PURIFICATION AND PARTIAL CHARACTERIZATION OF *PFHRP-II* PROTEIN OF *PLASMODIUM FALCIPARUM*

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Abstract. The human malarial parasite *Plasmodium falciparum* secretes various intra-and extra-cellular proteins during its asexual life cycle in human RBC. Histidine rich protein-II *(HRP-II)* is one of the most prominent proteins, found to be secreted by *P. falciparum* throughout the asexual cycle with the peak during mature schizont stage of the parasite development in human IRBC. The high histidine content (35% of the total amino acids in protein) of this protein suggested the potential to bind divalent metal ions. We have demonstrated by metal chelate chromatography, an extraordinary capacity of *HRP-II* to bind nickel ions (Ni^{++}) and employed this characteristic to purify the extra-cellular *HRP-II* protein secreted by *P. falciparum* from culture supernatant. The identity of the purified protein was verified by the relative molecular weight on SDS-PAGE, by reacting with polyclonal antibodies directed against it using Western blot technique.

INTRODUCTION

Malaria has probably had a greater impact on world history than any other infectious disease. It has been responsible for the outcome of wars, population movements, and the growth and development of various nations throughout the world. More than 300 million individuals throughout the world are infected with malaria, and more than 1 million people a year, most of whom are children, are being killed by the disease. It is still a very common disease in many parts of the world, particularly in tropical and subtropical areas. Of the four most common species that infect humans, *P. vivax* and *P. falciparum* account for 95% of infections. *P. vivax* has the widest distribution, extending throughout the tropics, subtropics and temperate zones. *P. falciparum* is generally confined to the tropics. There has been a definite increase in the number of cases of *P. falciparum* malaria reported, which may be related to increased resistance to chloroquine. Malaria prevention is difficult, and no drug is universally effective. Vaccine development studies are ongoing, but malarial vaccines are not yet in general use (Garcia and Bruckner, 1997).

In many developing countries, resources for malaria diagnosis are sparse or unavailable. Small numbers of trained microscopists and microscopes often limits local clinics examining blood smears from a large number of patients suspected of having malaria. Consequently, malaria diagnosis is often made only on the basis of clinical symptoms although this is, at best, 50% accurate (Anonymous, 1994).

Specific diagnosis of malaria is, usually made by microscopic examination. However, microscopic examination even by expert microscopists is time consuming, labor-intensive, and it is difficult to diagnose mixed *P. falciparum* and *P. vivax* infections, later being present predominantly in ring stages. Microscopic examination will not be cost-effective in malaria control in an area with very low but persistent transmission, because the cost to diagnose one positive slide will be high. There is therefore a need to develop alternative diagnostic methods to detect cases especially those with low-grade parasitemia to supplement and perhaps to replace microscopy in malaria control programs. (Tharavanij, 1990).

A key feature of the new WHO Global Malaria Control Strategy (WHO, 1991) is the rapid diagnosis of malaria at the village and district level, so that effective treatment can be adminis-

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tered quickly to reduce morbidity and mortality. The need for rapid diagnosis of *P. falciparum* is most acute because of the severe nature of this infection and its non-specific symptomatology.

Human malarial parasite *Plasmodium falciparum* is the most pathogenic. *Plasmodium falciparum* infected erythrocytes (IRBCs) synthesize several histidine rich proteins (HRPs) that accumulate high levels of histidine but very low levels of amino acids such as isoleucine or methionine (Howard *et al,* 1986). Three such proteins, designated *P. falciparum* histidine-rich proteins (HRP) I, II and III, are synthesized by asexual parasites within red blood cells. *PfHRP-I* is found associated with the cytoskeleton of IRBC and is localized under knob-like protrusions of the infected cell membranes (Rock *et al*, 1987; Panton *et al*, 1989; Parra *et al*, 1993). *PfHRP-II* is expressed by both, knob-positive (k+) and knob-negative (K-) IRBCs. HRP-II is synthesized throughout the asexual cycle, transported in concentrated 'packets' through the red cell cytoplasm and released from intact infected cells into the culture medium (Howard *et al*, 1986). Extracellular HRP-II is water soluble (Rock *et al*, 1987). Sequencing of the genomic DNA has shown that HRP-II contains 34% histidine, as well as relatively high contents of alanine and aspartic acid (37% and 10% respectively). HRP-II contains many tandem repeats of the sequences AHH and AHHAAD (Rock *et al*, 1987). HRP-II is very similar in sequence to HRP-III and both HRP-II and HRP-III are expressed simultaneously by some asexual *P. falciparum* parsites (Panton *et al*, 1989).

