted purified PfCS protein (10, 5 and 1 μg/ml), or as a negative control, LSA-C [20] recombinant protein in culture medium, were placed per well. Immediately thereafter 20 000 sporozoites in 50 μl of medium were added to each well. NFS1 (100 μg/ml) monoclonal antibody [19] was used as a positive control. Each protein concentration was tested in triplicate and the experiments were repeated twice. HepG2 cells, protein and sporozoites were incubated for 3 h. The cells were washed twice with PBS, fixed with cold methanol and washed again with PBS before analysis. Sporozoites that had invaded the HepG2 cells were detected by using a monoclonal antibody, NFS1 (10 μg/ml), as a primary antibody (30 min, RT) and an anti-mouse IgG peroxidase conjugate as a secondary antibody (30 min, RT).

### *2.7. Binding of PfCS protein to HepG2 cells*

HepG2 A16 cells were grown on Labtek slides. Culture medium containing 10 μg/ml PfCS protein, or as a negative control, LSA-C [20] recombinant protein, was placed per well. HepG2 cells, protein and sporozoites were incubated for 30 min. Cells were washed twice with PBS, fixed with cold methanol and washed again with PBS before analysis. Protein that had bound to the HepG2 cells was detected by using a monoclonal antibody, NFS1 (10 μg/ml), as a primary antibody (30 min, RT) and an anti-mouse IgG peroxidase conjugate as a secondary antibody (30 min, RT).

## 3. Results and discussion

### *3.1. Extraction, solubilization, and denaturation of the PfCS protein*

The PfCS protein produced in this study was found both in insoluble inclusion bodies and in the soluble protein fraction (data not shown). Therefore, in order to obtain the highest yield, the PfCS protein was purified from the total bacterial protein extract rather than only the inclusion bodies being isolated. Guanidine and urea were used rather than a detergent for the solubilization of existing inclusion bodies, in order to avoid the difficulty of detergent removal or its reduction to acceptable regulatory levels for vaccine use.

### *3.2. Immobilized metal affinity chromatography on Ni–NTA superflow resin*

Immobilization of the PfCS protein during the solubilization step presumably restricts interaction with neighboring molecules, preventing aggregation and promoting refolding. For optimization and reproducibility of Ni–NTA separation we determined the capacity of the column for PfCS protein and developed refolding conditions. We found lot-to-lot variability in ligand capacity of commercial Ni–NTA resins (data not shown). Therefore, to be assured of more consistent $Ni^{2+}$ loading we choose to strip and recharge the resin before each experiment. This resulted in fraction peaks that were sharp and more symmetrical and contained a higher concentration of the desired PfCS protein. We found that the optimal sample loading volume on 28 ml of Ni–NTA resin was 100 ml of the suspension containing approximately 10 g (wet weight) of lysed cells, or roughly 3 ml of Ni–NTA resin needed for each 1 g of wet cell paste. Non-specific binding on the Ni–NTA resin was reduced by including 1 m*M* imidazole in the Ni–NTA loading and refolding buffers (N3 and N4). To optimize refolding to yield a soluble product we investigated different gradient slopes and different time lengths of refolding. Optimal refolding–renatu-

ration on the Ni–NTA column took place by changing buffer from 6 *M* Gu-HCl to 8 *M* urea following by refolding–renaturation on Ni–NTA column with two subsequent linear urea gradients. The first gradient reduced the urea concentration from 8 to 4 *M* in 30 min. This was followed by the second gradient to reduce the urea concentration from 4 to 0 *M* in 3 h. Most of the PfCS protein was then eluted at 50 m*M* imidazole with buffer N5. This protein was soluble and stayed in solution without the requirement of a denaturant. Fractions eluted at 300 m*M*, 500 m*M* and 1 *M* imidazole did not contain PfCS protein. As a result, 150 mg of total protein could be recovered from 10 g of wet paste in a single sharp peak using 28 g of Ni–NTA resin. This represented a yield of 12.5% calculated from the dry weight of the total in the paste (Table 1). This protein peak, while enriched for the PfCS protein, was still contaminated with many bacterial proteins (Fig. 1A, lane 3). The endotoxin content of this peak fraction was 3500 EU/50 μg of protein.

