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Stabilization of a chimeric malaria antigen in separation and purification through efficient inhibition of protease activity by imidazole

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ABSTRACT

A chimeric antigen M.RCAg-1 of *Plasmodium falciparum* expressed in *Escherichia coli* was previously demonstrated inhibiting the growth of malaria parasites *in vitro*, but its further development has been retarded by the antigen's instability during the downstream process. In this study, it was definitely demonstrated the instability was caused by the susceptibility of M.RCAg-1 to metalloprotease(s) released from the disintegrated host cells. Interestingly, imidazole showed better inhibition effects on the degradation than EDTA. Hence, a purification procedure was successfully developed to produce M.RCAg-1 with a purity of up to 95% and an overall recovery of nearly 600 mg/L culture. When performed this protocol following the Good Manufacturing Practice regulations, the endotoxin level, the host protein content and residual DNA level, all met the FDA standards. MALDI-TOF MS demonstrated a consistent molecular weight with the theoretical value and CD revealed a mainly disordered random coil secondary structure. Immunizing mice with M.RCAg-1 with Freund's adjuvant elicited high levels of specific antibodies. Moreover, M.RCAg-1 itself could be stable at 4 °C for up to 6 months. Our results would provide an efficient and robust protocol for the large-scale production of M.RCAg-1 which would warrant the further development of this promising malaria vaccine candidate.

1. Introduction

Malaria is an infectious disease of high morbidity and mortality in human populations in tropical countries. The most severe human malaria is caused by the infection of *Plasmodium falciparum* parasites [1,2]. Developing of prophylactic vaccines is believed to be the most crucial way for the global elimination of malaria [3]. However, the complex life cycle of the parasites poses unique challenges for the development of a valid malaria vaccine even after decades of endeavors [4].

Forty years ago, the radiation-inactivated *Plasmodium falciparum* sporozoites were demonstrated to confer complete protection against malaria infection [5], but the safety concerns and practical difficulties in large-scale production of sporozoites retarded their step going forward to practical applications [6]. There are more than 5000 proteins related to the parasite whose life cycle includes three stages: pre-erythrocytic, erythrocytic, and transmission [7,8]. Some of these proteins or protein fragments have been identified as predominant antigenic epitopes which could induce obvious immune responses [9]. Such proteins or protein fragments were named subunit antigens, including CSP [10], AMA1 [11], MSP1 [12], and so on [13]. Recently, the first malaria vaccine RTS,S/AS01B, a subunit vaccine based on CSP incorporated into a viral-like particle, has finished its Phase III clinical trial, but it just demonstrated an efficacy ranging from 26 to 50% in infants and young children against malaria [14–16].

Theoretically, a multistage and multi-epitope vaccine, which integrates several predominant epitopes from different stages and strains of the parasites, is expected to provide more effective protection against malaria infection than a single epitope [17]. Hence, several multistage and multi-epitope vaccine candidates have been constructed which have shown good protection against malaria invasion [18,19]. Such chimeric subunit vaccines are generally expressed by recombinant host cells [20,21]. Various expression systems including *E.coli* [22], *yeast* [19], baculovirus-infected insect cells [17], or transgenic plant [23] are used to produce these chimeric protein-based antigens of malaria. Among these expression systems, *E.coli* is an excellent host because of its high protein yield and low cost [24]. For any system, developing a

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repeatable and scalable purification process is a crucial step for the comprehensive evaluation of these chimeric proteins as valid malaria vaccine candidates and for the subsequent industrial production [25,26]. Until now, a number of multi-epitope vaccine candidates for malaria were designed, constructed, prepared at lab-scale and have demonstrated good protective immune responses [9]. However, as non-naturally evolved proteins, it is still a great challenge to characterize and produce such chimeric proteins at large-scale.

In our previous study, a multi-epitope chimeric antigen M.RCAg-1 was demonstrated the great efficacy of inhibiting the parasites growth in animal models [27]. M.RCAg-1 was composed of eleven key epitope peptides of *Plasmodium falciparum* and could be cheaply expressed in *E.coli* in soluble form. However, this chimeric protein would degrade fast after cell disintegration, making it very difficult to develop a feasible purification strategy to obtain enough qualified protein for the physicochemical characterization, toxicological and clinical studies. Here, by optimizing the basic buffer components to efficiently inhibit the proteases from host cell disintegration, we developed a scheme for the large-scale production of M.RCAg-1 with good reproducibility and high recovery. Also, purity, endotoxin level, host protein content and the residual DNA for the final sample under GMP conditions all met the pharmacopoeia criterion. Moreover, some special features were also disclosed for the chimeric protein by MALDI-TOF, CD and HPSEC analysis.

2. Materials and methods

2.1. Construction and expression of M.RCAg-1 in E.coli

E.coli BL21 (DE3) containing the recombinant plasmid of M.RCAg-1 was constructed and friendly provided by Institute of Basic Medical Sciences (China) [28]. Fermentation was carried out in a 40-L fermenter (BIOSTAT C-plus, Sartorius, Germany), containing 30 L LB medium with 0.8% glycerol and 100 μg/mL kanamycin monosulfate. Fermentation was run for 9 h at 37 °C (agitation 200–800 rpm, airflow at 4.0–20 L/min, dissolved oxygen levels maintained at 30%). When the dissolved oxygen was observed to increase, fed-batch cultivation was started to keep a constant specific growth rate. After the cells were grown to exponential phase (OD_{600nm} of 15), 1 mM isopropyl-β-Dthiogalactopyranoside (IPTG) was added to induce target protein expression. After another 4 h, the cells were harvested by centrifugation (Thermo, USA) and the cell pellet was aliquoted and stored at −70 °C.