HRP-II is of particular interest because half of this protein is rapidly exported from infected cells into the extra-cellular medium (Howard *et al*, 1986). Previous studies on the endogenous histidine rich protein of human serum and rabbit serum, called histidine rich glycoprotein, show that this protein binds some divalent metal ions but not others (Panton *et al*, 1989). Previous study on the metal binding properties, especially the capacity of zinc affinity chromatography to be used for the purification of parasite protein *PfHRP-II* show that this protein binds to Zn ions. Zn chelate affinity chromatography could be used for purification of *PfHRP-II* from culture superna-

tants/ extracts of parasitized cells.

It is therefore of particular interest to determine whether *PfHRP-II* released in culture supernatant has similar binding properties to nickel ion in Ni-NTA agarose as that of other 6X-His tagged proteins, which could be useful for further study of *PfHRP-II* after purification using Ni-column. The purification of HRP-II protein from *in vitro* culture supernatant may play a major role in the reagent development for malaria diagnostics and malaria vaccine studies.

Here we describe the extraordinary affinity of HRP-II for Ni-immobilized on metal chelate chromatography columns, and utilize this property to isolate and partially purify this malarial protein from culture supernatant.

MATERIALS AND METHODS

Malaria culture

The culture adapted strain of *P. falciparum*, obtained from Malaria Research Center, New Delhi, by the kind courtesy of Dr CR Pillai, were cultured in RPMI-1640 with 10% AB positive human serum using the method of Trager and Johnson (1978), with minor modifications. The culture vials were monitored for parasitemia, after staining at 24 hours intervals. The media was changed and added to the blood cells whenever necessary.

Preparation of *PfHRP-II* antigen from culture supernatant

The culture supernatant from the vials with 0.1 - 10% parasitemia, were collected daily in different screw capped tubes and centrifuged at $500g$ for 10 minutes. The supernatant was collected in screw-capped vials and stored at -20°C before further processing and was used for purification of *PfHRP-II* antigen.

Purification of *PfHRP-II* antigen from culture supernatant

Two hundred ml batch of culture supernatant was thawed quickly and centrifuged at 1500 rpm for 10 minutes. Fifty mM of imidazole was added in each batch of 200 ml of centrifuged culture supernatant and incubated at 4°C in continuous stirring condition at 100 rpm for 12 hours. The super-

natant was centrifuged at 1500 rpm for 10 minutes and processed for purification of *PfHRP-II* antigen using affinity chromatography through *Ni-NTA* matrix (Qiagen, USA) packed column. The protein adsorbed in *Ni-NTA* agarose packed column, was eluted using a gradient of 0 - 750 mM of imidazole containing wash buffer. Elutes were collected in 15 ml screw capped tubes and stored at -20°C until further testing. The collected fractions of elute, wash W_1, wash W_2 and flow-through were tested for the presence of *PfHRP-II* antigen. (Parasight-f test, B & D, USA). The collected fractions of elute, wash W_1, wash W_2 and flow-through were also tested for purity and molecular Weight of the antigen present in different fractions using SDS-PAGE and Western blotting (Harlow and David, 1989). The fraction containing the purified antigen (*PfHRP-II*) was pooled, dialyzed and concentrated using the Amicon apparatus. The concentration of the antigen in solution was determined using the standard Micro BCA Assay method and was stored in small aliquots at -20°C containing 0.1% PMSF as a protease inhibitor.

