Chapter: V

Overexpression, purification, immunological characterization and localization of the recombinant protein.
Introduction:

Production of large-scale malaria antigens was made possible by the development of a variety of expression systems by which heterologous protein could be easily expressed and purified. The selection of an expression system for a particular antigen depends on the quantity and quality of the desired product. Eukaryotic expression systems harbor potential post translation machinery, but the disadvantages are expensive, complicated and often result in poor yield of recombinant protein. Prokaryotic systems, although they too have some limitations, are commonly used because they are convenient to handle, less expensive, easily available and most importantly yield very high amount of recombinant proteins. In prokaryotic expression systems, the foreign DNA is cloned into a plasmid and the recombinant protein is expressed in a bacterial host after transforming the plasmid into it. The Glutathion S- Transferase (GST) Gene Fusion System (pGEX plasmid expression vector) is designed for inducible, high level of intracellular expression of foreign genes or gene fragments as a fusion protein with Schistosoma japonicum GST, which was introduced by Smith and Jhonson (1988). It permits one-step purification of fusion protein from crude cellular lysates and used by many investigators to over express and purify the target recombinant protein of malarial parasite (Frangioni and Neel, 1993; Ma et al., 1996; Kang et al., 1998; Rotman et al., 1999; Chatterjee et al., 2000).

In this chapter, the insert released from the immuno-reactive clone (1132 bp insert - KP1) was sub-cloned into pGEX plasmid expression vector; the recombinant fusion protein was overexpressed in E. coli and purified using affinity purification column. Polyclonal antibodies were raised against the fusion protein in Balb/c mice and were used to identify the
native protein from the malaria parasite lysate, study its effect on the in-vitro growth of the parasite and localization of protein in the blood stages of the parasites.

Experimental Methodology:

1. Subcloning of KP insert into pGEX- 4T 1 vector.

a. Preparation of pGEX 4T 1 vector arm for cloning:

Smith and Jhonson (1988) constructed the pGEX series of expression vectors that direct the synthesis of foreign polypeptide in E. coli as a fusion protein with the 26-kDa Glutathion S-Transferase (GST). A schematic presentation of the expression vector (pGEX) is given in Fig 4.1. The pGEX vector feature a tac promotor for chemically inducible, high level expression and the expressed fusion protein can be purified in a single step using Glutathion Sepharose- 4B that preserve the antigenicity and functionality of the protein. Among the pGEX series, pGEX-4T-1 plasmid was selected to subclone the insert into the EcoR I site, which was in translational reading frame with the KP-insert. The pGEX 4T-1 plasmid was obtained commercially (Amersham Pharmacia, USA) and a medium scale plasmid DNA preparation was carried out using midi plasmid DNA preparation kit (Qiagen, USA). The procedure of plasmid DNA extraction was similar to that described in previous chapter. The plasmid was digested with EcoR I and the vector DNA was prepared as follow:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
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<tbody>
<tr>
<td>Plasmid DNA</td>
<td>5 µl (5 µg)</td>
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<tr>
<td>EcoR I enzyme</td>
<td>1 µl (20 units)</td>
</tr>
<tr>
<td>EcoR I buffer</td>
<td>5 µl</td>
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<tr>
<td>Sterile Water</td>
<td>39 µl</td>
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<td>Total</td>
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The above reaction was incubated for 4 hour at 37° C, then 1 μl of Calf Intestinal Alkaline phosphatase (CIAP – 20 units) was added and further incubated for one hour at 37° C. The reaction mixture was kept at 72° C for 10 minutes, extracted with phenol: chloroform, DNA was ethanol precipitated and washed with 70% ethanol. The DNA pellet was air dried, dissolved in 100 μl of distilled water and used as vector arms for subcloning. The DNA was stored at -20° C until further use.

b. Ligation of KP 1 insert in frame in to the pGEX-4T-1 vector:

The insert DNA purified from the immunoreactive λgt11 phage DNA by EcoR I digestion was used for subcloning into the expression vector pGEX 4T-1 at EcoR I insertional sites. Around 300 ng of insert was ligated to 100 ng of pGEX 4T-1 arms in presence of T4 DNA ligase (10 units) in 20 μl of reaction mixture. For control pGEX 4T-1 vector arm alone was ligated to assess the self-ligation ratio. The reaction mixture was incubated at 16° C for 16 hours followed by 10 minutes at 70° C. The ligated mixture was used to transform into the chemical competent DH5α cell. The transformants were selected on LB-ampicillin (100μg/ml) plate, picked up individually and inoculated in to 5 ml of LB-broth containing ampicillin (100μg/ml). The liquid cultures were incubated on a rotary shaker (200 rpm) at 37° C overnight. Glycerol stocks of the individual over night cultures were prepared and stored. Plasmid DNA were prepared from 1.5 ml of same cultures by the method described earlier digested with EcoR I restriction enzyme, resolved on 1% agarose gel to identify the plasmid containing the insert.

It is important to identify the orientation of the subcloned insert in the expression vector to over express the protein in right frame. Recombinant plasmids that showed the
presence of an insert of 1.1 kb by restriction analysis were further digested with BamH I restriction enzyme to identify the orientation of the cloned insert. The reaction mix for digestion contained 1 µg of plasmid DNA, 1 µl BamH I (20 units), 2 µl of 10X buffer (NE buffer 2), BSA 0.2 µl (1 mg/ml) and sterile water to final 20 µl reaction mixture. The reaction mixture was incubated at 37°C for four hours and then the digested DNA was resolved in 1% agarose gel to identify pGEX-KP construct with proper orientation.

II. Small scale expression of recombinant clones:

A single colony of DH5α cells containing the recombinant plasmids (pGEX-KP1) was inoculated into 5 ml of LB-Ampicillin (100 µg/ml). Non-recombinant plasmid was also processed in the same way as control. The inoculated cultures were incubated at 37°C on a rotary shaker for 2 hours and then aliquots of 1 ml culture were taken out from individual tubes before inductions (pre-induction). The remaining 4 ml cultures were induced with 0.1 mM IPTG at 37°C for 2 hours. One ml of induced cultures were transferred to microcentrifuge tubes, centrifuged (6000 rpm/5 min) and to the pellet 100 µl of 1X gel loading buffer [50mM Tris.Cl, pH 6.8; 100 mM Dithiotheritol (DTT); 2% SDS; 0.1% bromophenol blue; 10% glycerol] was added and boiled for 5 minutes. The samples were centrifuged and supernatants were resolved on a 12% Sodium dodecyl sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

a. SDS-polyacrylamide gel analysis of the expressed fusion protein:

The induced and over expressed recombinant protein was analysed by SDS- PAGE. In this experiment, 12 % SDS-polyacrylamide gels were prepared (Maniatis et al, 1982) and
run on a SDS-PAGE mini protein electrophoresis apparatus (Bio-Rad, USA). The procedure for gel preparation and electrophoresis was as follows:

1. The resolving gel solution (12%) was prepared by mixing water (6.6 ml), 30% Acrylamide mix (8.8 ml), 1.5 M Tris.HCl pH 8.8 (5 ml), 10% SDS (0.2 ml), 10% APS (0.2 ml) and TEMED (0.008 ml). The solution was quickly poured in between the cleaned glass plate assembly with a spacer of 1.5 mm thickness and 2 cm space for stacking gel was left out. Immediately 1 ml of water saturated butanol solution was layered over the resolving gel and allowed the gel to polymerize for 30 minutes.

2 After the gel was polymerized completely, the top of the gel was rinsed three times with water. Stacking gel solution (5%) was prepared by adding water (2.77 ml), acrylamide mix (0.83 ml), 0.5 M Tris.cl pH 6.8 (1.26 ml), 10% SDS (0.05 ml), 10% APS (0.05 ml) and TEMED (0.005 ml) and poured over the resolving gel. The comb was inserted and the stacking gel was allowed to polymerize for 15 minutes. The comb was removed from the stacking gel and the wells were washed with deionized water. The gel with its assembly placed inside the tank and upper and lower chambers were filled with 1X running buffer (0.025 M Tris; 0.1 M glycine, pH 8.3 and 1% SDS).

3 The sample was loaded in the wells and run at a constant current (20 mA) until the bromophenol blue dye front reached the bottom of the gel. The gel was removed from the apparatus, stained with Coomassie stain for 15-20 minutes and destained with several changes of destaining solution (30% methonal; 10% glacial acetic acid and 60% water) until the background was clear and protein bands were visible.
b. Immuno-blotting to identify the expressed GST fusion recombinant protein:

The crude induced and pre-induced whole cell lysate were resolved by 10% SDS-PAGE and electroblotted onto nitrocellulose paper briefly as follows. Six sheets of Whatman filter paper (3 mm) were cut according to the size of the gel and dipped in the transfer buffer (0.0025 M Tris, 0.19 M Glycine, pH 8.3). Three such sheets, one above the other, were placed on the electro-blotting transfer unit (LKB, Pharmacia) and the air bubbles were removed by rolling over a glass pipette. The nitrocellulose membrane pre-wetted in deionized water was placed on top of these sheets. The SDS-PAGE gel was then placed over the nitrocellulose membrane. Another set of three sheets wetted in transfer buffer was placed over the gel one by one and trapped air bubbles were removed as before. The lid was placed on the electroblotting unit, setup was connected to the power supply and run at 90 volt for 2 hours. After the electrophoretic transfer was completed the orientation of the gel and the wells were marked on the nitrocellulose paper and removed from the assembly. The electroblotted membrane was stained with Ponceau S (0.5 % Ponceau S, 1 % glacial acetic acid in water) for 2 to 3 minute and destained with water until the transferred protein bands were clear. The position of the molecular weight of the protein marker was marked and stain from the membrane was completely removed by shaking slowly in deionized water for 2-3 minutes.

The blotted nitrocellulose membrane was blocked for 2 hours in blocking solution [TBST (150 mM NaCl, 50 mM Tris.HCl, pH 7.4 and 0.1% Tween 20) contain 5% Bovine serum albumin (BSA)] on a shaker at room temperature. The blocked membrane was washed thrice with TBST for 2-3 minutes and incubated with 10 ml of primary antibody solution (1:5000 dilution of Anti-GST Monoclonal antibodies in TBST with 1% BSA). The incubation
was carried out for 1 hour at room temperature and the membrane was washed as stated before to remove unbound antibodies. Then secondary antibody solution (1:5000 dilution of Anti-goat IgG whole molecule – Alkaline Phosphatases in TBST with 1% BSA) was added and incubated for 45 minutes. The membrane was washed with five changes of washing solution to remove any traces of unbound conjugate and incubated with freshly prepared NBT/BCIP solution at room temperature. The reaction was covered with aluminum foil and after 20-30 minutes the reaction was stopped by adding PBS containing 20 mM EDTA. The expression of the pGEX-4T1 -KP construct was indicated by strong immunoreactive band with anti-GST antibodies.

III. Purification of recombinant GST-fusion proteins:

The recombinant plasmid with right orientation of the cloned insert and that showed expression of the parasite polypeptide in the above experiments was taken for medium scale production and purification of the fusions protein following the method of Fragioni and Neel, 1993, which describes the solubilization and purification of enzymatically active GST fusion recombinant protein. Twenty ml of overnight culture of the expression clone was inoculated into 1000 ml of LB broth containing 100 μg/ml Ampicillin and grown for 90 minutes. Bacteria were induced with 0.1 mM IPTG (freshly made) for 4 h at 37°C and pelleted by centrifugation at 7000 g in a GS-3 rotor (Sorvall) for 7 min. The pellet was washed with 60 ml of ice cold STE buffer (10 mM Tris HCl, pH 8.0; 150 m NaCl; 1 mM EDTA). The pellet was resuspended in 60 ml of STE buffer containing Lysozyme (100 μg/ml) and incubated for 15 minutes on ice. Dithiothreitol (DTT) was then added to a final concentration of 5 mM. The bacterial cells were lysed by addition of N-laurylsarcosine (Sarkosyl) to a final concentration of 1.5 % from the 10% stock prepared in STE buffer. After sonication on ice
for 1 minute, the lysate was clarified by centrifugation at 10,000 g, 5 minutes at 4°C) in a SS-34 rotor (Sorvall). Supernatant was transferred to a fresh tube, triton-X 100 was added to final 2% concentration followed by 500 μl washed and preswollen glutathione agarose bead suspension (50% v/v in PBS). The mixture was incubated at 4°C on an Orbiton rotor for 2 hours, centrifuged at 1000 g for 10 min and washed thrice in 50 ml ice cold PBS. The bound recombinant fusion protein was eluted with 10 mM glutathion in 50 mM Tris.HCl, pH 8.0. The eluted protein was subjected for Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) for analysis and protein estimation.

IV. Immunization of mice with purified recombinant GST fusion protein:

Inbred Balb/c female mice of 6-8 weeks were used for raising antibodies against fusion protein. All mice were bled before immunization and sera were separated and stored at −20°C until further use. Mice were divided into three groups (10 mice/group) and immunized with GST fusion recombinant protein, GST alone and one set was left as a uninoculated control. Antigens were administrated intraperitoneally in each mouse with 10 μg of the antigen (in PBS) in Complete Freunds Adjuvants (CFA), followed by 10 μg antigen on 25th and 50th days with Incomplete Freunds Adjuvants (IFA) to boost the responses. Sera was collected after two weeks of each immunization and stored at −20°C for further use.

Enzyme Linked Immuno-Sorbent Assay (ELISA) was carried out to check the titer of antibodies in the sera of mice against the immunized proteins. Briefly, 10 μg/ml of fusion protein and GST were diluted in coating buffer (0.015 m Na₂ CO₃, 0.035 M NaHCO₃, pH 9.6) and a 50 μl aliquot of this solution coated into the microtiter plate wells overnight at 4°C in a humid chamber. Then the wells were washed with washing solution (PB containing
0.05% Tween 20) twice. The sera samples were made in to various dilutions in dilution buffer (PBS containing 0.5% BSA) and added to wells in duplicate and incubated for 2 hours at room temperature. Then wells were washed thrice with washing buffer, 50 µl of secondary antibody solution (1: 5000 dilution of horseradish peroxidase conjugated goat anti-mouse IgG whole molecule in dilution buffer) was added and incubated for 45 minutes. Unbound antibodies were removed by washing the wells as before. Enzyme reaction was developed by adding 50 µl of substrate solution [50 mg Ortho-phenylenediamine dihydrochloride (OPD), in 50 ml of 0.1 M citrate buffer, 50 µl of 30% H2O2]. Reaction was allowed to develop for 2-3 min and then stopped with 50 µl of 8 M H2SO4. Optical density was read at 490 nm using an ELISA reader (Molecular Devices, UV max).

V. Identification of native protein in the parasite lysate:

To identify the presence native protein in the natural life cycle of the malaria parasite, antigen extract of *P. falciparum* were prepared, resolved on SDS-PAGE, electoblotted on to nitrocellulose membrane and probed with antisera raised against the fusion protein. The experimental procedure followed is described below.

*P. falciparum* was cultured *in-vitro* as described in the earlier chapter was used as source of parasite material. The parasitized RBCs were lysed with 0.15 % saponin, followed by centrifugation (500 g for 10 min at 4°C) to pellet down the parasites. The parasite pellet was washed twice with ice cold PBS, resuspended in double the volume of extraction buffer (50 mM Tris.Cl, pH 7.5, 5 MM EDTA, 5% Triton-X 100, 10 mM Idoacetamide, 1 mM PMSF, 1 mg/ml of pepstatin, 20 mg/ml Leupeptin), sonicated and freeze thawed in liquid nitrogen. The suspension was centrifuged (1200 g for 10 min) at cold condition, the supernatant
was transferred to a separate tube and a 10 μl aliquot was subjected for SDS-PAGE analysis. The resolved protein was electroblotted on to nitrocellulose membrane for western blot analysis. The *P. falciparum* antigen electro-blotted membrane was stained with Ponceau S, the molecular weight marker and orientation was marked (lower side and upper side in each lane) and each lane was separated to probe with different primary antibodies. The procedure followed for western blotting was that described in earlier section with only changes of primary and secondary antibodies and their dilutions, which are as follows:

1. Antisera against purified recombinant *P. falciparum* GST fusion protein at 1: 300 dilution, antisera raised against GST at 1: 300 dilution and normal (control) mice sera at 1: 300 dilution.

2. The second antibodies used for the primary antibodies were antimouse IgG–alkaline phosphatase (at 1: 3000 dilution for primary antibody raised in mice). The incubation, washing and color development were carried as described in earlier chapter for immunoblotting.

VI. Intracellular *P. falciparum* growth inhibitory effect of the antibodies against fusion protein:

The *in-vitro* cultured *P. falciparum* were synchronized twice using sorbitol to obtain homogenous stage of parasites, which is essential for inhibition experiments. Total IgG were purified from the a. normal mice serum, mice serum raised against purified GST fusion protein and GST protein respectively using protein A column purification procedure. In this method, protein A lyophilized powder was dissolved in 16ml of 10 mM Tris pH 8.0 and
allowed it to swell overnight. Then it was washed with same buffer 4 times and columns were prepared. The pH of sera samples (300 μl each) was adjusted to pH 8.0 by adding 30 μl of 1M Tris pH 8.0. The antibody solutions were passed through the column and washed with 10 bed volumes of 100 mM Tris pH 8.0 followed by 10 bed volumes of 10 mM Tris pH 8.0. The antibodies were eluted by adding 500 μl of 100 mM glycine (pH 3.0) and collected in a 1.5 ml eppendorf tube containing 50 ml of 1M Tris pH 8.0. The purified IgG (500 μl) were heat inactivated (56°C for 30 minutes) and incubated overnight in 50 μl of washed human RBC (O+) at 4°C to adsorb antibodies against human erythrocytes. Then the tube containing this mixture was centrifuged. The supernatant was collected in a separate tube and O.D of the solution was measured at 280nm to determine the concentration. The sera samples were diluted in RPMI and filter sterilized before subjecting to inhibition assay.

The inhibitory effect of purified IgG specific to purified recombinant fusion protein (GST-KP) was assessed by measuring the incorporation of radiolabelled nucleic acid precursor, [3H] hypoxanthine. Synchronized parasites at 1% parasitemia were seeded into 96 wells tissue culture plate at 3% haematocrit in a total volume of 100 μl. The antibody dilution (concentration 10 μg/ml) in 100 μl volume was added in triplicates and the plates were incubated at 37°C and 5% CO2 for 24 hours. Wells added with anti-GST antibodies served as a control. In addition to it, heparin (concentration of 0.5 mg/ml) was included as a positive control as it is a known inhibitor for parasite invasion. [3H] hypoxanthine was then added to each well at a concentration of 1μCi/well and the plates were incubated further for 18 hours. The cells were harvested and the cell associated radioactivity was measured by scintillation counting.
VII. Immunofluorescent antibody tests (IFA):

To identify the sub-cellular localization of the cloned protein in the blood stages of
the parasite Immunofluorescence Antibody test (IFA) was carried out using antibody raised
against the recombinant protein. In this experiment, the parasitized erythrocytes were applied
on to microscope slide wells, air-dried, and fixed with 90% acetone, and 10% methanol. The
wells were incubated with primary antibodies (anti- GST-KP antibodies, anti-GST antibodies
as control and normal mice sera) at a dilution of 1: 50. The secondary antibody reagent was
fluorescein-labeled goat serum IgG (diluted 1: 100) antibody to mouse immunoglobulin.
The wells were incubated for 2 hour with primary antibodies and for 1 hour with secondary
antibodies in a humid chamber at room temperature. After each incubation, the wells were
washed in phosphate buffer saline containing 0.05% Tween 20. The wells were mounted in
PBS/glycerol after another wash and observed under fluorescent microscope.

Results:

a. Sub-cloning in pGEX- 4T-1 expression vector, overexpression and purification of
recombinant polypeptide:

The structural map with restriction sites of GST fusion expression vector pGEX-4T-1
(Fig 5.1) and the KP 1 inset (Fig 5.2) were presented. The insert was ligated into the pGEX
vector, transformed in chemical competent cells, and mini plasmid DNA were prepared from
fifty transformants. Plasmid DNA was digested with restriction enzyme, EcoR I and twelve
colonies were found to contain the KP 1 insert (Fig 5.3). To identify the recombinant with
properly oriented insert for expression, the recombinant plasmids DNA were digested with
BamH I restriction enzyme. The restriction analysis map of the insert as well as pGEX 4T-1
expression vector shows that there was single BamH I restriction site in both insert as well
vector. The BamHI digestion of the recombinant resulted in the release of a 277 bp BamHI fragment from the rightly orientated insert in comparison to 800 bp BamHI fragment in wrongly ligated insert. One of the recombinant clones (No 114) showed rightly orientated KP insert (Fig 5.4) and subjected to recombinant polypeptide expression. The broth culture of the clone was induced with IPTG (1 mM) to overexpress the cloned protein, the cell lysate prepared out of it was directly resolved by SDS-PAGE (Fig 5.5). Approximately 70 kDa polypeptide was expressed in fusion with 28 kDa glutathione S-transferase (GST) was seen in induced recombinant colony as a fat band. The 70 kDa fusion protein was affinity purified on Sepharose Agarose (Fig 5.6) and reacted with anti-GST monoclonal antibodies (Fig 5.7). The medium scale (1000 ml) culture yielded approximately 0.4 mg fusion protein. The purified protein was used for raising polyclonal antibodies in Balb/c mice.

b. Presence of native protein in *P. falciparum* lysate:

In order to identify native protein of karyopherin in *P. falciparum* parasite lysate immunoblotting was carried out using antibodies raised against purified fusion protein. The *P. falciparum* parasite lysate was prepared, resolved by SDS-PAGE and transferred to nitrocellulose by standard method. The blot was probed with antiserum against GST-fusion protein along with appropriate control anti-GST antibodies and normal mice sera. The antibodies used for immunoscreening (anti- *P. yoelii* mice sera and immune sera from endemic area and normal human sera) were also used to probe the lysate. Antiserum against GST fusion recombinant protein (partial fragment of karyopherin) specifically recognized a 120-kDa protein band in *P. falciparum* lysate (Fig 5.8). The identified protein bands in the lysate of *P. falciparum* corresponded to the size of
karyopherin beta reported for organism like Yeast, *C. elegans* and human. The 120-kDa protein band was not reactive with anti-GST serum suggesting that the 120-kDa protein band is specific to protein of 1.1 kb clone. The anti-*P. yoelii* and endemic human sera recognized several protein bands in the *P. falciparum* lysate. The result indicates the approximate size of the native protein and its expression during the life cycle of the parasite.

c. Immunological characterization of the cloned protein:

Reactivity of antibodies raised against cloned recombinant protein (clone 1) in the growth and development of *P. falciparum* was studied *in-vitro* using standard method of inhibition assays. The *in-vitro* inhibition of parasite growth observed was 20% more than the control set of experiments (Fig 5.9). This experiment suggested that the antibodies against karyopherin have role in providing partial protection against parasite infection in *in-vitro* conditions.

d. Sub cellular localization of Karyopherin in the parasite:

Immunofluorescence Antibody test (IFA) was carried out to localize the antigen on the parasite (Taylor *et al.*, 1981). The protein was localized in *P. falciparum* blood stages using anti-GST fusion recombinant (karyopherin) specific antibodies (diluted to 1: 100) and fluorescein-labelled anti-mouse IgG (diluted to 1: 200). Control with anti-GST alone and normal mice sera were also included in the experiments. The fluorescence was observed on the schizonts stages, which indicate that the protein is expressed in blood stages of the parasite life cycle (Fig 5.10). Further immuno-electron microscopy and
confocal microscopy characterization is essential to precisely localize the protein on the parasite.

Discussions:

The cross reactive *P. falciparum* 1132 insert (KP1) identified by immunoscreeing was expressed by cloning in pGEX expression vector as a fusion protein with 28 kDa glutathione S-transferase (GST). pGEX vector has been used to express malaria recombinant proteins other workers (Kilbey *et al.*, 1993, Ma *et al.*, 1996, Kang *et al.*, 1998, Rotman *et al.*, 1999, Chatterjee *et al.*, 2000) In the original description of the pGEX system, it was suggested that most of the GST fusion protein would be soluble (Smith and Johnson, 1988) However, in practice many GST fusion proteins are partially or completely insoluble after lysis in non-ionic detergent In 1993, Frangoni and Neel, 1993 developed method to solubilize and purify enzymatic active protein by adding sarkosyl in the solubilization procedures. We followed their method exactly to express, solubilize and purify the GST recombinant protein as soluble fraction But we were able to partially solubilize the protein and purify the quantity required for immunological studies.

The mice antiserum raised against fusion peptide (GST-KP with a molecular weight of 72 kDa) recognized approximately 120 kDa protein from *P. falciparum* lysate. The protein size corresponded to all the known karyopherin beta of other organisms such as yeast, the free living nematode, *C. elegans* and human. An important finding of this study is that the protein is expressed in blood stages of the malarial parasite. As blood stages are the epicenter for all pathogenic conditions of malaria, and that the karyopherin is expressed in this stage and is a vital protein for the parasite survival it assumes a lot of significance as a
vaccine/drug target. Most importantly, it is conserved across different *Plasmodia* species as seen in earlier chapters. The existence of different epitopes common to *P. yoelii* and *P. falciparum* were reported earlier (Taylor *et al.*, 1981, Ray *et al.*, 1994, Ma *et al.*, 1996). These results are further suggestive of its role as a target candidate for vaccine/drug development.

In the absence of any definitive assay to correlate immune responses to a given antigen with protection against the blood stage malaria infection, inhibition of parasite growth has been often used by many workers as one of the criteria for judging the potential of the antigen for a vaccine candidate against blood stages (Shi *et al.*, 1999, Nikoderm *et al.*, 2000). The inhibition assay showed partial inhibition under *in-vitro* conditions. The expression of full-length protein and antibodies against it may be necessary to study the inhibitory effect of the protein to derive any conclusion on protection. The other reason may be that antibodies raised against recombinant fusion protein may be less immunogenic against the natural proteins. It is also observed that several vaccine target antigens contain regions that constitute immunodominant determinants, had potential to divert immune response to more conserved and possible critical epitopes (Wrightsman *et al.*, 1994, Anders, 1986, De La Cruz *et al.*, 1989). In malaria it takes very long and continuous exposure to develop immunity, which also indicates that the critical antigens are less immunogenic and require a long and continuous challenge to build up protective immunity. The immunofluorescence assay using the antibodies against expressed fusion protein lighted up schizonts stages of the parasites. The precise organellar location of the protein in parasite by using other methods like electron microscopy and its function by gene knockout is essential and is a future plan for the project.
A. Figure 5.1. Genetic map of Glutathion fusion protein expression vector, pGEX-4T showing multiple cloning sites

B. Figure 5.2. Restrictions map of the insert KP1, showing a unique BamH I site at 227 bp. Presence of BamH I site unique site in insert as well in the pGEX expression vector was useful to identify right orientations recombinant expression pGEX- KP1 construct by enzymatic digestion. BamH I restriction digestion of the pGEX- KP1 construct DNA will release 277 bp in right orientated insert.

C. Figure 5.3. Sub-cloning of KP1 insert into pGEX-4T1 vector at EcoR I sites. Agarose gel electrophoresis of EcoR I digested recombinant plasmid (Lane 1) showing KP1 insert subcloned into the pGEX-4T1 plasmid. Lane M, was loaded with 1 Kb DNA size marker.

D. Figure 5.4. Confirmation of the right orientation of the sub-cloned KP insert into pGEX-4T1 at EcoR I sites. The recombinant plasmids (released 1132 bp inserts by EcoR I digestions) were digested with BamH I and analyzed on agarose gel electrophoresis. The gel picture shows, the wrong oriented recombinant plasmid released 855 bp (Lane 1) and in proper orientated recombinant plasmid showed 277 bp (Lane 2). Lane M shows the 1 Kb DNA size marker.
A. Figure 5.5. Coomassie blue stained SDS polyacrylamide gel showing expression of partial fragment of karyopherin (KP insert) as GST-fusion protein in \textit{E. coli}. The DH5\textalpha{} competent cells were transformed with non-recombinant pGEX 4T1 (Control) and recombinant pGEX 4T 1-KP construct (recombinant plasmid with right orientation of sub-cloned KP insert). Single colony were picked up from both non-recombinant and were induced with 0.1 mM IPTG for 3 hours and bacterial lysates were loaded in the wells of SDS-polyacrylamide gel, stained with Coomassie brilliant blue. Lane 1 (Uninduced) and lane 2 (Induced) are non-recombinant (Control) bacterial lysate. Lane 3 (Uninduced) and lane 4 (Induced) are recombinant bacterial lysate.

B. Figure 5.6. Coomassie blue stained SDS polyacrylamide gel showing affinity purified GST fusion recombinant protein. The recombinant GST fusion protein was expressed in medium scale and affinity purified using sepharose 4B column by batch purification method. Lane M (Molecular mass standards), lane 1-3 are the fractions (No 1, 2 and 3) of purified GST-fusion protein.

C. Figure 5.7. Western blot analysis purified GST fusion recombinant protein. Affinity purified recombinant protein (Lane 2) and purified GST (Lane 1) were probed with anti-GST monoclonal primary antibodies (developed in goat), followed by anti- goat alkaline phosphates conjugates and the color was developed by BCIP/NBT substrate solution. Lane M shows the molecular mass standards.
Figure 5.8. Identification of native karyopherin protein in the blood stage lysate of the *P. falciparum* by Western blot analysis. *P. falciparum* parasite lysate were subjected to SDS-PAGE, blotted to nitrocellulose membrane and probed with normal mice serum (Lane 1: control), mice antiserum against GST protein (Lane 2: GST control) and mice antiserum against purified recombinant GST fusion protein (Lane 3). Then the blots were incubated in anti- mouse IgG (whole molecule) alkaline phosphates conjugates, washed and color was developed by BCIP/NBT substrate solution. The antibodies against recombinant protein recognized a 120-kDa band. Lane M shows the molecular mass standards.
Figure 5.9. Bar diagram shows intra erythrocytic *P. falciparum* growth inhibitory effect of the antibodies against fusion protein (bar A) and GST antibodies (control bar B). The inhibitory effect of purified IgG specific to purified recombinant fusion protein (GST-KP) was assessed by measuring the incorporation of radiolabelled nucleic acid precursor, [³H] hypoxanthine. Synchronized parasites at 1% parasitemia were seeded into 96 wells tissue culture plate at 3% haematocrit in a total volume of 100 μl. The antibody dilution (concentration 10 μg/ml) in 100 μl volume was added in triplicates and the plates were incubated at 37°C and 5% CO₂ for 24 hours. Wells added with anti-GST antibodies served as a control. In addition to it, heparin (concentration of 0.5 mg/ml) was included as a positive control as it is a known inhibitor for parasite invasion. [³H] hypoxanthine was then added to each well at a concentration of 1μCi/well and the plates were incubated further for 18 hours. The cells were harvested and the cell associated radioactivity was measured by scintillation counting. The % inhibition was calculated as 100[(Count of the Test antibodies treated well – Count of the normal mouse treated well)/Count of the normal mouse treated well Control]. The wells added with antibodies against fusion protein showed 42.2% (SD +/- 5.4, Bar A) and 23.1% with anti-GST antibodies (SD +/- 6.5, Bar B). The intra erythrocytic inhibitory effect of antibodies against fusion protein was significant (The P value was 0.0100608 at 95% confidence limit). Heparin treated wells showed 80% inhibition (not showed in bar diagram).
Figure 5.10. Immunofluorescence localization of karyopherin in *P. falciparum* blood stages. *P. falciparum* infected red cells were harvested from the *in-vitro* culture and processed for Immunofluorescence assay. The infected RBC smear were air-dried, fixed in chilled acetone, incubated with primary with mice sera against recombinant GST-fusion protein (a), mice sera against GST protein (GST control: b) and normal mice serum (control: c) followed by incubation in anti-mouse IgG – FITC conjugate solution. The slides were washes and observed under UV light.