2.2. Effects of protease inhibitors on the degradation of M.RCAg-1

An aliquot of cell pellet was re-suspended in phosphate buffer (20 mM PB, pH 7.4) at 1:10 (w/v), and protease inhibitors of 1 mM PMSF, 1 mM EDTA, 0.1 mM TPCK, 0.1 mM TLCK and 10 mM imidazole were separately added to the buffer to investigate the effects on inhibiting the degradation of M.RCAg-1. Cells were orderly lysed by sonication in ice-bath. The lysates were clarified by centrifugation (10, 000 rpm, 20 min, 4 °C), and the supernatants were stored at room temperature and subjected to SDS-PAGE analysis at fixed time points of 0 h, 6 h, 16 h, 36 h and 48 h respectively.

2.3. Development of a chromatographic purification procedure

70 g cell pellet (wet weight) was re-suspended in 500 mL buffer A (20 mM PB, 1 mM EDTA, 10 mM imidazole, pH 7.4) and disintegrated by a high pressure homogenizer with a pressure of about 800 bar for three cycles (APV-2000, German). After centrifugation, the supernatant was treated with 0.12% (v/v) polyethylenimine (PEI) (Sigma, USA) to remove nucleic acids from host cells. After centrifugation, the supernatant was prepared for the following purification step.

Purification of M.RCAg-1 was performed on an ÄKTA explorer 100 (GE Healthcare, USA) through a three-step chromatography procedure. The supernatant after PEI precipitation was firstly loaded onto the column containing cOmplete His-tag purification resin (Roche, Switzerland) pre-equilibrated by buffer B (with 0.5 M NaCl in buffer A). The column was washed by 3 CV of buffer B and the adsorbed protein was eluted by buffer C (with 0.5 M imidazole in Buffer B). The eluted protein was concentrated using a 10-kDa cut-off, 0.1 M^2 polyethersulfone (PES) ultrafiltration cassette (GE Healthcare, USA), and then loaded onto the second column containing Superdex 200 (XK 1000 × 50 mm ID, GE healthcare), which was pre-equilibrated by buffer D (10 mM PB, 1 mM EDTA, pH 7.4). The target peak was collected and further polished by a polymixin B column (PMB) (Pierce, USA), which was pre-equilibrated by buffer D. The resin was regenerated by buffer E (10 mM PB, 1% sodium deoxycholate, pH 7.4) before use. For each step, the collected fractions were analyzed for protein purity, recovery, and endotoxin level. Protein concentration was determined by the Bradford Coomassie brilliant blue assay using bovine serum albumin (BSA) as the standard [29]. Finally, the protein solution was filtrated through a 0.22 μm filter unit (Millipore, USA) and stored at −20 °C.

2.4. SDS-PAGE and western blot

The SDS-PAGE and Western blot were both performed under reducing condition and all samples were pre-treated with 1% (v/v) β-mercaptoethanol. For SDS-PAGE analysis, about 10 μg protein was subjected to the gel and the purity of intended protein was estimated by densitometry. The gels were stained with coomassie blue R250 (Sigma, USA). For immunoblotting, the SDS-PAGE separated proteins were electronically transferred to polyvinylidene fluoride membranes (PVDF, Amersham Biosciences) based on the method with slight modifications [30]. The PVDF membranes were blocked with 5% non-fat milk for 1 h and incubated with anti-chicken M.RCAg-1 IgY (prepared in our lab) overnight at 4 °C. The PVDF membranes were washed with PBST (PBS containing 0.05% Tween-20) followed by incubation with 1: 1000 dilution of HRP conjugated secondary antibody (Beyotime, China) at 37 °C for 1 h. Then the PVDF membranes were incubated with fixative solution and developing solution (Thermo, USA). The standard *E.coli* host proteins (National Institutes for Food and Drug Control, China) were used as the negative control.

2.5. Analysis and characterization of the purified M.RCAg-1

2.5.1. HPSEC

500 μL protein solution was loaded onto the column of Superdex™ 200 10/300 GL (300 × 10 mm ID, GE Healthcare), which was pre-equilibrated with 20 mM PB, 0.15 M Na_2SO_4, pH 7.4, and eluted at 0.5 mL/min. Absorbance was recorded at 280 nm. For the samples treated with 1% (w/v) sodium dodecyl sulfate (SDS) or 5 mM dithiotretiol (DTT), the buffer of HPSEC contained 1% SDS or 5 mM DTT in addition, respectively. The protein marker was also run with the elution buffer of 20 mM PB, 0.15 M Na_2SO_4, pH 7.4.

2.5.2. MALDI-TOF MS

The molecular weight of the purified M.RCAg-1 was determined by matix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS, Bruker Daltronics, USA) using sinapinic acid as matrix.

2.5.3. Circular dichroism (CD)

To investigate the secondary structure of purified M.RCAg-1, far-UV CD analysis of M.RCAg-1 was performed on J-810 spectrometer (Jasco, Japan) at room temperature using a 1.0 mm path length quartz cuvette. The signal was recorded from 260 nm to 200 nm at a scanning rate of 1000 nm/min and 1.0 nm resolution. The final signals were adjusted by subtracting the buffer signal. The CD signal was analyzed by Spectra Manager™ software (Jasco, Japan).