RESULTS

The metal binding character of *PfHRP-II* was examined by metal-chelate affinity chromatography of culture supernatants. The eluted solution was collected in fractions of 1 ml each in each tube and measured absorbance at 280 nm. Presence of *Pf HRP-II* in each fraction was tested using a commercial antigen capture assay (Parasight-f test). Fig 1, shows the elution profile of *PfHRP-II* from the culture supernatant. The profile shows two peaks of adsorption at 280 nm. The presence of *PfHRP-II* was detected in fractions 3, 4, 5 and 6.

Analysis of the antigen *PfHRP-II* by SDS-PAGE

The fractions which showed positive in commercial antigen testing system were pooled and analyzed in 10% SDS-PAGE. (Fig 2), which resolved as a 72 kDa band. This was, further confirmed by Western blotting using commercially available antibody. In an average, 300 - 400 μg of *Pf HRP-II* could be purified from 200 ml of culture supernatant.
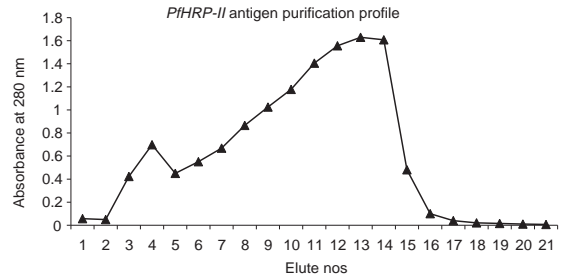

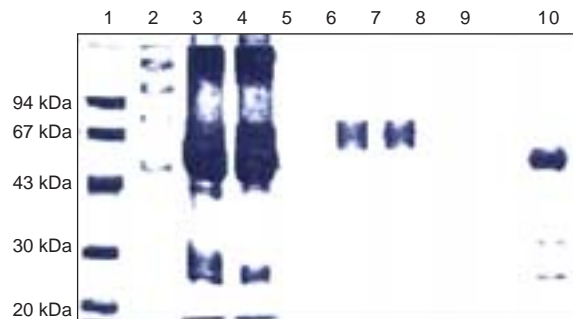Fig 1–Chromatogram showing purification profile of *PfHRP-II* antigen.

Fig 2–SDS-PAGE of purified *PfHRP-II*.

Lane 1: Mol wt marker (14 - 94 kDa); lane 2: mol wt marker (55 - 245 kDa); lane 3: load (*P. falciparum* culture soup); lane 4: flow through; lane 5: blank; lane 6: purified *Pf HRP-II* (elute 4); lane 7: purified *Pf HRP-II* (elute 5); lane 8: Blank; lane 9: blank; lane10: BSA (fraction-V).

Western blot for confirmation of the purity of the antigen *PfHRP-II*

The protein revealed in the SDS-PAGE, was conformed by Western blotting, which blotted a single band around 72 kDa, in only the lane in which *PfHRP-II* was run but did not blot in the lane in which *rKAHRP* (recombinant *PfHRP-I*) was run, confirming its purity.

DISCUSSION

Malaria probably has a greater impact on world history than any other infectious disease. In many developing countries, resources for malaria diagnosis are sparse or unavailable. Small numbers of trained microscopists and micro-
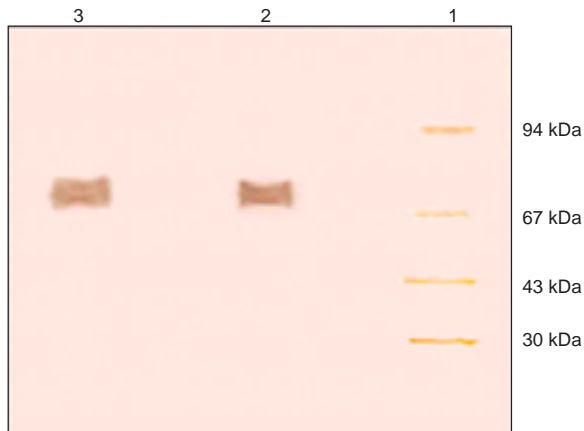
Fig 3–Western blotting with the commercial antibody for confirmation of purified *PfHRP-II*.

Lane1: mol wt marker (14 - 94 kDa); lane 2: *Pf HRP-II;* lane 3: *Pf HRP-II;* primary antibody: rabbit anti- *PfHRP-II* IgG (1:1000) (commercial); conjugate: goat anti-rabbit IgG HRP (1:5,000); substrate: DAB + H_2O_2+ PBS.

scopes often limits local clinics examining blood smears from a large number of patients suspected of having malaria. Consequently, malaria diagnosis is often made only on the basis of clinical symptoms although this is, at best, 50% accurate (Anonymous, 1986).

A key feature of the new WHO Global Malaria Control Strategy (Anonymous, 1991) is the rapid diagnosis of malaria at the village and district level, so that effective treatment can be administered quickly to reduce morbidity and mortality. The need for rapid diagnosis of *P. falciparum* is most acute because of the severe nature of this infection and its non-specific symptomatology.

In this study, method for *in-vitro* culture of *P. falciparum*, and maintenance of culture adopted Indian wild strain of *P. falciparum in vitro* were standardized. Parasite culture at 0.1- 10 % parasitemia were maintained *in vitro* using the standard methodology (Pillai and Ugha, 1996) and spent culture supernatant *P. falciparum* were collected and stored at -20°C, for the purification of *PfHRP-II* antigen.

In this study, we have standardized a novel method, and buffer system, for the purification of extra-cellular *PfHRP-II* antigen secreted by the

parasites in culture soup during the asexual cycle of life cycle. We have purified the native extra-cellular *PfHRP-II* antigen from the culture soup, in good quantity and quality.

Panton and group (1989) have demonstrated by metal chelate chromatography, an extraordinary capacity of HRP-II to bind zinc ions (ZN^{+2}) and employed this characteristic to isolate the extra-cellular protein. The identity of the purified protein was verified by relative molecular weight on denaturing polyacrylamide gels, by reacting with monoclonal antibodies and mono-specific rabbit antiserum.

Panton and his group (1989) have also found that HRP-II binds even more strongly to a column charged with Cu^{+2}, requiring a minimum of 450 mM imidazole to release it.

In this study, we have employed the extraordinary capacity of HRP-II antigen of *P. falciparum,* to bind metal ions like, Zn^{+2}, Cu^{+2}, Ni^{+2}. We used a commercially available matrix charged with Ni^{+2} ion, originally manufactured for the purification of recombinant protein tagged with six histidine residues, expressed in bacterial system (*E. coli*). We designed and standardized novel buffer system and the protocol to purify native *PfHRP-II* protein which contains only four histidine residues (at one stretch), from *P. falciparum* culture supernatant.

In our study, we took a Ni-NTA agarose packed column and purified the *PfHRP-II* antigen from the *P. falciparum* culture supernatant with the methodology mentioned in the materials and methods section. The fractions of the elute were tested for the presence of *PfHRP-II* using a commercial test, Parasight-f test and fractions showing a positive reaction were pooled together and dialyzed against PBS pH 7.2. An aliquot of each pool was analyzed using 10 % SDS-PAGE.

Washing the column with a low molar concentration of imidazole with detergent Triton X-100 (0. 1%) was able to reduce the non-specific binding of protein from the culture supernatant into the Ni -NTA agarose column. Even-though the 0-750 mM imidazole was used for elution of *PfHRP-II*, most of the *PfHRP-II* eluted at a low concentration of imidazole. This shows the weak binding of *PfHRP-II* in the Ni-NTA agarose column, in comparison to many histidine tagged re-

combinant proteins. This may be due to the presence of only four histidine at a stretch in the polypeptide chain of *PfHRP-II*.

The SDS-PAGE picture in our study is also similar to the study of Panton *et al* (1989) *ie PfHRP-II* antigen we purified has a molecular weight of 70-72 kDa, which is similar to that of Panton's study.

The purified *PfHRP-II* reacted very strongly with commercial polyclonal antibody. This antigen is purified and hence could be used for malaria research including polyclonal/monoclonal antibody production for diagnostic development, for the study of malaria pathogenesis or vaccine studies.

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