Optimized Method for Purification of Expressed Plasmodium Vivax Duffy Binding Protein-II (PvDBP-II): Implication for Vivax Malaria Vaccine Development

Vahideh Valizadeh¹, Sedigheh Mirkazemi¹, Behrouz Vaziri², Sedigheh Zakeri³, Navid D. Djadid¹

1 Malaria and Vector Research Group (MVRG), Biotechnology Research Center (BRC), Pasteur Institute of Iran, Tehran, Iran. 2 Department of Medical Biotechnology, Biotechnology Research Center (BRC), Pasteur Institute of Iran, Tehran, Iran.

ABSTRACT

Introduction: The purity and correct folding of a recombinant protein is critical for any structural, biochemical and vaccine design studies. Plasmodium vivax Duffy binding protein-II is a leading vaccine candidate for vivax malaria. In the present study, the purification process of recombinant DBP-IX (a variant form of PvDBP-II) was optimized to achieve the highest yield and purity. Moreover, naturally-acquired IgG antibodies to the expressed protein have been evaluated. Methods: DBP-IX was cloned and expressed as a his-tagged protein in E. coli. The recombinant protein was purified using Ni-NTA agarose and different purification parameters were optimized to achieve the highest yield and purity. The quality of the purified rDBP-IX was assessed by different procedures such as SDS-PAGE gel analysis in both reducing and non-reducing conditions, followed by indirect immunofluorescence antibody test and ELISA using the sera of P. vivax infected patients (n= 202). Results: DBP-IX was successfully cloned, expressed and optimally purified. Differential mobility of the rDBP-IX on the SDS-PAGE gel in reducing and non-reducing conditions, confirmed the presence of disulphide bonds. In addition, anti-rPvDBP-IX antibody produced in mice recognized the native PvDBP-II, suggesting that epitopes in the recombinant protein were similar to the corresponding native form. Finally by performing ELISA experiments, it was demonstrated that natural P. vivax infection produces IgG against rDBP-IX (42.1%) while cytophilic IgG1 antibody (35.4%) was the predominantly detected IgG subclass. Conclusion: The results indicated that the optimally-purified rDBP-IX was of a quality that could be used in vaccine development research and immunological studies of vivax malaria.

KEYWORDS: Plasmodium vivax, Duffy binding protein, Recombinant protein, Vaccine

INTRODUCTION

Historically, vaccines have been one of the most effective and simply administered tools for controlling and preventing of infectious diseases such as small pox, rabies, plague, cholera and typhoid [1]. One of the neglected life-threatening diseases is malaria which occurs in nearly 100 countries worldwide and infects at least 300 million people living in tropical regions, annually [2]. Malaria is preventable, treatable and history shows that it can be eliminated. Potential means that could facilitate malaria eradication include highly sensitive diagnostic tests, single-dose treatments and vaccines.