### *3.3. Anion-exchange chromatography on Q Sepharose fast flow resin*

Because ion-exchange chromatography separates biomolecules based on surface charge, which can vary with buffer pH, an attempt was made to improve the binding of PfCS to Q Sepharose by first optimizing pH and then determining optimal salt concentration. Aliquots of the protein-containing sample eluted from the Ni–NTA column were mixed in microfuge tubes with a small amount of ion-exchange resin that had been equilibrated with a series of buffers of different pH. The optimal pH was chosen by monitoring the levels of PfCS protein and endotoxin in the supernatant. Total protein presence was estimated by the method of Bradford [16]. Western blot revealed that more *E. coli* proteins eluted at pH 6.5 to 7.5 than at pH 8.0 (data not shown). The study was repeated with another series of tubes containing Q Sepharose resin equilibrated with a series of salt (NaCl) solutions of different concentrations, all at pH 8.0. Because of these studies, 1×Q2 buffer (10 m*M* phosphate loading buffer at pH 8.0, 20 m*M* NaCl) was developed for the Q Sepharose column. Despite manufacturers recommendations for the binding capacity of Q Sepharose Fast Flow resin being up to 120 mg of human serum albumin for each millilitre of gel, we found only 0.5 mg of Ni–NTA eluted protein would bind per 1 ml of gel. Subsequently, 22 ml of Q Sepharose resin was used to purify each 12.5 mg of protein eluted in the Ni–NTA main fraction. Using a step gradient, three major peaks were obtained (Fig. 1B), a flow through peak at 20 m*M* NaCl (Q1), a peak at approximately 150 m*M* NaCl (Q2) and another peak at 1 *M* NaCl (Q3). Western blot analysis and LAL tests of these fractions revealed that the flow through and 1 *M* NaCl eluted peaks consisted of *E. coli* proteins and endotoxin. The 150 m*M* NaCl peak contained the PfCS protein with low amount of *E. coli* protein contamination (Fig. 1) and low of endotoxin levels (10 EU/50 μg protein). For scale-up of the purification of PfCS from 10 g of wet paste, it required the use of 300 ml of Q Sepharose

Table 1
Purification of recombinant PfCS protein

| Sample | Total protein weight (mg) | Calculated protein yield (%) | Purity of PfCS protein[a] (%) | Endotoxin level (EU/50 μg protein) |
|---|---|---|---|---|
| Wet cell paste (10 g) (2000 mg dw=20% of ww) | 1200 (estimated as 60% of dw) | 100.0 | <1 | nd |
| Ni–NTA (50 m*M* imidazole fraction) | 150 | 12.5 | 55 | 3500 |
| Q Sepharose (150 m*M* NaCl fraction) | 20 | 1.6 | >95 | 10 |

ww=Wet weight; dw=dry weight; nd=not done; EU=endotoxin units.
[a] Estimated from Coomassie blue stained SDS–PAGE.

resin to assure minimal protein loss due to column overloading. Following these procedures, 20 mg of PfCS protein could be recovered from 10 g of wet cell paste.

### 3.4. SDS–PAGE and Western blot analysis

The apparent molecular mass of PfCS protein observed by SDS–PAGE, 62 kDa, is different from the deduced molecular weigh of 47 kDa; this aberration has previously been reported [2,3]. The expression of a small amount of PfCS protein can be seen in Coomassie Blue stained gels of total *E. coli* cell lysate after induction (Fig. 2). Although the majority of the protein forms inclusion bodies, it is still toxic to the cell. The growth of the culture stops shortly after induction, barely allowing for a doubling in cell density. For that reason we allowed the cell to grow to a high density before induction and then harvested 1 h after induction of recombinant gene expression.

We achieved enrichment of the PfCS protein after Ni–NTA affinity (Fig. 1B). Further separation and enrichment was achieved on Q Sepharose and the resulting PfCS protein preparation was significantly free of *E. coli* proteins (Fig. 3) and endotoxins.

### 3.5. ISI

The recombinant PfCS protein bound to hepatoma cells in culture (Fig. 4) and inhibited the invasion of sporozoites in a concentration dependent manner (Fig. 5). At 10 μg/ml, the PfCS protein inhibited invasion greater than 80% compared with the control protein.

## 4. Conclusion

We report a method to obtain a significant amount of a highly purified recombinant PfCS protein from *E. coli* (20 mg/10 g wet cell paste) that is functional active, in so far as it binds to heptocytes and interferes with sporozoite invasion. We cloned the entire gene, except for the putative signal sequence,

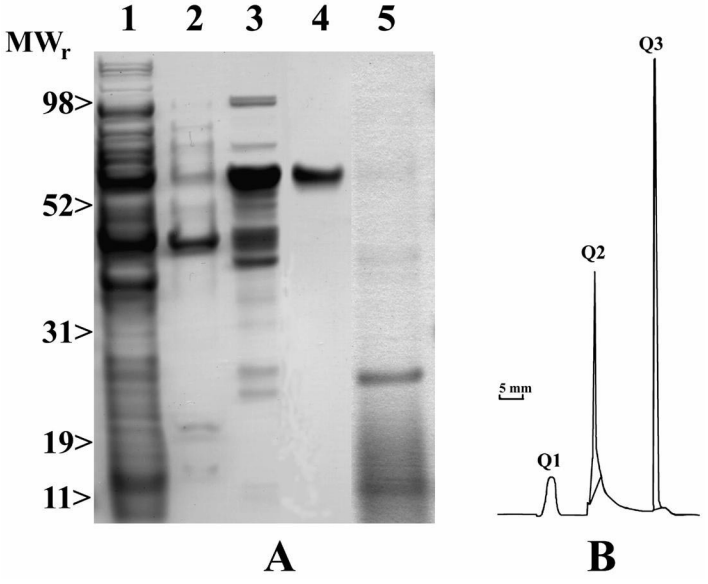


Fig. 2. Purification of PfCS protein. (A) Coomassie blue stained 4–12% SDS–PAGE of select fractions during the purification process: (1) bacterial cell lysate loaded on Ni–NTA, (2) flow through from Ni–NTA, (3) fraction eluted at 50 m*M* imidazole from Ni–NTA, (4) fraction eluted at 150 m*M* NaCl from Q Sepharose Fast Flow, (5) fraction eluted at 1 *M* NaCl from Q Sepharose Fast Flow. Relative molecular masses are indicated ($\times 10^{-3}$). (B) Chromatogram from Q Sepharose Fast Flow at 280 nm: (Q1) flow through, (Q2) fraction eluted at 150 m*M* NaCl, (Q3) fraction eluted at 1 *M* NaCl. The PfCS protein elutes in peak Q2 and is shown in Fig. 1A, lane 4. Flow-rate=6 ml/min, chart speed=1 mm/min.

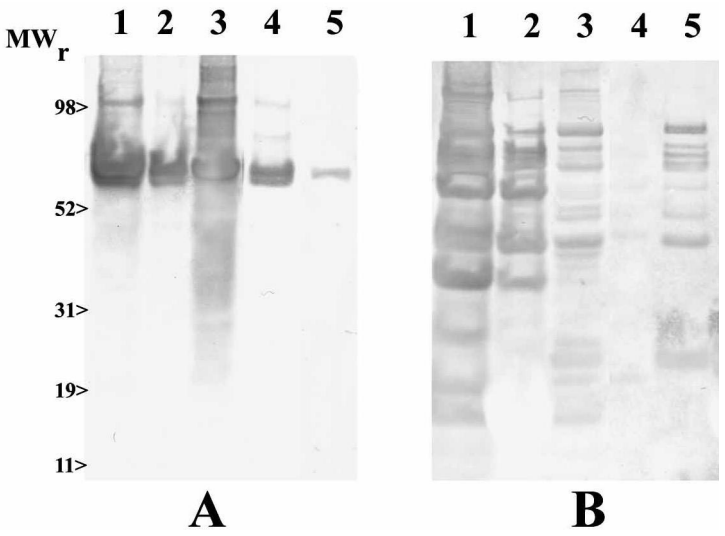


Fig. 3. Western blot analysis of PfCS protein. Lanes in both gels are transfers of duplicate SDS–PAGE gels shown in Fig. 2A. (A) Primary antibody was mouse anti-PfCS C-terminal peptide polyclonal antibody diluted 1:20 000. Bound antibodies were detected with a goat anti-mouse IgG AP conjugate diluted 1:10 000. (B) Primary antibody was rabbit anti-*E. coli* diluted 1:5000. Bound antibodies were detected with a goat anti-rabbit IgG AP conjugate diluted 1:10 000. Relative molecular weights are indicated ($\times 10^{-3}$).

into a plasmid vector under tight regulation of recombinant gene expression. Growing the bacteria to a high density before induction allowed increased total accumulation of the PfCS protein before it became toxic to the culture. The combination of microfluidization and solubilization in chaotropic guanidine hydrochloride ensured maximum recovery of the PfCS protein. The combination of Ni–NTA affinity chromatography allowing on-column refolding resulted in an aqueous soluble PfCS protein that could be further purified by anion-exchange chromatography. Furthermore, development of conditions that allowed application of the Ni–NTA eluate onto the anion-exchange column without prior dialysis or buffer exchange resulted in a fast, efficient, economical and reproducible purification procedure. This protocol resulted in significant lowering of endotoxin levels below those required by the US Food and Drug Administration for clinical grade vaccines, a major obstacle in protein production and purification

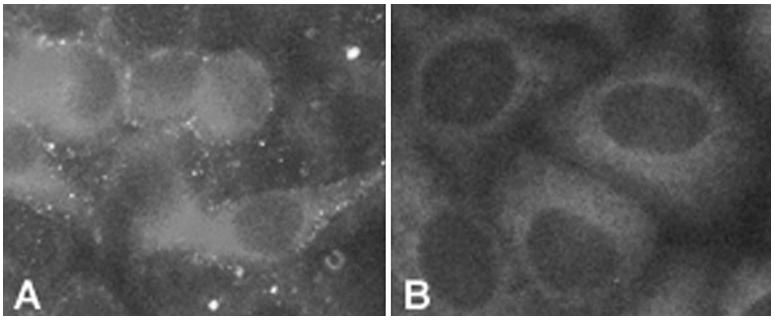


Fig. 4. PfCS protein binding to HepG2. (A) PfCS protein (10 μg/ml) or (B) LSA-C (10 μg/ml) were incubated with HepG2 cells in culture for 30 min. HepG2 cells were rinsed and probed with mAb NFS1.

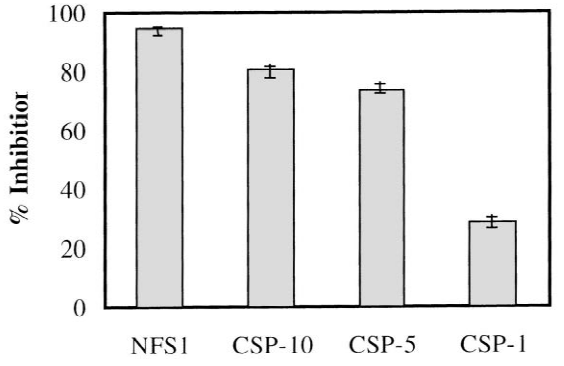


Fig. 5. Percent inhibition of sporozoites into HepG2 cells in the presence of decreasing concentrations of recombinant CSP or monoclonal antibody NFS1. CSP-10 (10 μg/ml), CSP-5 (5 μg/ml), CSP-1 (1 μg/ml). A recombinant LSA-C protein used as a negative control at equal concentrations showed no inhibition. Values are the mean of three experiments.

of potential vaccine material. The protein shows no loss of solubility or stability and remains immunoreactive for at least 1 year when stored at −20°C with the addition of 1 m*M* EDTA and 10% glycerol (v/v).

## References

[1] V. Nussenzweig, R.S. Nussenzweig, Cell 42 (1985) 401.
[2] J.B. Dame, J.L. Williams, T.F. McCutchan, J.L. Weber, R.A. Wirtz, W.T. Hockmeyer, W.L. Maloy, J.D. Haynes, I. Schneider, D. Roberts, G.S. Sanders, E.P. Reddy, C.L. Diggs, L.H. Miller, Science 225 (1984) 593.
[3] J.F. Young, W.T. Hockmeyer, M. Gross, W.R Ballou, R.A. Wirtz, J.H. Trosper, R.L. Beaudoin, M.R. Hollingdale, C.L. Diggs, Science 228 (1985) 958.
[4] J.A. Tine, D.E. Lanar, D. Smith, B.T. Wellde, P. Schultheiss, L.A. Ware, E. Kauffman, R.A. Wirtz, C. de Taisne, G.S.N. Hui, S.P. Chang, P. Church, D.C. Kaslow, M.R. Hollingdale, S.L. Hoffman, K.P. Guito, W.R. Ballou, J.C. Sadoff, E. Paoletti, J. Infect. Immol. 64 (1996) 3833.
[5] J.A. Stoute, K.E. Kester, U. Krzych, B.T. Wellde, T. Hall, K. White, G. Glenn, C.F. Ockenhouse, N. Garcon, R. Schwenk, D.E. Lanar, P. Sun, P. Momin, R.A. Wirtz, C. Golenda, M. Slaoui, G. Wortmann, C. Holland, M. Dowler, J. Cohen, W.R. Ballou, J. Infect. Dis. 178 (1998) 1139.
[6] T.P. Le, K.M. Coonan, R.C. Hedstrom, Y. Charoenvit, M. Sedegah, J.E. Epstein, S. Kumar, R. Wang, D.L. Doolan, J.D. Maguire, S.E. Parker, P. Hobart, J. Norman, S.L. Hoffman, Vaccine 18 (2000) 1893.
[7] C. Cerami, F. Kwaky-Berko, V. Nussenzveig, Mol. Biochem. Parasitol. 54 (1992) 1.
[8] D. Rathore, T.F. McCutchan, PNAS 97 (2000) 8530.
[9] The QIAexpressionist, Qiagen, March 1999, A handbook for high-level expression and purification of 6×-His-tagged proteins.
[10] H. Rogl, K. Kosemund, W. Kuhlbrandt, I. Collinson, FEBS Lett. 432 (1998) 21.
[11] A.E. Frankel, J. Ramage, A. Latimer, T. Feely, S. Delatte, P. Hall, E. Tagge, R. Kreitman, M. Willingham, Protein Express. Purif. 16 (1999) 190.
[12] P. Gupta, S.M. Waheed, R. Bhatnagar, Protein Express. Purif. 16 (1999) 369.
[13] A. Holzinger, K.S. Phillips, T.E. Weaver, BioTechniques 20 (1996) 804.
[14] B.J. Takacs, M. Girard, J. Immunol. Methods 143 (1991) 231.
[15] O.H. Lowry, N.J. Rosenbrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[16] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[17] Vector NTI, version 5, Informax, Rockville, MD (1999).
[18] P. Friberger, M. Knos, L. Mellstam, A quantitative endotoxin assay utilizing LAL and a chromogenic substrate, in: S.W. Watson, J. Levin, T.J. Novitsky (Eds.), Endotoxins and Their Detection With the Limulus Amebocyte Lysate Test, Prog. Clin. Biol. Res., Vol. 93, Alan R. Liss, NY, 1982, p. 195.
[19] Y. Charoenvit, V. Fallarme, W.O. Rogers, J.B. Sacci Jr., M. Kaur, J.C. Aguiar, L.F. Yuan, G. Corradin, E. Andersen, B. Wizel, R.A. Houghten, A. Oloo, P. De La Vega, S.L. Hoffman, Infect. Immol. 65 (1997) 3430.
[20] J.D. Kurtis, D.E. Lanar, M. Opollo, P.E. Duffy, Infect. Immol. 67 (1999) 3424.