Today, research in the field of malaria vaccine development has accelerated considerably and increased funding and studies are driving the discoveries of new vaccines, especially the recombinant subunit ones [3]. Plasmodium vivax has three distinct stages in its life cycle. One of the most important stages at which an individual would start to experience clinical symptoms is the erythrocytic stage. Different antigens from this stage are evaluated as vaccine candidates and one of the most promising is Duffy binding protein [4, 5]. This protein consists of seven regions and a cysteine rich domain, referred to as region II which has a fundamental role in adhesion of the parasite to the red blood cells and initiation of the invasion. PvDBP-II is 330 amino acids in length and contains 12 conserved cysteine residues. It is postulated that the interaction of PvDBP-II with the Duffy antigen receptor for chemokines
was grown in Luria broth (pH 7.2) containing ampicillin (100 μg/ml) and kanamycin (25 μg/mL) at 37°C with shaking (150 rpm) until an optical density (OD) of 0.6-0.7 at 600 nm was reached. The expression of recombinant DBP-IX (rDBP-IX) was induced with 1 mM isopropyl-β-thio galacto pyranoside (IPTG, Sigma-Aldrich, USA). The culture was grown for another 4 h and the cells were harvested by centrifugation and kept at -80°C until use. **Optimizing the purification process of recombinant PvDBP-IX** DBP-IX contains six disulphide bonds and its expression in Escherichia coli results in formation of inclusion bodies. Therefore, the first step in purification of rDBP-IX was dissolving the cell pellets in a denaturation buffer. For this purpose, two distinct chaotropic agents were examined for solubilizing the inclusion bodies in lysis buffer and efficacy of each buffer was compared. The first solution containing 6M GTC, 20mM Tris–HCl, 500mM NaCl, 10mM Imidazole (30mM imidazole was also checked) and 1mM phenyl methane sulfonyl fluoride (PMSF) as protease inhibitor (pH 7.9). In the second solution, 8M urea was used instead of GTC while all other reagents were the same. The cells were lysed on ice by sonication (Ultra schalpprozesor, Germany) with 12 cycles; each consisted of 20-sec pulses with 40-sec intervals. The bacterial lysate was centrifuged at 12,000 xg at 4°C for 15 min. The supernatant was incubated with Ni2+ nitritetriacetic acid agarose resin (NITAgarose, Qiagen, Germany) at 4°C for 90,120 and 150 min. In order to remove the unbound proteins and non-specifically bound proteins, the resin was packed into a columns and washed with a 10-column volume of wash buffer containing 8M urea, 20mM Tris–HCl, 500mM NaCl, and different concentrations of imidazole (30, 40 and 60mM) and eluted separately in elution buffer (pH 7.9). In addition to reducing the non-specific protein bindings, the concentration of NaCl was increased to 1M in the lysis and wash buffers and the bound protein was eluted with elution buffer containing 8M urea, 20mM Tris–HCl, 500mM NaCl and different concentrations of imidazole (30, 250 and 500mM). For correction refolding of the expressed protein and preventing the formation of protein aggregates, the chaotropic agents were removed gradually (6 and 4M urea) and the results were assessed by SDS-PAGE in both reducing and non-reducing conditions. For complete removal of urea, fractions containing rDBP-IX was desalted with Econo-Pac 10DG columns (BioRad, USA) according to the manufacturer’s manual and concentrated with a concentrator (Eppendorf, Hamburg, Germany). **Purity analysis and confirmation of recombinant protein** The eluted proteins were analyzed under both reducing [with 1% SDS and 2% 2ME (β–mercapto ethanol)] and non-reducing [with 1% SDS and without 2ME] conditions by SDS-PAGE (12%). Western blot assay was carried out by standard protocols using anti-His antibody (Penta His Antibody, Qiagen) as well as P. vivax–infected and normal human sera in both reducing and non-reducing conditions to confirm the purified recombinant proteins. **Indirect immune fluorescence antibody test (IFAT)** The similarity of epitopes in the recombinant protein to its corresponding native form was checked by immune fluorescent antibody test (IFAT) using the ability of the anti-DBP-IX sera to recognize the native form of the PvDBP-IX antigen on the merozoite surface. Anti-sera against rDBP-IX was produced by immunizing mice with...
purified DBP-IX in combination with complete Freund’s adjuvant and the sera used for recognition of native DBP-II on \textit{P. vivax} parasites. Briefly, multi-spot slides of the parasites were prepared from \textit{P. vivax} infected patients, air-dried and then fixed in cold acetone for 10 min. The spots were blocked with 5% BSA diluted in PBS in a wet chamber at room temperature for 30 min. After washing three times with PBS (pH-7.4), 25 \mu L mouse sera diluted (1:200, 1:100 and 1:50) in PBS were added to the spots and incubated in a wet chamber for 30 min. Slides were washed three times with PBS (pH-7.4) and each well was covered with 25 \mu L of the fluorescein-conjugated anti-mouse or anti-human (in case of positive control) polyvalent IgG (1:40) and then left in a wet chamber for another 30 min. After washing three times with PBS, coverslips were placed on each slide and examined under a fluorescence microscope (Nikon E200, Tokyo, Japan) with an oil immersion objective (100x). The serum samples obtained from normal mice and also the two known human sera infected with \textit{P. vivax} were used as negative and positive controls, respectively.

**Measurement of IgG and subclasses antibody responses to recombinant DBP-IX antigen by ELISA**

To characterize the naturally acquired immune responses towards the target protein anti-PvDBP-II IgG, IgG1 and IgG3 subclasses were determined in the sera of \textit{P. vivax} infected patients by ELISA. Briefly, maxisorp flat-bottomed, 96-well microplates (Grainer, Germany) were coated with 250 ng/well of the rDBP-IX in 0.06 M carbonate buffer (pH 9.6) and incubated overnight at 4°C. After washing with PBS containing 0.05% Tween 20 (PBS-T), microplates were blocked with 200 \mu L PBS containing 2.5% bovine serum albumin (BSA, pH-7.4) for two hours at room temperature (RT). The plates were then washed and incubated with 100 \mu L plasma of each patient (n=202) at 1:100 dilution for 90 min at RT. After washing with PBS-T, the plates were incubated with horseradish peroxidase-labeled anti-human IgG (1:30,000; Sigma-Aldrich, USA). The plates were washed again with PBS-T, and the presence of bound IgG was detected using o-phenylene diamine dihydro chloride-H2O2 (Sigma, USA) as substrate and stopped with 2NH2SO4. The absorbance at 492 nm was measured by using a microplate reader (Biotech, USA), and the mean value of each pair of wells was calculated. To detect subclasses of human IgG, an ELISA was performed as described above, but secondary antibodies were added at a dilution of 1:2000 using biotin-conjugated isotype-specific antihuman IgG subclass antibodies (Sigma-Aldrich, USA) at RT for one hour. After washing steps, streptavidin–peroxidase conjugate (Sigma-Aldrich, USA) was added at a dilution of 1:2500 and incubated at RT for 1 h. The enzyme reaction was developed with OPD–H2O2 (Sigma-Aldrich, USA) and stopped with 2NH2SO4. A pool of immune sera (n=5) from adult Iranian malaria patients residing in \textit{P. vivax} endemic region and 35 serum samples from healthy non-exposed Iranians from outside the malaria endemic regions were used as positive and negative controls, respectively. The cutoff value for positive plasma was considered as the mean plus 3 standard deviation (SD) of optical density (OD) readings of the negative samples.

**RESULTS**

Expression and optimizing purification process of recombinant PvDBP-IX

Transfection of pQE30-DBP-IX into \textit{E. coli} M15 competent bacteria resulted in production of the recombinant protein with the expected sizes of ~47 kDa as revealed by SDS-PAGE.

**Fig. 1.** SDS-PAGE analyses of eluted DBP-IX using different chaotropic reagents in the lysis buffer. (A) lysis buffer containing 6M guanidinium thiocyanate (GITC), (B) lysis buffer containing 8M urea.

**Fig. 2.** SDS-PAGE analyses of eluted DBP-IX using different concentration of imidazole in the elution buffer.

**Fig. 3.** SDS-PAGE analyses of recombinant DBP-IX purified with the optimized purification protocol under reducing (+2ME) and non-reducing (-2ME) conditions.
The concentration of imidazole in the elution buffer was also optimized and our results showed that the yield of the purified protein was markedly higher when 250 mM imidazole was used (Fig. 2), but the difference between 250 and 500 mM imidazole was not significant. Therefore, 250 mM imidazole was chosen for the optimized purification protocol. Additionally, in order to prevent aggregate formation, the concentration of the chaotrope agent was decreased and the highest yield was obtained by 6 M urea.

Table 1. Summary of purification optimization parameters of expressed PvDBP-IX in the prokaryotic system.

<table>
<thead>
<tr>
<th>Optimized parameters</th>
<th>Chaotropic agent</th>
<th>NaCl</th>
<th>Imidazole</th>
<th>Final optimized condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis buffer</td>
<td>8 M urea 6 M GITC</td>
<td>500 mM</td>
<td>10 mM</td>
<td>8 M urea, 1 mM DTT, 1 M NaCl, 30 mM Imidazole</td>
</tr>
<tr>
<td>Wash buffer</td>
<td>8 M</td>
<td>500 mM</td>
<td>40 mM</td>
<td>6 M urea, 1 M NaCl, 60 mM Imidazole</td>
</tr>
<tr>
<td>Elution Buffer</td>
<td>8 M</td>
<td>300 mM</td>
<td>150 mM</td>
<td>4 M urea, 300 mM NaCl, 250 mM Imidazole</td>
</tr>
</tbody>
</table>

*The optimum incubation time with Ni-NTA resin was 120 min

1GITC: Guanidinium thiocyanate

**Fig. 4.** Confirmation of purified DBP-IX protein by Western blot analyses under both reducing (+2ME) and non-reducing (-2ME) conditions: (A) using P. vivax-infected human sera; (B) Anti-His antibody; (C) Normal human sera.

**Fig. 5.** IFAT results demonstrating the recognition of native PvDBP-II on the surface of P. vivax merozoites using anti-rPvDBP-IX anti mouse antibody (A) and no detection by normal mouse sera (B).

**Fig. 6.** The prevalence of IgG and its subclasses (IgG1 and IgG3) responses to the DBP-IX antigen among patients sera (n=202) with P. vivax infection from south eastern regions of Iran. Cut-off values were 0.34, 0.26 and 0.24 for IgG, IgG1 and IgG3, respectively.

The ability of the sera from mice immunized with the recombinant protein to detect the native DBP-II on the P. vivax merozoites was confirmed by IFAT. Anti-rDBP-IX antibody
produced in mice recognized the native protein present on the surface of *P. vivax* merozoite with high intensity (Fig. 5A). Moreover, the control sera did not react with the native protein on *P. vivax* parasit (Fig. 5B), confirming that there are common epitopes presented in the recombinant and the corresponding native form of the protein.

**Measurement of IgG and IgG subclasses antibody responses to recombinant rDBP-IX antigen by ELISA**

Naturally-acquired anti-DBP-II IgG responses to the purified target protein were examined in the sera from 202 patients and the results showed that natural *P. vivax* infection produces IgG against rDBP-IX in 42.1% (cut off=0.34) of patients. Moreover, analysis of the antibody responses to DBP-IX showed high (5%), medium (19.3%) and low (17.8%) positive as well as negative (57.9%) responses and the mean OD±SD values were1.3±0.26, 0.68±0.13, 0.44±0.02 and 0.27±0.06 in each group, respectively. None of the sera from healthy individuals (control group) contained IgG antibodies to rDBP-IX which confirms the specificity of the present results. Furthermore, sera that were positive for total anti-DBP-IgG were characterized for IgG subclass responses to DBP-IX antigen. In individuals who were infected with *P. vivax*, the IgG1 (35.4%, mean OD490=0.77) was the predominant subclass whereas IgG3 (15.4%, mean OD490=0.55) was the second most prevalent subclass (Fig. 6).

**DISCUSSION**

Vaccines have been the most useful medical tools to combat infectious diseases during the last century. However, there is no licensed vaccine against any human parasitic disease such as malaria which causes massive human suffering. However, a malaria vaccine is essential if the goal of malaria eradication is to be realized [16]. In recent years, attempts to develop a useful vaccine for malaria and especially vivax malaria have been increasing significantly and scientists are using diverse technologies to isolate and deliver specific antigens for this purpose instead of attempting to produce a live attenuated vaccine [17]. Meanwhile, the purity and correct folding of a recombinant protein are essential factors which should be considered for structural, biochemical and developmental studies of the vaccines.

PvDBP-II is the most important blood stage vaccine candidate antigen for vivax malaria. It has been studied extensively by many researchers using sera from the infected patients [18, 19] and laboratory animals which had been immunized by recombinant forms of the target protein [10]. In all these studies, recombinant forms of PvDBP-II have been produced and purified with different protocols using affinity tags. In a number of studies, rPvDBP-II was expressed as a soluble glutathione S-transferase (GST) fusion protein [8, 18, 20, 21]. Although such GST-tagged proteins were produced in soluble forms, their GST component had to be removed from DBP-II, because of its possible interference with the co-purified recombinant protein in the immunological assays. The cleavage of GST from DBP-II by thrombin should be followed by its removal from the mixture which would make the purification procedure cumbersome and time consuming. In the present study, rDBP-IX was expressed as an N-terminal his-tagged fusion protein and the purification process was optimized to obtain a higher yield of the purified protein.

Furthermore, in the previous studies in which rPvDBP-II were expressed as a his-tagged protein, the inclusion bodies were dissolved using 6 M GITC in the solubilizing buffer [22-24, 26]. However, in the present investigation, by comparing 6MGITCand 8M urea as chaotropic agents, we concluded that 8M urea was more effective in dissolving the inclusion bodies and the obtained yield of the recombinant protein was much higher (i.e. 67% increase), although some non-specific protein bands were observed with this method. In our optimization procedures to remove non-specific proteins, different concentration of imidazole and NaCl in the lysis and wash buffers were also compared.

In summary, in the present investigation, different purification parameters were optimized to achieve the highest purity and yield of a properly folded rDBP-IX. Our aim was to optimize the purification of this recombinant protein by examining different parameters which could lead to the higher quality of the target protein. Based on our IFAT and ELISA results, we envisage this recombinant protein is suitable for use in vaccine developing research and immunological studies for vivax malaria.

**AUTHOR’S CONTRIBUTION**

VV (Ph.D. student) contributed in the laboratory work, analysis of the data and drafted the paper. SM helped with the experimental assays and BV gave critical suggestions for optimizing the protein purification procedure. SZ designed the work, supervised the study and critically revised the manuscript. NDD helped with data analysis and critical reading of the manuscript.
ACKNOWLEDGEMENTS

This study was financially supported by Pasteur Institute of Iran grant to S. Zakeri (project no. 590). We are grateful to all study participants including their parents/guardians as well as health management teams of Zahedan University of Medical Sciences, staff in Public Health Department, Sistan and Baluchistan Province and Chabahar District for their assistance.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES