CHARACTERIZATION OF THE MITOCHONDRIAL *PLASMODIUM FALCIPARUM* HEAT SHOCK PROTEIN 70

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ABSTRACT

Malaria remains a global health problem and accounts for many deaths and illnesses in sub-Saharan Africa. *Plasmodium falciparum*, the causative agent of the most fatal form of malaria, expresses a repertoire of heat shock proteins for cytoprotection, survival and pathogenesis. The parasite genome encodes six Hsp70 proteins found in various cell compartments. However, the putative parasite mitochondrial Hsp70 (PfHsp70-3) has not been investigated. The J-proteins, Pfj1 and PFF1415c, were proposed to function as co-chaperones of PfHsp70-3. The biochemical characterization of PfHsp70-3 was initially complicated by the fact that the protein was insoluble when expressed in *E. coli* cells. Various approaches to solubilize it resulted in inactive protein. A general characteristic of eukaryotic mitochondrial Hsp70s is their insolubility and their reliance on an Hsp70 escort protein (Hep) for solubility and ultimate functions. In this study, a putative Hep protein was identified in the genome of *P. falciparum* that is referred to as PfHep1. Co-expression of PfHep1 with PfHsp70-3 resulted in soluble and biochemically active PfHsp70-3.

Size exclusion chromatography was employed to separate PfHsp70-3 from PfHep1after co-expression. PfHep1 suppressed thermally induced aggregation of PfHsp70-3 but not the aggregation of malate dehydrogenase or citrate synthase, thus showing specificity for PfHsp70-3. Zinc ions were also found to be essential for maintaining the functions of PfHep1, as EDTA chelation abrogated its abilities to suppress the aggregation of PfHsp70-3. Furthermore, PfHep1 did not stimulate the basal ATPase or increase refoldase activities of PfHsp70-3 hence displaying no co-chaperone roles. The full-length putative mitochondrial type I J protein, Pfj1, could not be produced in *E.coli* but a truncated protein containing the J-domain was produced which stimulated both the ATPase and refoldase activities of PfHsp70-3.

Further, this study demonstrated that both PfHep1 and PfHsp70-3 localized to the mitochondrion in the erythrocytic stage of *P. falciparum* development thus confirming *in silico* predictions of their localization. Besides, PfHsp70-3 was expressed during all stages of the intraerythrocytic cycle of parasite development and was heat inducible. Generally, the data obtained in this study will enhance the existing knowledge on the biology of the parasite mitochondrial chaperone functions and open the possible avenue of drug targeting considering the specificity of PfHsp70-3 and PfHep1 partnerships.

DECLARATION

I, David Onchong'a Nyakundi, declare that this thesis is my own work, submitted in fulfillment of the award of Doctor of Philosophy (Biotechnology) in the faculty of Science Rhodes University. Any other work done by other researchers included in this thesis, individuals responsible for such work have been acknowledged accordingly. This thesis has not been submitted for any other degree for examination in any other university.

David Onchong'a Nyakundi

Dated this _**December**___Day of __**9th**__**2016**__ at __**Rhodes University**

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DEDICATION

This thesis is dedicated;

To my elder brother Charles Ondieki Nyakundi, whose great love and unfainting sacrifices for his siblings saw me taking the first step into the academic journey that none of us ever knew could end up touching the arch of excellence that curved distantly at the horizon. I LOVE YOU MY HERO.

And

To the two greatest women, my mother Seraphine Nyakundi and my wife Dr. Zawadi Nh'ulubhili whose refreshing presence in my life provided the inspiration that carried me through to the end of my difficult academic journey. I LOVE YOU MY HEROINES.

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"*Ebenezer" hitherto hath Jehovah helped* **me (1Samuel 7:12)**

I would like to sincerely appreciate my supervisor, Dr. Aileen Boshoff for accepting me to join her research lab and for providing quality and professional supervisory assistance in my research work. Her motherly care, sincere interest on my work and constant encouragements made it possible for me to come to the summit of the once seemingly insurmountable academic mountain.

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To the only wise God our Saviour, [be] glory and majesty, dominion and power, both now and forever. Amen (**Jude 1:25**)

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List of research outputs

Publications

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LIST OF ABBREVIATIONS

A	Absorbance
A_{412}	Absorbance at 412 nanometres
A_{600}	Absorbance at 360nm
ACT	ART combination therapies
ADP	Adenosine diphosphate
AMA	Apical Membrane Antigen
Amp^R	Ampicillin resistance (β-lactamase gene)
APS	Ammonium persulphate
ARTs	Artemisinin and its derivatives
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BLAST	Basic Local Alignment Search Tool
Bp	Base pairs
BSA	Bovine Serum Albumin
CHIP	C-terminus-Hsp70-Interacting Protein
CM^R	Chloramphenical resistance (gene)
CXXC	C-cysteine-X-any residue- C-cysteine
CyRPA	cysteine rich protective antigen
DAPI	4,6-Diamidine-2-phenylindole dihydrochloride
DNA	Deoxyribonucleic Acid
DnaJ	A family of proteins characterized by J domain
DnaK	Prokaryotic Hsp70
DTT	Dithiothreitol
E.coli	*Escherichia coli*
EBA	Erythrocyte-Binding Antigen
EDTA	Ethylene glycol tetra-acetic acid
EEVD	Glutamate-Glutamate-Valine-Aspartate (Glu-Glu-Val-Asp) motif
EEVN	Glutamate-Glutamate-Valine-Asparagine (Glu-Glu-Val-Asn) motif
ER	Endoplasmic Reticulum
Gm	Gram(s)

G	Gravitational force
GF	Region Glycine-Phenylalanine rich region
GFP	Green fluorescence protein
GLURP	glutamte-rich protein
Hep	Hsp70 escort protein
Hop	Hsp70/Hsp90 organising protein
HPD	Histidine-Proline-Aspartic acid motif
Hsc70	70 kDa Heat shock cognate protein
Hsp	Heat shock protein
Hsp	Heat shock protein
Hsp110	110 kDa heat shock protein
Hsp40	40 kDa heat shock protein
Hsp70	70 kDa heat shock protein
Hsp90	90 kDa heat shock protein
IPTG	Isopropyl-β-D-thiogalactopyranoside
J domain	Conserved domain in DnaJ and DnaJ-like proteins which interact with Hsp70s
KAHRP	Knob-associated histidine-rich protein
Kan^R	Kanamycin resistance
Kb	Kilo base pairs
kDa	Kilo Daltons
L	Litre(s)
M	Molar
MDH	Malate dehydrogenase
Mg	Milligram(s)
ML	millilitre (s)
mM	Millimolar
mol	Mole(s)
MR4	Reference Reagents Resource
n	Nano
NaCl	Sodium chloride
NBD	Nucleotide Binding Domain

NCBI	national Center for Biotechnology Information
NEF	nucleotide exchange factor
NEF	nucleotide exchange factors
Ng	Nanogram(s)
NIAID	National Institute of Allergy and Infectious Diseases
ONPG	ortho-nitrophenyl-β-galactoside
OD	optical density
P. falciparum	*Plasmodium falciparum*
P. knowlesi	*Plasmodium knowlesi*
P. malariae	*Plasmodium malariae*
P. ovale	*Plasmodium ovale*
P. vivax	*Plasmodium vivax*
P. yoelii	*Plasmodium yoelii yoelii*
PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PEXEL	Plasmodium export element
PfEMP	*Plasmodium falciparum* Erythrocyte Membrane Protein
PfHsp	PfHsp *Plasmodium falciparum* Hsp
Pi	Inorganic phosphate
PMSF	Phenyl Methyl Sulfonyl Fluoride
PNEP	Negative Exported Proteins
PV	Parasitophorous vacuole
RESA	Ring-infected erythrocyte surface proteins
RNA	Ribonucelic acid
SBD	Substrate Binding Domain
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis
SERA	serine repeat antigen
TBS	Tris-Buffered Saline
TBS-T	Tris Buffer Saline – Tween 20

TBV	transmission blocking vaccines
TDR	Tropical Disease Research
TEMED	N,N,N',N'-tetramethylethylenediamine
TIGR	The Institute for Genomic Research
TIM	translocase of the inner membrane
TOM	translocase of the outer membrane
TPR	Tetratricopeptide Repeat
UV	Ultraviolet
v/v	volume per volume
VTS	vacuolar transport signal
w/v	Weight to volume ratio
WHO	World Health Organization
YT	Yeast- Tryptone media
α	Alpha
β	Beta
µ	Micro
µg	Microgram(s)
µl	Microlitre(s)
µM	Micromolar
<	Less than
%	percent
>	More than
°C	Degrees Celsius
6xHis	Hexahistidine tag
Zf-DNL	Zinc finger domain

CHAPTER ONE

Literature review and study background

1.1.Malaria infection

Notwithstanding the great strides made in malaria control for the past decades, it still remains the most devastating infectious disease and major cause of morbidity and mortality to mankind (Molina et al. 2014; Murray et al. 2012). Approximately 1.2 billion people across the world are at risk of malaria with 219 million new infections and more than 0.6 million deaths reported annually (WHO 2014; Mendis et al. 2001). More than 90% of the total global malaria cases are reported from the African continent especially in sub-Saharan Africa with children (Akachi et al. 2011), pregnant mothers and the elderly at the highest risk (Rowe et al. 2009; WHO 2012).

Malaria, in many of these affected regions, is aggravated by factors such as the inability of the relevant countries to transparently plan and implement public health policies (Fukuyama et al. 2004), economic status of individuals (Deaton, 2003) and environmental factors such as temperature (Garg et al. 2009), rainfall (Martens et al. 2000) and altitude (Bødker, 2003). Other factors include HIV infections (Craig et al. 2004), poor housing, lack of sanitation and poor drainage of stagnant water from agricultural activities that increase vector breeding (Martens et al. 2000; Boussalis et al. 2012). Governments from these endemic regions direct much of their resources towards the efforts of combating the disease, resulting in negative economic output (Chima et al. 2003) and aggravation of poverty (Sachs and Malaney, 2002). The spread of drug resistant parasite strains, the failure of vector control programs, insecticide resistance and lack of a vaccine have further worsened the effects of malaria on economic development and human health (Foley & Tilley 1998; Alonso, 2011).

It is estimated that malaria results in global economic losses amounting to 12 billion USA dollars every year as well as rising health costs (www.rollbackmalaria.org.keyfacts). The global public health burden posed by malaria has galvanized international community organizations responsible for donations to prioritize reduction of infection incidences with the ultimate aim of reducing additional financial budget expenditures in malaria endemic regions due to it more lethal effects (The Global Fund to Fight AIDS, Tuberculosis and Malaria; GFTAM, 2007; Snow et al. 2005).

1.1.1 Malaria causing parasites and vectors

Malaria is caused by infection with unicellular protozoan parasites belonging to the genus *Plasmodium* and transmitted by the bite of infected vectors, which are female Anopheles species mosquitoes. The parasite belongs to the phylum apicomplexa that constitutes a group of obligate intracellular organisms, many of which grow and replicate within the parasitophorous vacuole (PV), a membrane bound compartment that is segregated from most cellular trafficking pathways (Ward et al. 1993; Morrissette and Sibley, 2002). Apicomplexan parasites are characterized by the presence of an apical complex structure, which is a remnant plastid that fulfils important metabolic roles (Morrissette and Sibley, 2002). In addition, the phylum possesses unique secretory organelles such as micronemes and rhoptries essential for host cell invasion and egress, which together constitute the apical complex (Meissner, 2013).

Human malaria is caused by infection of five eukaryotic *Plasmodium* species that include *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae,* and *P. knowlesi* (cox et al. 2010). Recently, based on molecular techniques, two species of *P. ovale* (*P. ovale wallikeri*, and *P. ovale curtisi*) were reported to infect humans (Calderaro et al. 2013). *P. falciparum* causes the most lethal form of malaria known as cerebral malaria (Storm et al. 2014) and accounts for more than 90% of the world's malaria mortality (Wells et al. 2010; Snow, 2015). *P. vivax* is the most geographically widespread of the five human malaria parasites causing nearly half of malaria cases outside sub-

Saharan Africa (Mueller et al. 2009; Baird, 2013). The parasite has shown the highest resistance against malaria drugs such as chloroquine (Melo et al. 2014; Añez et al. 2015).

P.ovale causes benign tertian malaria commonly found in the tropics within Africa and Asia, and is easily treatable with conventional antimalarials (Martens et al. 2000; Alemu et al. 2013). However, due to its low density, it is often misdiagnosed owing to less sensitivity and unspecificity of common diagnostic methods used that show reduced detection of the parasite (Bigaillon et al. 2005; Miller et al. 2015). *P. malariae* is wide spread throughout sub-Saharan Africa, Southeast Asia, Indonesia, and in many of the islands of the western Pacific where it mostly affects children (Collins et al. 2005). The parasite has low parasitemia due to its tendency to develop in older erythrocytes and, unlike other human malaria causing parasites; it persists in the blood stage for almost a life time in the human host (Roucher et al. 2014). Malaria caused by *P. knowlesi* is primarily a zoonotic disease and the parasite is often termed as a malaria parasite of old world monkeys (Ramasamy, 2014). *P. knowlesi* infects humans accidentally due to trespassing the parasite's transmission habitats (Singh et al. 2004). Generally, *P. falciparum* and *P. vivax* parasites remain the major cause of the global burden of malaria and it is believed that it would take a much longer time to eradicate the two species (Okie, 2008). Indeed, *P. falciparum* parasite species eradication has proved difficult despite more than a century of research findings (Rowe et al. 2009).

There are over five hundred species of Anopheles vector species with over sixty of them transmitting malaria-causing parasites (Arrow et al. 2004). The most common species are *Anopheles gambiae, A. arabiensis, A. obscursus, A. guadrimacutis, A. nili,* and *A. moucheti*. Malaria disease distribution and endemicity is determined by the distribution of the vector and parasite species (Zofou et al. 2014). For instance, *P. falciparum* remains entrenched in Africa because of optimal environmental conditions for the most efficient Anopheline mosquito vectors. The occurrence of *P. falciparum* parasite and Anopheline vectors in the sub-saharan regions of Africa has made malaria endemic in these regions owing to the efficiency of both the parasite and the vector (Baird, 2013).

1.1.2 *P. falciparum* life cycle & clinical symptoms of malaria.

Malaria parasite life cycle involves two hosts, humans and female *Anopheles* mosquitoes. In humans, the parasites develop and multiply asexually first in the liver cells and then in the erythrocytes of the blood (Prudêncio et al. 2006). In the blood, the parasites undergo asexual multiplication inside the erythrocytes, destroying them and releasing merozoites that continue the cycle by invading other erythrocytes. Some parasites that differentiate into gametocytes are picked up by a female *Anopheles* mosquito during a blood meal. These gametocytes initiate the sexual phase of developmental cycle in the mosquito (Figure 1.1) (Fujioka & Aikawa, 2000).

Malaria infection in human begins when sporozoites in the infected mosquito vector get injected into the blood stream (Vaughanet al. 2013). The sporozoites eventually invade liver hepatocytes and undergo asexual reproduction giving rise to merozoites, where each sporozoite gives rise to approximately 1000 merozoites that are contained in vesicles called merosomes (Amino et al. 2006; Duffy et al. 2012). The merozoites get released into the blood and invade the erythrocytes to initiate the second phase of asexual reproduction (Figure 1.1) (Baer et al. 2007). In the erythrocytes, the parasite goes through several stages, from ring to trophozoite and finally to schizont stage. After erythrocyte invasion, the parasite's metabolic rates slow down and it manifests as unfilled cytoplasm, generally referred to as ring stage or early trophozoite stage (Figure 1.1) (Tilley et al. 2011).

Ring stage infected erythrocytes freely circulate in the peripheral blood making them easily detectable in infected patients (Tilley et al. 2011). The parasite increases its metabolic rate from the 24^{th} to 40^{th} hour post invasion and consequently increases the rate of hemoglobin uptake forming late trophozoites. Trophozoites are relatively mature parasites characterized by the presence of hemozoin pigments as a result of hemoglobin digestion (Prudêncio et al. 2006). Parasites at trophozoite stage are rarely observed in the circulation of infected patients because of their adherence to endothelial cells and consequent sequestration away from circulation (White et

al. 2014). After the 40th hour post invasion, the parasite undergoes cytokinesis to form schizonts that contain a number of daughter parasites generally referred to as merozoites.

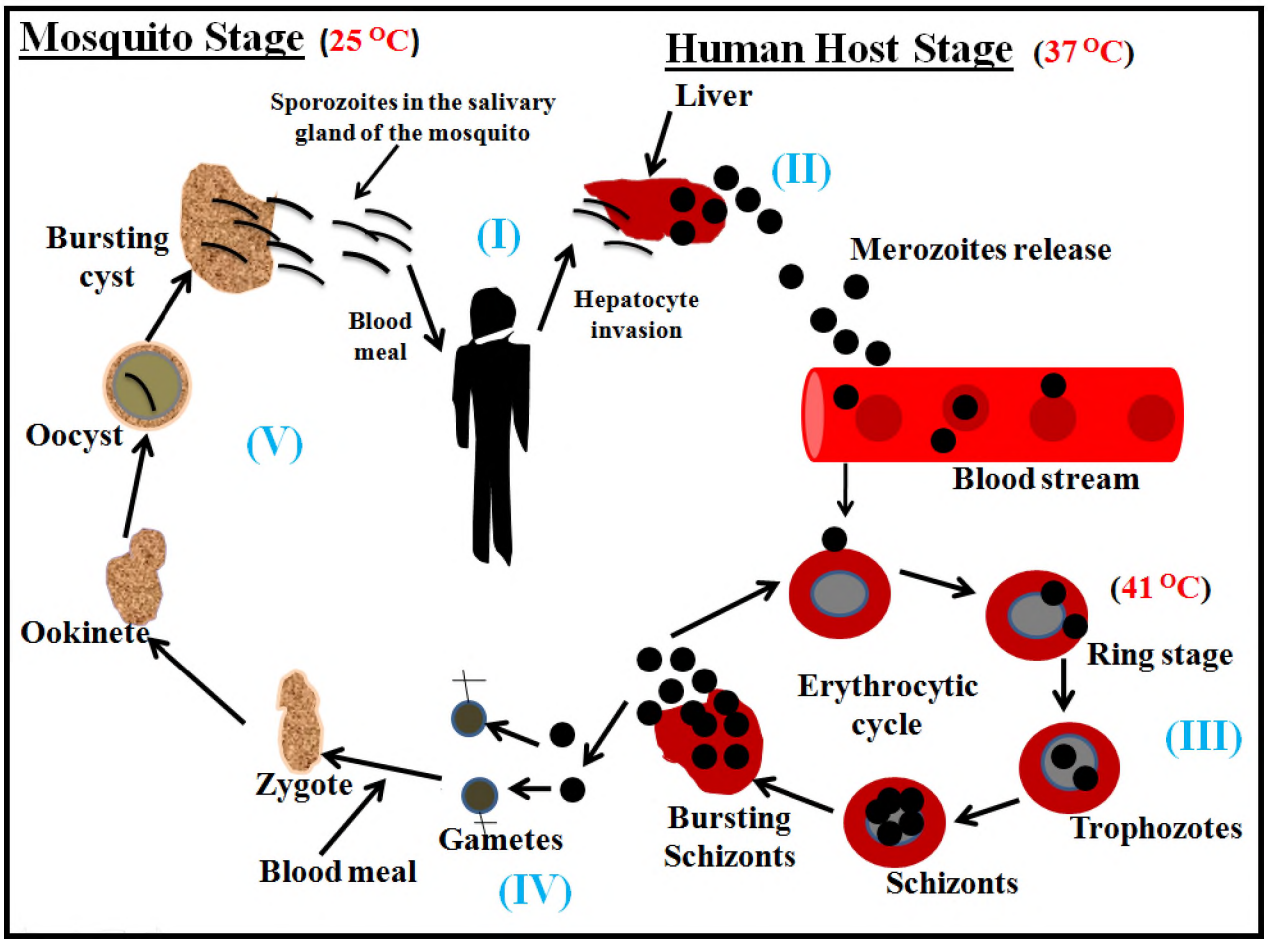

Figure 1.1 : The life cycle of *P. falciparum*: (I) During a blood meal on humans, Anopheles mosquito injects sporozoitse into the body. The sporozoites move to the liver and invade hepatocytes where they develop to produce merozoites. (II) The merozoites from the liver get released into the blood stream. (III) Merozoites invade erythrocytes and grow into trophozoites via ring stage and mature into schizonts. Mature schizonts burst to release merozoites that re-invade new erythrocytes while some develop into gametocytes. (IV)The gametocytes are taken up by a blood feeding mosquito into the gut where they develop into mature male and female gametes. (V) The fertilized zygotes develop into ookinetes and oocysts and finally sporozoites that migrate to the salivary glands of the mosquito ready to be injected back to the human host during mosquito blood meal (Figure prepared by author).

The schizont bursts and releases the merozoites that will invade new erythrocytes (Duffy et al. 2012). The process of erythrocyte invasion and infection is indefinitely repeated and it is responsible for malaria clinical symptoms characterized by high fever in patients (Cox-Singh and Balbir, 2008). A small percentage of merozoites differentiate into gametes (Alano, 2007) that circulate in the blood until they are taken up by a female anopheline mosquito when taking a blood meal to initiate the sexual phase inside the mosquito gut (Baker, 2010). Gametes mature and fertilization occurs to form a motile zygote (ookinete) within the lumen of the mosquito gut (Matuschewski, 2006). The ookinete penetrates the gut and develops into oocysts that undergo further development to form sporozoites that will migrate to the salivary glands of the mosquito ready to be injected into a new host when the mosquito feeds on blood (Figure 1.1) (Cox, 2010; Fujioka & Aikawa, 2002). Only a small a number of sporozoites ($\leq$ 200) are ejected from the salivary ducts even though the vector mosquitoes can carry as many as thousands of them in the salivary glands (Medica and Sinnis, 2005).

The parasite's presence in the host system causes inflammatory responses that lead to high fever and in some cases life-threatening sickness characterized by severe anemia, unconsciousness, breathing difficulties, failure of a number of body organs, coma and death (Dondorp et al. 2008; Marsh et al. 1995). The severe clinical manifestations are due to high parasitemia and sequestration of mature *P. falciparum*-infected erythrocytes in micro-vascular areas throughout the body (Miller et al. 2002). Intracellular parasites remodel erythrocytes and make them rigid and poorly deformable with the tendency to adhere to a variety of cell types, hence facilitating cell rosseting and sequestration (Cowman et al. 2012).

Remodeling of erythrocytes is an adaptation believed to help the parasite to evade the host's splenic clearance mechanisms that remove aged or damaged erythrocytes (Mebius and Georg, 2005). Sequestration can lead to cerebral malaria if it does occur in the brain (Jain et al. 2013) and can cause complications in metabolic disturbances such as lactic acidosis (Planche and Krishna, 2006) due to general micro-vascular obstruction (Dondorp et al. 2008). Malaria symptoms can also manifest as vomiting, joint aches, myalgia and associated metabolic

complications (Planche and Krishna, 2006). However, the manifestations and severity of the disease depends on many other factors, such as transmission intensity, infecting parasite species and a myriad of host factors such as the level of natural and acquired immunity, age, genetic constitution, general health and nutritional status amongst others (Shelton et al. 2015).

1.2 Malaria prevention, control and management

The control of malaria represents one of the greatest challenges in global public health management in the 21st century. The disease has had a major impact on public health and socio-economic development due to lack of effective control measures. The integrated use of physical, biological and standard chemicals to eradicate mosquito vectors together with prophylactic and chemotherapeutic agents to control the parasites, have a long and proven track-record of saving lives. Despite challenges facing most malaria control strategies, mortality has been reduced by 48% between 2000 and 2015 as a result of a major scale-up of vector control interventions, diagnostic testing, and treatment with artemisinin-based combination therapy (WHO, 2015).

1.2.1 Vector control

The ultimate objective of malaria vector control is to reduce vectorial capacity of vector populations below the critical threshold needed to achieve a malaria reproduction rate in a given area (malERA, 2011). National Malaria Control Programs (NMCP) throughout Africa have embarked on emphasizing vector control as an integral component (Barat et al. 2004). Control strategies such as indoor residual insecticide sprays (Eisele et al. 2010) and long-lasting insecticide-treated nets have proved effective in reducing vector survival (Lengeler, 2004; Enayati et al. 2010). This has in turn reduced mortality rates especially among children less than five years of age (Akachi et al. 2011). However, these methods of vector control are not effective in areas like sub-Saharan Africa which have higher transmission rates with estimated entomological inoculation rates of 1000 infective bites per person annually (Hay et al. 2000; Kelly-Hope et al. 2009).

Dichlorodiphenyltrichloroethane (DDT) is one of the pesticides that is commonly used for the control of malaria vectors, however, the vectors have been reported to exhibit resistance against it (Mabaso et al. 2004). DDT has also been shown to exhibit deleterious effects such as hepatic and central nervous system toxicity, estrogenic and anti-androgenic effects, possible carcinogenicity (Loomis et al. 2015) , shortening of lactation period and cause of preterm delivery (Kezioset al. 2013). The use of DDT is also thought to have caused mutations in the malaria vector leading to cross resistance to conventional insecticides. However, the use of DDT as a chemical pesticide remains relatively high due to its low cost in comparison to alternative insecticides (Roberts et al. 2000; Van den Berg, 2009).

The current biological control of malaria causing vectors is dependent on pyrethroid-derived pesticides (Zaim et al. 2000). However, over-reliance on pyrethroids has led to widespread vector resistance, hence calling for more research to identify new pesticides with novel modes of actions that can circumvent the emerging resistances (Ranson et al. 2009). Most of the promising biological insecticides are unlikely to play any active role in operational control of vectors owing to the fact that the formulations have short residue shelf life under operational conditions which is a major barrier against commercialization (malERA, 2011). There is a need for economic investment in the production of new pesticides to avoid over-reliance on the current ones in the market and to curb vector resistance (Ranson et al. 2011).

Malaria vectors can also be eradicated at the larval stage by the use of biological control agents such as predators that include aquatic plants, fish, fungal pathogens and bacterial pathogens such as *Bacillus thuringiensis israelensis* and *Bacillus sphaericus* (Kamareddine 2012). Unlike adult vectors, larval immobility makes them readily accessible and hence targeting them will kill mosquito vectors collectively before they spread to human habitations (Killeen et al. 2002; Fillinger et al. 2011). However, the application of biological control agents, which suppress larval populations in the lab settings, often fail to do so under field conditions and are mostly

vector and habitat specific (Das &Amalraj 1997; Walker, 2002). Generally, the use of combined vector control methods has proved life saving but the full benefits of integrating these control measures into national health and community mechanisms are lagging behind, especially in sub-Saharan Africa (Castro et al. 2009; Ichimori et al. 2014). The selection of a correct vector control strategy is further hampered by a large number of vector species populations (Guelbeogo et al. 2009) as well as limited understanding of the ecological behavior and population structure of some of the major vectors such as *A. gambiae* (malERA, 2011).

1.2.2 Parasite Control

The use of chemotherapies remains the mainstay of malaria control and management that has proved effective when administered in time (Whitty et al. 2008). A number of chemotherapies have been employed for treatment of malaria cases including chloroquine, quinine, sulfadoxine-pyrimethamine and artemisinins (Delves et al. 2012). Most of these drugs are active either against schizonts at tissue level (schizonticidal) or against gametes (gametocidal) that prevent transmission (Chou and Fitch, 1993; Cowman et al. 1994). Chloroquine and sulfadoxine-pyrimethamine were the drugs of choice in the 1950s and 1980s respectively (WHO, 1999; Bloland et al. 1993; Talisuna et al. 2004). Sulfadoxine-pyrimethamine exerts its anti-parasitic activities through inhibition of the formation of hemozoin from reactive heme cofactors leading to the accumulation of free toxic heme in the parasite's cells and eventual death of the parasite (Bray et al. 1998). However, the use of drugs was deemphasized in the market due to parasite resistance and more use of artemisinin derived chemotherapies was encouraged (Wellems and Plowe, 2001). Furthermore, *P. falciparum* parasite is also resistant against Malarone, a combination of atovaquone and proguanil (Nixon et al. 2013).

Artemisinin and its derivatives (collectively referred to as ARTs) have proven successful chemotherapies in malaria endemic areas of the world, thereby reducing the parasite burden and providing prompt therapy for severe infections (White et al. 2015). The ARTs are used as first-line therapy (Sinclair et al. 2009) and are active against the trophozoite ring stage of the parasite

development (Witkowski et al. 2013; White et al. 2015). The mechanism by which the ARTs function is poorly understood but they are believed to cleave the Endoperoxide Bridge by a source of Fe^{2+} or heme. The cleavage results in the formation of oxyradicals that re-arrange into primary or secondary carbon-centered radicals that alkylate parasite proteins leading to death of the parasite (Eckstein-Ludwig et al. 2003; Ismail et al. 2016).However, the ARTs have a short life span and therefore are normally co-administered with longer half-life partner drugs in ART combination therapies (ACT) to prevent recrudescence and slow down the emergence of resistance (White, 2013). Indeed the continued use of oral artemisinin-based monotherapies is considered to be a major contributing factor to the development of resistance to artemisinin derivatives, hence calling for the withdrawal of production and marketing of these oral monotherapies and promotion of access to quality-assured ACTs (WHO, 2016).The ACT drugs in the market include; artesunate–sulfalene–pyrimethamine, artemether-lumefantrine, artesunate–sulfadoxine–pyrimethamine, dihydroartemisinin–piperaquine, artesunate–amodiaquine and artesunate–mefloquine (Nosten and White, 2007; WHO, 2011b). Regardless of the precautions undertaken under ACTs administrations and uses, countries from Asia have reported parasite resistance (Ashley et al. 2014). Many efforts are underway to contain the resistance (Dogovski et al. 2015) since present antimalarial control is almost entirely dependent on ACTs and any emergence of resistance will roll back the gains already made in malaria control efforts (White et al. 2015). Besides, there is a challenge of scaling up the use of ACTs and implementing sustainability in a cost-effective and equitable manner (White et al. 2011). With the problem of drug resistance on the rise, there is an urgent need to explore more drug targets involved in important metabolic pathways of the parasite (Schwartz et al. 2012).

1.3 Malaria immunity and vaccine development

Clinical immunity to malaria is slow to develop and short lived because of extensive diversity and variations coupled with complex immune-protective mechanisms found in *Plasmodium* parasites (Cowman et al. 2012; Wright et al. 2014). Both adults and children from endemic regions can acquire partial immunity to malaria through infective bites but the immunity reduces the frequency of clinical attacks but not infection (Cox, et al. 1994; Doolan et al. 2009). It has

been demonstrated that children who acquire immunity against malaria during the first five years show reduced disease symptoms, regardless of high parasitemia (Snow & marsh, 2002). However, people who rarely experience infectious bites may not develop naturally acquired partial immunity regardless of their age (Doolan et al. 2009). Parasite variations and complexities facilitate its escape from host immune detection hence making it difficult for the host to develop protective immunity. Further, acquisition of long-lived protective immunity would require repeated infection by all variant strains in a given geographical area (Crompton et al. 2014). It has been noted that the rate at which people acquire protective immunity depends largely on the number of infecting parasites and the genetic diversity of the parasite population they are exposed to in a given locality (Krause et al. 2007).

The polymorphic nature of the surface proteins of the malaria parasite has hampered meaningful development towards an effective global malaria vaccine (Vaughan et al. 2008). The current vaccine candidates that are under trial have less than 30% efficacy (Mata et al. 2013). For this reason, there is no effective licensed vaccine that can prevent infections of malaria parasites (Geels et al. 2011; Bairwa et al. 2012). The vaccines under development and trial target the parasite's pre-erythrocytic, blood stage and transmission stages of development (Thera and Plowe, 2012). The pre-erythrocytic vaccines prevent sporozoites from entering the liver or protect hepatocytes in the event of parasite entry (Schwenk and Richie, 2011). The vaccine; RTS, S/AS01 also known as Mosquirix^{TM} is an example of pre-erythrocytic vaccine that is under phase III clinical trials and is aimed at protection against malaria infection but not transmission (White, 2011; Ouattara et al. 2015). The use of Mosquirix^{TM} vaccine against infants aged 6–12 weeks, showed a reduction of clinical malaria by 26% over a period of 38 months post immunization (Kester et al. 2009; Favuzza et al. 2016; Gosling and von Seidlein, 2016). The limited efficacy of this vaccine is due to narrow protective capacity of the induced immune responses relying mostly on the recognition of a few or perhaps single epitopes (Long and Zavala, 2016).

Blood stage vaccines target merozoite antigens expressed on the surface of infected red blood cells and are designed to elicit anti-invasion and anti-disease responses (Gosling, and von Seidlein, 2016). The vaccines are based on some protein candidate antigens that include apical membrane antigen 1 (AMA1) (Laurens et al. 2013), erythrocyte-binding antigen-175 (EBA-175) (El Sahly et. al. 2010), glutamate-rich protein (GLURP) (Ogutu et al. 2009), merozoite surface protein 2 (MSP2) (McCarthy et al. 2011), merozoites surface protein 3 (MSP3) (Jepsen et al. 2013) and serine repeat antigen 5 (SERA5) (Palacpac et al. 2013). Another highly conserved protein in *P. falciparum* isolates known as cysteine rich protective antigen (PfCyRPA) has been characterized and identified as a leading blood stage malaria vaccine candidate (Proietti and Doolan, 2014). The protein exhibits remarkable properties such as the ability to elicit antibodies that inhibit *in vitro* and *in vivo* parasite growth (Dreyer et al. 2012). However, the blood stage vaccines have not shown effective protection probably due to their highly polymorphic nature and antigenic complexity nature of the parasite (Takala and Plowe, 2009; Riley and Stewart, 2013).

Other vaccines that block transmission, collectively known as transmission blocking vaccines (TBV), utilize parasite molecules unique to the sexual or mosquito stages, such as components of gametes, zygotes, or ookinetes (Nikolaeva et al. 2015). The designed vaccines are based on antibodies against antigens found on the surface of gametocytes and prevent fertilization in mosquitoes after a blood meal hence preventing sexual development (Hirai and Mori, 2010; Carter, 2001). Major proteins that have been investigated include sexual stage antigens Pfs25 found primarily on ookinetes; Pfs230 and Pfs48/45 found on gametes and zygotes respectively and involved in fertilization (Wu et al. 2015). Human clinical trials of vaccines made from these proteins are in early stages, but it has become apparent that in order to reduce transmissions, high antibody titers are required in laboratory set up assays (Long and Zavala, 2016). Transmission blocking vaccines will have an advantage of contributing to removal of reservoir populations of parasites in semi-immune individuals who carry the parasite but do not exhibit malaria symptoms (malERA, 2011). Whole sporozoite parasites that are attenuated by use of drugs or irradiations (Renia et al. 2013) can also be used as vaccines to offer transient protection for about 10 months (Hoffman et al. 2002; Vaughan et al. 2013). However, to this end, the production of an effective vaccine has remained elusive due to short-lived antimalarial immunity and the

evasion of the parasite from host immune surveillance through adhesion to vascular tissues (Ho and White, 1999; Grau and Craig, 2012).

1.4. Heat shock proteins: molecular chaperones of proteostasis

Molecular chaperones are a ubiquitous and evolutionarily conserved class of proteins that are essential for maintaining protein homeostastasis, also called proteostasis (Powers et al. 2009). They function in a coordinated manner to prevent protein aggregation and either refold unfolded proteins or direct them for degradation through the main proteolytic ubiquitin proteasome system or the autophagy lysosome systems (Roth and Balch, 2011; Trougakos, 2013). In particular, molecular chaperones assist newly synthesized proteins to reach their native conformational structure while protecting them from various types of stress and protein aggregation (Bukau et al. 2006; Hartl and Hayer-Hartl, 2009). Proper protein folding usually occurs when the hydrophobic amino acid side chains are buried at the center of the protein (Dobson, 2003; Kim et al. 2013) and any exposure of these hydrophobic stretches can lead to cellular stress through protein aggregation and eventual inactivation of essential components of the cellular machinery (Bednarska et al. 2013). Molecular chaperones detect and associate with these hydrophobic surface areas and block them from interaction with other proteins and biological membranes (Walter and Buchner, 2002; Bukau et al. 2006).

A number of molecular chaperones have been identified and characterized as proteins induced upon heat stress and were originally classified as heat shock proteins (Hsps) (Craig, 1985; Ritossa, 1962). However, many other proteotoxic insults such as heavy metals, oxidative stress, hyperthermia, nutrient deficiencies, osmotic pressures, dehydration, UV radiation, toxins of viral and bacterial infections and increased protein expressions can induce heat shock protein expression in the cell (Åkerfelt et al. 2010; Edkins and Boshoff, 2014). Physiological heat shock response is a highly conserved mechanism in all organisms implying its importance in the survival of organisms in a stressful environment (Feder and Hofmann, 1999; Åkerfelt et al. 2010).The Hsps are among the most highly expressed cellular proteins accounting for 1–2% of

the total protein under non-stressful conditions (Lencioni et al. 2007; Dhamad et al. 2016). Organisms significantly increase the production of Hsps in the event of heat shock cellular stress (Morano et al. 2012; van Oosten-Hawle and Morimoto, 2014). However, a number of Hsps are constitutively expressed and are known as heat shock cognate (Hsc) proteins that play roles in general housekeeping and maintenance of proteostasis (Hartl and Hayer-Hartl, 2002; Karagozand Rudiger, 2015).

The Hsps are classified primarily on the basis of their molecular mass (in kilodaltons, kDa) and sequence homology. However, due to the increased number of Hsps and discrepancies in their nomenclature, human Hsps have been renamed (Kampinga et al. 2009), with the names of the human proteins shown in parentheses. The major Hsps include Hsp110 (HSPH), Hsp90 (HSPC), Hsp70 (HSPA), Hsp60 (HSPD), Hsp40/J proteins (DNAJ) and small Hsp family (HSPB). The functions of many of the heat shock proteins are regulated by co-chaperones and co-factors. During their functional activities, Hsps interact with different chaperones, co-chaperones and nucleotide exchange factors to form functional complex machinery that recognizes and temporarily binds proteins during synthesis and folding (Mayer, 2010). The interaction of Hsps with their client proteins and co-chaperones is often regulated through the hydrolysis of nucleotides (Dragovic et al. 2006).

1.4.1 Hsps70 chaperone system

The Hsp70 family is the most highly conserved and well characterized family of Hsps (Rohde et al. 2005). The proteins are highly ubiquitous in eukaryotic cells and found in several cellular compartments where they play essential roles in cell viability (Daugaard et al. 2007). They occupy a central role in cellular chaperone networking and protein homeostasis (Meimaridou et al. 2009). Hsp70s co-operate with other chaperones and co-chaperones in carrying out a number of cellular functions that include protein translocation, folding of nascent polypeptides, protein assembly, prevention of protein aggregation and targeting proteins for degradation (Tomala and Korona 2008; Frydman, 2001; Mayer and Bukau, 2005). This places Hsp70 at the core of

proteostasis and therefore one of the most important molecular chaperones for organisms from bacteria to humans, with the exception of some archaea (Macario and de Macario, 1999; Buchberger et al. 2010). Indeed the over-expression of both stress inducible and constitutively expressed Hsp70 homologues help the cell to withstand cellular stresses and maintain general cellular physiology (Brodsky and Skach, 2011; Hartl et al. 2011).

All Hsp70 proteins share a common structural architecture comprising of a 45 kDa N-terminal ATPase domain or nucleotide binding domain (NBD) that is linked to a 25 kDa C-terminal substrate binding domain (SBD) via a linker region (Figure 1.2) (Zhu et al. 1996; Mayer and Bukau, 2005). The C-terminal domain is composed of 18-kDa β-sandwich sub-domain forming a hydrophobic binding pocket, and a C-terminal 10-kDa α-helical sub-domain that acts as a lid over the binding pocket (Zhu et al. 1996; Nicolaı et al. 2013). In addition, eukaryotic Hsp70s found in the cytosol contain an EEVD-motif at the C-terminal which mediates the binding of TPR-containing co-chaperones, such as C-terminus-Hsp70-Interacting Protein (CHIP) and Hsp70-Hsp90 Organizing Protein (HOP) (Scheufler et al. 2000; Brinker et al. 2002).

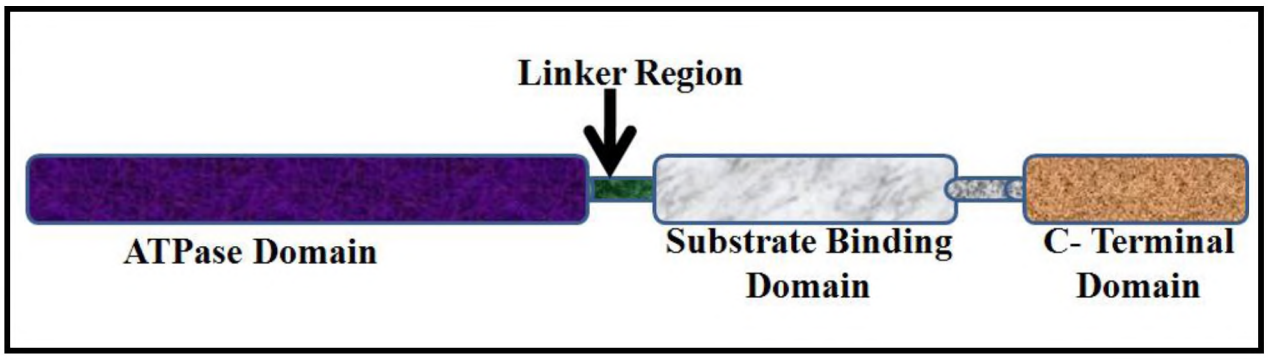

Figure 1.2 : Schematic representation of the domains present in Hsp70: Hsp70 domain organization showing ATPase and substrate binding domains joined together by a linker region. The ATPase domain is important for its chaperone activity and interaction with Hsp40 co-chaperones (Figure prepared by author).

The functions of Hsp70 rely on interdomain allostery where ATP hydrolysis in the NBD controls thermodynamics and kinetics of substrate binding and release (Chiappori, 2013). Hsp70s bind

with low substrate binding affinity and fast exchange rates when the ATPase domain is occupied by ATP molecules and with higher substrate affinity and slower exchange rates when the chaperone is in the ADP-bound state (Sharma and Masison, 2009). The ATP bound conformation "traps" client proteins within the protein binding domain followed by effecting the relevant changes on the client protein (Batista et al. 2015). The basal ATP activities of Hsp70 is usually low and co-chaperones and co-factors couple with substrates to increase the rate of ATP hydrolysis (Mayer, 2010).

There are a number of co-chaperones for Hsp70, with the majority belonging to the Hsp40/DnaJ/J protein family (Fan et al. 2003; Kampinga and Craig, 2010). Besides stimulating ATP activities of Hsp70s, J proteins deliver client substrates and recruit Hsp70 to the site of reaction (Langer et al. 1992; Kampinga and Craig, 2010). The dissociation of ADP is stimulated by NEFs which enhances substrate dissociation and subsequent recycling of Hsp70 chaperone molecules (Kampinga and Craig, 2010). A number of proteins that serve as NEFs for Hsp70s have been identified and GrpE serves as a NEF for bacterial Hsp70 (DnaK).The eukaryotic cytosol and endoplasmic reticulum contain HspBP1/Fes1p homologs that function as NEFs (Kabani et al. 2002; Kim et al. 2013). Hsp110/Grp170 family proteins also serve as NEFs for eukaryotic Hsp70s. The Hsp110 proteins are larger than Hsp70 proteins and have an extended substrate binding domain and a longer flexible C-terminus (Schuermann et al. 2008; Cyr et al. 2008). Some Hsp110s can bind unfolded proteins but will require Hsp70 to fold them (Dragovic et al. 2006; Verghese et al. 2012). Furthermore, unlike the canonical Hsp70s, Hsp110s cannot employ a nucleotide-dependent, peptide-binding release cycle (Polier at al. 2008). Hsp110s are absent from prokaryotes and are the third most abundant Hsps in mammalian tissues (Easton et al. 2000). BAG domain proteins also serve as NEFs of eukaryotic Hsp70s and there is a single BAG-type NEF (scSnl1) in yeast and six members (hBAG-1, hBAG-2, hBAG-3, hBAG-4, hBAG-5, and hBAG-6) in humans (Takayama and Reed, 2002).

1.4.2 Hsp70 escort protein

Mitochondrial Hsp70s are unique in that they rely on the presence of an Hsp70 escort protein (Hep) for structural integrity in the mitochondrial matrix. The function of the mitochondria is critically dependent on mitochondrial Hsp70 (mtHsp70) (Craig et al. 1988) and it plays a major role in the translocation of nuclear encoded proteins across the mitochondrial membranes and folding of proteins in the matrix (Kang et al. 1990; Matouschek et al. 2000). The Hep protein was first discovered in yeast mitochondria, where it was shown to prevent aggregation of mitochondrial Hsp70s and to maintain them in an active state (Sichting et al. 2005). Yeast cells deprived of Hep1 accumulate insoluble mitochondrial Hsp70 (mtHsp70) and generally exhibit mitochondrial defects similar to those observed upon mtHsp70 deletion (Sanjuan Szklarz et al. 2005; Blamowska et al. 2010). Hep orthologues are conserved in many eukaryotic species including protozoa, but they are not found in prokaryotes (Sichting et al. 2005). Hep proteins were also discovered in chloroplasts with one orthologue identified in *Chlamydomonas reinhardtii* (Willmund et al. 2008), and two in *Aradopsis thaliana* (Kluth et al 2012). An orthologue of Hep has also been reported and characterized in *Leishmania braziliensis* (Dores silver et al. 2015).

Yeast Hep1, also called Zim17/Tim15, possesses an essential zinc finger domain similar to that of bacterial DnaJ and mutation of the Hep1 lead to aggregation of yeast mitochondrial Hsp70s (Ssc1 and Ssq1) proteins (Sanjuan Szklarz et al. 2005). Despite the fact that the zinc-finger motifs in Hep1 resemble those of other J proteins, the overall folding pattern of Hep1 is different from the cysteine-rich domain of DnaJ (Martinez-Yamout et al. 2000; Momose et al. 2007). The zinc finger contains one tetracysteine motif that binds to the zinc ions which is critical for its functions and structure (Yamamoto et al. 2005; Momose et al. 2007; Dores-silva et al. 2015).

The N-terminal ATPase domain of mtHsp70 in association with the interdomain linker is prone to aggregation, while the ATPase domain and C-terminal substrate binding domain are both soluble when expressed separately (Sanjuán Szklarz et al. 2005; Sichting et al. 2007). The inter-domain linker attached to the ATPase domain is the minimal binding entity required by Hep1 to interact and keep mtHsp70 soluble and active (Blamowska et al. 2012). Furthermore, the proper

folding of mtHsp70 is independent of other mitochondrial chaperone system suggesting that Hep1 is the major, if not only, protein mediating the proper folding of mtHsp70 (Blamowska et al. 2012). Hep1 only binds to nucleotide-free mtHsp70 and is released upon nucleotide binding (Blamowska et al. 2010). Among the three yeast mitochondrial Hsp70s parologues: Ssc1, Ssq1 and Ssc3/Ecm10 (Kominek et al. 2013; Baumann et al. 2000), only Ssc1 and Ssq1 produce aggregation-prone conformers of the ATPase domain that require Hep1 to maintain solubility (Yamamoto et al. 2005). The third member, Ssc3, does not interact with Hep1 and remains insoluble upon co-expression with Hep1. More so, non-mitochondrial Hsp70s targeted to a Hep1 deficient mitochondrial matrix, remain soluble, an indication that the propensity to aggregate is a specific feature of mitochondrial Hsp70 proteins rather than a common feature of all Hsp70 proteins (Blamowska et al. 2010). Both human and *L. braziliensis* Hep1 proteins have been reported to have the ability to stimulate the ATPase activity of mtHsp70 and this was not observed in yeast or any other Hep1 orthologue (Claros and Vincens, 1996; Sigrist et al. 2009; Zhai et al. 2011; Dores-silver et. al. 2016). Therefore, human and *L. braziliensis* Hep1 displayed the features of a Type I J-protein in stimulating the ATPase activity of their respective mtHsp70. Furthermore, the human Hep1 functioned as a holdase by binding unfolded proteins such as rhodanese (Zhai et al. 2011).

Nuclear magnetic resonance (NMR) structural elucidations have shown that Zim17 has an L-shape with two conserved zinc-finger motifs located at the end of the L sandwiched by two anti-parallel beta-sheets (Momose et al. 2007). The Zinc ion within the zinc finger domain promote Hep1's stable native functional structure and mediate both local and long range communication through the tetracysteine residues (Fraga et al. 2011). Yeast mutants harboring either a C75S or C100S mutation in the tetracysteine motif that is part of the zinc finger domain or zf-DNL (named after a short C-terminal motif in this domain) of Hep1 were found to be incapable of rescuing growth defects in cells lacking Hep1 (Yamamoto et al. 2005; Burri et al. 2004). This highlighted the importance of these residues and the entire motif in the overall function of Hep1. Yeast Hep1/Zim17 contains a large acidic groove at the convex face of the L where D111 and two positively charged residues, R106 and H107, are in close proximity to the zinc-finger motifs and these residues have been shown to play a critical role in interactions with mtHsp70 (Momose

et al. 2007; Fraga et al. 2013). Mutations of the key residues R81, H107, and D111 in human Hep1 decreased the binding affinities for HSPA9. Furthermore H107 played a critical role in stimulating the ATPase activity of HSPA9 (Sigrist et al. 2009).

Mitochondrial Hsp70 and its co-chaperones play essential roles in the import and folding of mitochondrial proteins. The inner and outer mitochondrial membranes possess a translocase of the outer membrane (TOM40) and a translocase of the inner membrane (TIM23) respectively, which facilitate the entry of precursor proteins into the mitochondrial matrix (Schilke et al. 1996; Matouschek et al. 2000). In yeast, the core component of the translocation of proteins into the matrix is Ssc1, assisted by the co-chaperones of the PAM complex (Pam 16-Pam18/Tim14) and Mge1/Yge1 which facilitates nucleotide exchange (Sanjuán Szklarz et al. 2005; Sichting et al. 2005; Blamowska et al. 2010). Cycles of ATP hydrolysis by Ssc1 are used to effectively drive the translocation of the substrate across the membrane. Tim14, anchored to the inner membrane in association with the TIM23 complex, is a type III J-protein that lacks the ability to bind substrates but can stimulate the ATPase activity of mtHsp70 during protein import.

Therefore it has been proposed that Zim17 and Tim14 function together as a "fractured" J protein, with the J-domain of Tim14 working in *trans* with the zinc finger domain of Zim17. Depletion of Zim17 decreases the rate of protein import into the mitochondria and *in vitro* assays show that Zim17 binds to unfolded proteins (Burri et al. 2004). Furthermore, mitochondria that lack Hep1 exhibit a reduced rate of protein import by the TIM23 complex, reduced Fe/S protein production, increased instability of nuclear genome, and the eventual death of the cell (Zhai et al. 2011). Zim17 appears to be replaced by Mdj1, the only Type I J-protein in the mitochondria, where it delivers substrate and stimulates the ATPase activity of Ssc1 in the matrix.

1.4.3 Hsp40 (J-proteins) co-chaperones

Hsp40s are a large family of proteins that are found in both eukaryotes and bacteria and mainly function to specify the cellular action of Hsp70 chaperone proteins (Fan et al. 2003). Hsp40s are appropriately referred to as J proteins, or DNAJ and DnaJ in humans and prokaryotes respectively due to the presence of a canonical J domain in their structure. J-proteins function together with both Hsp90 and Hsp70 chaperone cycle in regulating and controlling a number of physiological activities that include folding of nascent and damaged proteins, translocation of polypeptides across cellular membranes and degradation of misfolded proteins (Goh et al. 2004; Rabiller et al. 2010).

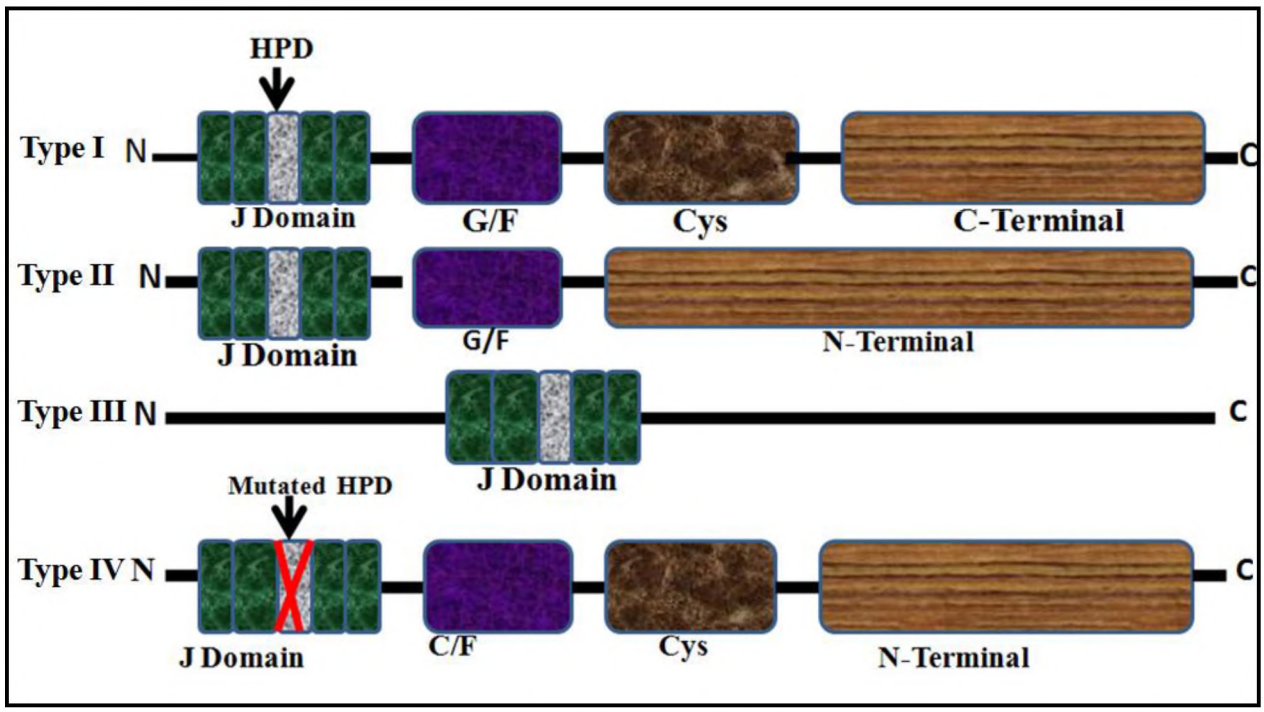

Figure 1.3 : Schematic representations of the structural domains of type I, II, III and IV Hsp40/J proteins: All Hsp40 types contain the signature J domain. In type I and II Hsp40s, the J domain is usually at the N-terminal, while in type III Hsp40s, the J domain can be found at any position in the protein. The glycine/ phenylalanine-rich region (G/F) is found in type I and II Hsp40s and functions as a hinge and regulates the specificity of Hsp70-Hsp40 interactions. The cysteine repeat region (Cys) found only in type I Hsp40, forms a zinc finger-like structure involved in substrate binding. The C-terminal regions of type I and II Hsp40 are involved in substrate recognition and binding. Type IV Hsp40s have a mutated or corrupted HPD motif in the J domain (Figure prepared by author).

The Hsp40s proteins mainly serve as co-chaperones of Hsp70 protein where they deliver non-native clients and regulate ATPase activities of Hsp70s (Summers et al. 2009). They are more

diverse in number, structure and functions and some of them exhibit chaperone properties where they bind to substrate proteins and prevent their aggregation independent of Hsp70s (Hageman et al. 2009; Mansson et al. 2013). Moreover, Hsp40s are often more abundant compared to Hsp70s, for instance there are 23 J proteins compared to 14 Hsp70s in yeast (Craig and Marszalek, 2014*)*.While the J-domain is both the defining domain and essential for functional interaction with Hsp70s, many of them bear little structural similarity outside the J- domain (Hageman J, Kampinga, 2009; Craig et al. 2006). In addition to the J-domain, members of J-protein families possess unique domain architectures that facilitate recognition of different client proteins and target particular Hsp70s (Hohfeld et al. 1998; Walsh et al. 2004).

Structurally, Hsp40 proteins possess an approximately 70 amino acid long J domain consisting of four α-helices (helices I – IV) with a highly conserved histidine-proline-aspartate (HPD) motif found between helices II and III (Figure 1.3) (Yochem et al. 1978; Cheetham and Caplan, 1998) that is essential for stimulation of Hsp70 ATPase activities (Hennessy et al. 2005). Mutation of the HPD motif destroys Hsp40's abilities to stimulate the ATPase activities and impairs their co-chaperone functions of Hsp70 (Hennessy et al. 2005). Some J-proteins contain additional domains like protein disulphide isomerase domain and ubiquitin interacting motifs that impart specific functional roles to these diverse protein family members (Kampinga and Craig 2010; Sarkar et al. 2013).

The J-proteins are classified into four subtypes based on the domain architecture of the Hsp40 homologue from *Escherichia coli* DnaJ (Kampinga et al. 2009) and the naming comes from the arrangement of the four α-helices that resemble the letter "J" (Pellecchia et al. 1996). Type I J proteins possess an N-terminal J domain, a glycine/phenylalanine (G/F) rich domain and a zinc-finger domain containing four cysteine/glycine repeats with a variable C-terminal domain. Type II J proteins possess all other domains found in type I but lack the zinc domain with cysteine rich region and type IIIs have their J domain located anywhere along the protein (Figure 1.3) (Cheetham and Caplan, 1989).

Type IVs constitute a more recently defined group of Hsp40s that contain a compromised variation of the canonical HPD motif (Figure 1.3). Even though J proteins have been canonically

classified as type I, II, III and IV (Cheetham and Caplan, 1998; Botha et al. 2007), some of them contain additional domains and possess different biochemical properties within individual classes (Hennessy et al. 2005; Kampinga and Craig, 2010). Besides stimulating ATPase activities of Hsp70s some type III J proteins carry out more specialized functions that include mRNA splicing and protein translation and are unable to function as typical co-chaperones (Cheetham and Caplan, 1998; Walsh et al. 2004; Nicoll et al. 2007). Interactions between J proteins and their respective partners occur at the HPD motif and since type IVs' HPD motifs are non functional and corrupt, they may use a different mechanism of interaction with their partner Hsp70s (Botha et al. 2007). The J-proteins have been shown to occupy a central role in molecular chaperone function systems and therefore it is expedient to characterize and analyse them in order to clarify more of their regulatory roles on Hsps.

1.4 Hsp70 and J-protein interactions

During the process of protein folding, Hsp70s depend on J-proteins where they form complex interacting machinery (Craig et al. 2006). The basal ATP activity of Hsp70 is usually low and in order for a functional Hsp70 folding cycle to occur, co-chaperones and nucleotide exchange factors (NEFs) must couple with the substrate to increase the rate of Hsp70s ATP hydrolysis (Mayer, 2010) (Figure. 1.4). J-proteins stimulate Hsp70 ATPase activity by temporarily interacting with them, and a single J-protein can serve more than one Hsp70 (Hennessy et al. 2005; Kampinga and Craig, 2010). Furthermore, J-proteins deliver client substrates and recruit Hsp70 to the site of the reaction (Langer et al. 1992; Kampinga and Craig, 2010). Hsp70 proteins have two distinct conformations, an ATP-bound state that temporarily interacts with substrates and an ADP-state that binds to the substrate (Misselwitz et al. 1998). Substrates interact with Hsp70 in an ATP-bound state where J-proteins place substrates into the peptide-binding cleft of an Hsp70 coupled with stimulation of ATP hydrolysis by J-domains of the J-proteins (Craig et al. 2006). The conversion of Hsp70 to the ADP-bound state due to hydrolysis stabilizes the interaction of Hsp70 with the substrate. The nucleotide exchange factors facilitate the release of ADP from Hsp70 leading to conformational changes that result in substrate dissociation (Bukau

et al. 2006). The NEFs also position Hsp70 for the next cycle of substrate binding reaction (Dragovic et al. 2006) (Figure. 1.4).

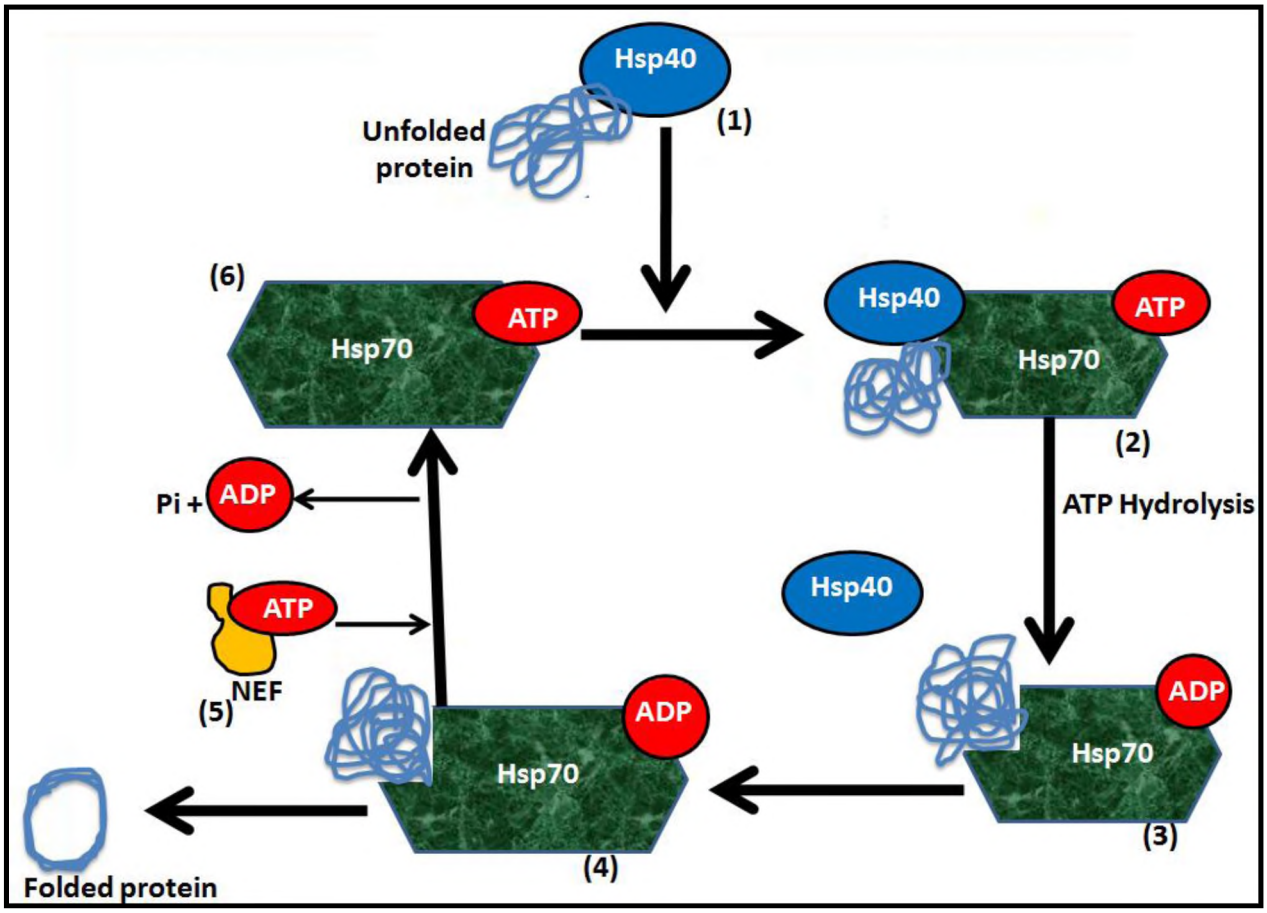

Figure 1.4 : Figure 1.4: Hsp70-J protein interaction cycle: (1) A J-domain containing Hsp40 protein interacts with the misfolded or unfolded polypeptide with a domain distinct from its Hsp70 binding site. (2)The Hsp40-substrate complex subsequently interacts with Hsp70 in its ATP-bound form and the substrate is passed from the J-domain of the Hsp40 to the SBD of Hsp70. (3) The J-domain interaction with Hsp70 stimulates hydrolysis of ATP, resulting in a conformational change in the SBD that closes the 'lid' and prevents substrate release (high affinity state) and subsequently releasing the Hsp40.(4,5) A nucleotide exchange factor (NEF) interacts with the ATPase domain of Hsp70 and stimulates exchange of ADP for ATP. (6) This in turn results in a conformational change in the SBD of Hsp70 that permits the release of the bound substrate while returning Hsp70 to its initial low affinity state (Figure prepared by author).

Newly synthesized polypeptides interact with Hsp70 to acquire a functional conformational structure but those proteins that fail to reach a native state in the cycling process by Hsp70 may be handed downstream to chaperonins/ Hsp60 for final folding (Frydman et al. 1994).

1.5 Hsp70 and Hsp90 interactions

Hsp70 and Hsp90 are major chaperones involved in protein quality control in regulating the functioning, trafficking and turnover of proteins involved in cell signaling (Pratt and Toft, 2003). They are also involved in transportation, folding, maturation, degradation (Scheufler et al. 2000; Young et al. 2001) and serving as a buffer of genetic variations by keeping mutant proteins in wild-type conformations (Lindquist, 1998; Krishna and Gloor, 2001). The Hsp90 proteins are conserved across many species alluding to their indispensable functions, however, prokaryotic Hsp90 orthologues do not seem to be essential (Johnson, 2012; Karagoz and Rudiger, 2015). Hsp90 isoforms found in eukaryotes are located in different cellular organelles with interchangeable functions (Krone and Sass 1994). There are three cytosolic isoforms, inducible Hsp90K, constitutive Hsp90L (Csermely et al. 1998) and cellular transformation associated isoforms Hsp90N (Grammatikakis et al 2002). Additional isoforms include Grp94 in the endoplasmic reticulum (Marzec et al. 2012), TRAP1/ Hsp75 in the mitochondria matrix and Hsp90C in the chloroplast (Csermely et al. 1998; Altieri et al. 2012) also exist.

Structurally, Hsp90 proteins possess N-terminal and C-terminal domains joined by a variable and charged linker that provides a binding site for client proteins (Pearl and Prodromou, 2001). The N-terminal provides a site for ATP hydrolysis (Prodromou, 2000; Mayer, 2010) followed by a 35 kDa middle domain (Nemoto and Sato, 1998), while the C-terminal contains a dimerization domain together with the EEVD motive that binds to co-chaperones (Pearl and Prodromou, 2006). Functionally, Hsp90 couples with Hsp70 to form a crucial cytosolic ATP-driven chaperone network partnership (Bukau et al. 2006; Wegele et al. 2004; Clare and Saibil, 2013). The Hsp90/Hsp70 partnership is controlled by co-chaperones and their activities are connected by the adaptor protein Hop (Hsp90-Hsp70 organizing protein) (Mayer, 2010; Li et al. 2012). Both Hsp90 and Hsp70 use their EEVD motifs at the C- terminus to bind to the tetratricopeptide

repeat (TPR) domains of Hop and facilitate the transfer of client proteins from Hsp70 to Hsp90 (Johnson et al, 1998; Schmid, 2012). The molecular basis for task distribution between Hsp90 and Hsp70 is not well known especially in the folding process (Karagoz et al. 2014). However, unlike Hsp70 that is thought to interact with client proteins at earlier folding stages (Rudiger et al. 1997), Hsp90 acts on clients during late folding stages (Pearl and Prodromou, 2006; Li et al. 2012; Taipale et al. 2010). In order for substrates to move from Hsp70 to Hsp90, ATP hydrolysis of Hsp90 becomes inevitable where Hsp90 acts as a substrate release factor for Hsp70 (Kirschke et al. 2014). In addition, Hsp90 does not seem to require specific binding sites on the client proteins thus enabling it to recognize a myriad of structurally diverse substrates (Echeverria and Picard, 2010; Street et al. 2011).

1.6 *P. falciparum* heat shock proteins

During developmental stages, malaria parasites experience heat shock episodes when shuttling between the cold blooded insect vector (25^{o}C) and warm blooded human host (37^{o}C) (Sherman, 1998; Bayoh and Lindsay, 2003) (Figure 1.1). Hsps play a major role in the adaptation and survival of the parasite in the human host especially during fever episodes that are a clinical hallmark of malaria where body temperatures rise sharply (Pavithra et al. 2007). The rise of temperature during fever episodes is believed to increase resilience of the parasite following physiological stress and the parasite adapts to the stress by increasing the expression of Hsps for cytoprotection (Pavithra et al. 2004; Pérez-Morales and Espinoza, 2015). The role of Hsps also becomes critical for the parasite during cellular stress induced by high protein turnover due to rapid multiplication rates in asexual blood stage development where more protein produced at this stage would require Hsps to properly fold them and prevent them from aggregation (Rathore et al. 2015).

Parasite heat shock proteins have also been shown to play a role in drug resistance by suppressing the host immune system and neutralizing oxidative stress often induced by antimalarial therapeutics (Akide-Ndunge et al. 2009). Moreover, the asparagine (Asn) rich proteome of the parasite is vulnerable to aggregation hence the need for Hsps to prevent probable aggregation of its proteins during heat stress (Aravind et al. 2003; Muralidharan et al. 2012).

Perhaps for these reasons, *P. falciparum* dedicates 2% of its total genome for encoding molecular chaperones (Acharya et al. 2007). Indeed, a number of *P. falciparum* Hsp70 and Hsp40s have been proposed to play a major role in parasite development and survival, particularly within the human host. This is based on the fact that Hsp70 chaperones and their Hsp40 co-chaperones are critical to the maintenance of cellular proteostasis through their role in the folding, refolding, aggregation suppression, translocation and degradation of proteins (Shonhai et al. 2011; Njunge et al. 2013; Pesce and Blatch, 2014).

The parasite exports a number of Hsp40 and Hsp70 proteins to the infected erythrocyte as part of its adaptation (Marti et al. 2004; Kulzer et al. 2012). Besides its own heat shock proteins, the parasite hijacks the host's chaperone machinery to aid in trafficking its own proteins to the erythrocyte (Oakley et al. 2011). Proteins that are trafficked to the host cells enable the parasite to remodel the host cell in such a way as to facilitate nutrient absorption and avert the host immune system (Maier et al. 2009). There are approximately 8% of proteins encoded in the parasite genome which are trafficked to the host cell and the majority of these proteins possess a signature export motif called vacuolar transport signal (VTS) (Hiller et al. 2004) or Plasmodium export element (PEXEL) (Marti et al. 2004). There are approximately 463 PEXEL containing proteins (Boddey et al. 2013), however, some exported proteins generally referred to as PEXEL Negative Exported Proteins (PNEPs) do not contain the conserved export motif (Heiber et al. 2013).

Some Hsp40 proteins have been shown to localize to discrete infected erythrocyte compartments consequently known as J-dots in *P. falciparum* (Kulzer et al. 2010). The J-dots contain a specialized family of protein known as *Plasmodium falciparum* Membrane Protein 1 (PfEMP1), restricted to *P. falciparum* parasite species. PfEMP1 is amongst the proteins transported onto the surface of infected erythrocytes that mediates cytoadhesion of infected erythrocytes to human endothelium (Rask et al. 2010). The parasite transported proteins localize and remodel the infected erythrocyte through formation of raised knob structures mainly associated with histidine-rich protein (KAHRP) (Taylor et al.1987; Külzer et al. 2012). The structures help infected erythrocytes to stick and adhere to vascular endothelium, thereby preventing them from splenic clearance (Acharya et al. 2007) thus promotes parasite infectivity and pathogenesis

(Banumathy et al. 2002; Maier et al. 2008). Another Hsp40 protein; Ring erythrocyte surface antigen (RESA) (PFA0110w) is similarly localized to the RBC membrane (Morahan et al. 2011). The protein binds to spectrin, and is believed to promote cytoadhesion and stabilization of the cytoskeleton against heat stress (Cooke et al. 2002; Silva et al. 2005). RESA also increases resistance to further parasite invasion (Pei et al. 2007). By preventing secondary erythrocyte invasion, RESA is proposed to provide a selective advantage on the parasite by enhancing parasite survival (Pei et al. 2007). The role of type IV HSP40; PF10_0381 in the formation of knobs has been demonstrated and targeted disruption of the protein resulted in decreased knob formations (Maier et al. 2008).

It is generally suggested that the J-proteins are involved in promoting and regulating a number of parasite-erythrocyte exported proteins (Banumathy et al. 2002; Lingelbach and Przyborski, 2006) due to their canonical functions of regulating the activities of Hsp70 in a manner that facilitates the folding of proteins under both normal and stress response conditions (Walsh et al. 2004). Generally, Hsp chaperone proteins play a crucial role during parasite developmental stages and expression levels of these chaperones during the human intra-erythrocytic imply that heat shock proteins could take part during adaptation of the parasite to the human host (Pavithra et al. 2007) and by extension promote pathogenesis.

1.6.1 *P. falciparum* Hsp70 family.

The *P. falciparum* genome encodes six Hsp70 (PfHsp70) homologues that localize in different cellular compartments where they carry out specialized and specific functions (Shonhai et al. 2011). Both PfHsp70-1 (PF3D7_0818900) and PfHsp70-z (PF3D7_0708800) are cytosolic Hsp70 proteins (Raviol et al. 2006; Shonhai et al. 2007). High levels of PfHsp70-1 are present throughout the erythrocytic stages of the parasite life cycle (Acharya et al. 2009), with increased levels at febrile temperatures (Kumar et al., 1991; Pesce et al. 2008). PfHsp70-1 is also observed to localize in the nucleus and PV (Patankar et al. 2001; Nyalwidhe and Lingelbach, 2006). Its

increased expression during drug induced stress points to its likely role in reversing the effects of oxidative damages caused by antimalarials (Akide-Ndunge et al. 2009).

The expression of PfHsp70-z is proposed to inhibit aggregation of malarial asparagine repeat-rich proteins (Muralidharan et al. 2012). The chaperone belongs to the HSP110 family and directly interacts with PfHsp70-1 in a nucleotide-dependent fashion hence most likely serving as a nucleotide exchange factor of PfHsp70-1 (Zininga et al. 2016). PfHsp70-z heat inducible properties enable it to play a vital role during the parasite stress response (Patankar et al. 2001). The expression of PfHsp70-z at all clinical stages of malaria provides further evidence on its involvement in malaria disease development (Pallavi et al. 2010). Further, its occurrence in chromosome seven together with *var* and *pfcrt* genes for drug resistance, points to its possible involvement in parasite drug resistance and virulence (Ecker et al. 2012; Picot et al. 2009). Selective inhibition of PfHsp70-1 by small molecule antimalarial inhibitor; malonganenone-A and 15-deoxy-spergualin compounds has been demonstrated (Cockburn et al. 2011; Ramya et al. 2007).Some compounds that selectively target the interaction of Hsp40 with Hsp70 in malaria parasites have been shown to exhibit limited cytoxicity to human cells, providing a potential avenue for drug target development (Cockburn et al. 2014).

PfHsp70-2/PfBiP (PF3D7_0917900) and PfHsp70-y (PF3D7_1344200) localize in the endoplasmic reticulum (Kumar et al. 1991; Shonnai et al. 2007). PfHsp70-2 is expressed in parasite blood stages where it plays a role in controlling protein quality and interacts with proteins bound for erythrocytes (Saridaki et al.2008). PfHsp70-2 has also been shown to encode a predicted apicoplast targeting signal, however, its C-terminal ER retrieval sequence is dominant (Heiny et al. 2012) making it more likely to reside in the ER than in the apicoplast (Przyborski et al. 2015). The protein has also been shown to possess chaperone properties (Ramya et al. 2006).PfHsp70-y belongs to the Grp110/Grp170 (Hsp110) group of chaperone proteins and shares structural similarities with PfHsp70-z (Shonhai, 2014), suggesting its possible role as a nucleotide exchange factor for the resident PfHsp70-2.

PfHsp70-x (PF3D7_0831700) is the only exported parasite Hsp70 protein. It localizes to the J-dots in the host cell cytosol and on the parasite PV (Külzer et al. 2012). PfHsp70-x associates

with *P. falciparum* erythrocyte membrane protein1 (PfEMP1), a major malaria virulence factor responsible for cytoadherance (Külzer et al. 2010) and there is a possibility of exported Hsp40s functionally associating with both PfHsp70-x and human Hsp70 (Hatherley et al. 2014). Recent studies demonstrated biochemical interactions between PfHsp70-x and the exported PfHsp40 molecular chaperones PFA0660w (Daniyan et al. 2016). PFA0660w play an important role in the trafficking and folding of exported proteins, including malaria pathogenesis factors (Pesce and Blatch, 2014).

PfHsp70-3 (PF3D7_113400) is predicted to reside in the mitochondria (Shonhai et al. 2007; Njunge et al. 2013). Generally, mitochondrial Hsp70s serve as a motor system which interacts with pre-proteins to facilitate their entry into the matrix (Neupert et al. 2002). Mature proteins are then expected to be processed and properly folded upon entry into the mitochondrial matrix with the aid of *P. falciparum* chaperonin (Hsp60/Hsp10) (Gyurkó et al. 2014). Despite the fact that PfHsp70-3 has been proposed to reside in the mitochondria, previous reports proposed it to occur in the Maurer's cleft where it possibly participates in exporting antigens to the erythrocyte surface (Shonhai et al. 2007;Vincensini et al. 2005).

1.6.2 *P. falciparum* PfHsp40 family.

The *P. falciparum* genome encodes 49 Hsp40 proteins, with 2 type Is, 8 type IIs, 26 type IIIs and 13 type IVs (Njunge et al. 2013; Botha et al. 2011). PfHsp40 (PF3D7_1437900), a type I J-protein, is similar to other eukaryotic Hsp40 co-chaperones and interacts with the parasite cytosolic Hsp70 in the usual protein folding process (Botha et al. 2007). The other Type I, Pfj1 (PF3D7_0409400) possesses structurally different features with an extended C-terminal (Nicoll et al. 2007). Eighteen PfHsp40s are predicted to harbor a PEXEL motif (Botha et al. 2007) and they are believed to be exported to the cytosol of the infected erythrocytes where they take part in host cell remodeling (Pesce et al. 2014). There are three type II PfHsp40s that are exported, (PFA0660w, PFB0090c and PFE0055c, where PFA0660w and PFE0055c play a role in J-dot formation (Külzer et al. 2010; Crabb et al. 1999; Miller et al. 2002). PFB0595w (PF3D7

0213100), a type II J-protein, was recently shown to reside in the cytosol and stimulated the ATPase activity of PfHsp70-1 (Njunge et al. 2015).

Type IV PfHsp40 proteins form the largest percentage of exported PfHsp40s where 11 of them (PFE0040c, PFL2550w, PF14_0013, PFA0675w, PFA0110w, PF11_0512, PFB0085c, PF10_0381, PF11_0035 and PFB0925w) have been identified to be exported (Maier et al. 2008). Indeed, half of the PfHsp40 exportome are type IVs (Pesce et al. 2014) and some members in the group carry out specific roles. For instance, PFL2550w Also called PfGECOis expressed in gametogenesis (Morahan et al. 2011) and PFA0110w protects the infected erythrocytes membrane during fever episodes (Silva et al. 2005). A number of type IV Hsp40s could function in a different manner from the canonical co-chaperones and most of them could be unlikely to interact with Hsp70s of both the parasite and host cell due to their corrupted HPD motif on the J-domain (Pesce et al. 2014). Exported Hsp40s are likely to functionally associate with both parasite and human exported PfHsp70s.

1.7 Functions of Mitochondria in *P.falciparum* life cycle

The *P. falciparum* parasite mitochondrion undergoes various morphological and numerical changes between the asexual and sexual developmental stages (MacRae et al. 2013). The parasite relies on anaerobic cytosolic glycolysis for energy production during the infective asexual stages but retains a single double membrane mitochondrion without a functional TCA cycle and oxidative phosphorylation due to lack of pyruvate dehydrogenase complex (Torrentino-Madamet et al. 2010). Even though the parasite mitochondrion contains some components of TCA cycle and oxidative Phosphorylation, its main function tends to be production of metabolites rather than ATP synthesis (Van Dooren et al. 2006; Deponte et al. 2012). The parasite gametes possess six similar mitochondrial organelles that appear morphologically different from those in the erythrocytic stage due to the change of environment from the mammalian host to the mosquito vector where the concentration of free oxygen is higher (Van Dooren et al. 2006). The functions of the parasite mitochondrion in the asexual stages are not well known but it could maintain respiratory transport chain for transportation of proteins and other metabolites (Krungkrai et al.

1991; MacRae et al. 2013). The respiratory transport chain process would also be needed for the re-oxidation of inner-membrane dehydrogenases, such as the dihydroorotate dehydrogenase involved in de novo pyrimidine biosynthesis (Ke et al. 2011; Painter et al. 2007).

The importance of the mitochondrion to the parasite is demonstrated by the use of antimalarial drugs, such as atovaquone and arthemeter, which inhibit the functions of the organelle leading to death of the parasite (Uyemura et al. 2000; Krungkrai, 2004). The parasite's erythrocytic development makes use of 80% of the host cell hemoglobin which is converted into a chemically reactive heme cofactor that can be highly toxic to the parasite if left to accumulate in the cell (Ke et al. 2014). To avoid the toxicity of heme accumulation, the parasite converts it into hemozoin (Kumar et al. 2007; Clark et al. 2014). The parasite mitochondrion helps in synthesizing three of the eight enzymes needed for the pathway of heme biosynthesis (Nagaraj et al. 2010). A recent study showed that erythrocytic heme biosynthesis in *P. falciparum* is dispensable but plays a vital role in the sexual stage thus making the mitochondrion organelle indispensable to the parasite at this stage of development (Ke et al. 2014).

The vast majority of mitochondrial proteins are synthesized on cytosolic ribosomes and subsequently imported into the organelle as precursor proteins (Hutu et al. 2008). This requires mitochondrial resident chaperones and co-chaperones to assist in translocating imported protein across the membrane and refolding them into their conformational structure while in the matrix (Neupert and Brunner, 2002). The ATP-driven pre-sequence translocase-associated motor (PAM) is essential for full translocation of preproteins into the matrix (Wiedemann et al. 2004). Mitochondrial heat shock protein 70 (mtHsp70) play an integral role as a part of the PAM component (Okamoto et al. 2002). PfHsp70-3 is the only proposed mitochondrial Hsp70 protein in *P. falciparum* which would therefore play an essential role in the importation and refolding of proteins in the parasite mitochondrial matrix (Seraphim et al. 2014). Furthermore, a number of co-chaperones have been predicted to reside in *P. falciparum* mitochondrion. It has been proposed that PfHsp70-3 together with its co-chaperones GrpE (PF3D7_1124700), Tim44 (PF3D7_1125400), and PfPam18 (PF3D7_0724400), could potentially form the motor system that transports preproteins and fold them in the mitochondrial matrix (Dolezal et al. 2005; Njunge et al. 2013).

1.8. Knowledge gap and motivation

The role of heat shock proteins in the survival and general pathogenesis of *P. falciparum* has been demonstrated (Pérez-Morales et al. 2015; Shonhai et al. 2011) and a number of these proteins have been considered as possible drug targets in the fight against malaria (Kumar et al. 2003; Bedin et al. 2004; Neckers and Tatu, 2008). The predictions of PfHsp70-3 localization in the parasite mitochondria have been challenged by reports of it being in the Maurer's cleft. There is a need for experimental validation on PfHsp70-3 localization and its molecular chaperone properties. Most of the characterized mtHsp70s have shown to possess aggregation propensities and dependence on an Hsp70 escort protein (Hep) for solubility and functional activities. Even though a putative orthologue of Hep has been indentified in *P. falciparum* (PfHep1) (Seraphim et al. 2014), it has not been biochemically characterized. Considering the central role mitochondrial Hsp70s play in transporting and folding matrix bound proteins to guarantee mitochondrial biogenesis and homeostasis, *P. falciparum*PfHsp70-3/J chaperone machinery could also prove critical to the organelle and hence the survival of the parasite. The predicted mitochondrial PfHsp40s/J-proteins that could serve as PfHsp70-3 co-chaperones have not been experimentally validated. STRING protein interaction network resources predicts PFF1415c (PF3D7_0629200) J-chaperone protein as an interacting partner of PfHsp70-3 (Szklarczyk et al. 2011) but this has not been proved experimentally. Pfj1 (PF3D7_0409400) is another J protein predicted to reside in the parasite mitochondria (Watanabe et al. 1997), however, the protein has also been reported to localize in the apicoplast (Kumar et al. 2010). This calls for validating experiments to ascertain Pfj1's role as a co-chaperone and it localization in the parasite.

1.9 Hypothesis

PfHsp70-3 is a mitochondrial Hsp70 in *P. falciparum* and both PfHep1 and Pfj1 function as its co-chaperones.

1.10 Broad Objectives

The study will attempt to biochemically and biologically characterize PfHsp70-3 to gain insights to its molecular chaperone properties. The study will also incorporate both Pfj1 and PfHep1 as possible PfHsp70-3 co-chaperones.

1.10.1 Specific Objectives and Approach

The specific objectives of this study are:

i) Bioinformatics analysis of PfHsp70-3 and PfHep1

The study aimed at conducting comparative sequence and phylogenetic analysis to infer to the properties and possible interactions between PfHsp70-3 and PfHep1. Functional domains and residues will be identified and cladistic relationship between species in relation to the two proteins will be analyzed. Protein models for PfHsp70-3 and PfHep1 will be generated closely related templates.

ii) Expression, solubilization and purification of PfHsp70-3, Pfj1 and PfHep1

The study aimed at over-expressing His-tagged PfHsp70-3, Pfj1 and PfHep1 in a bacterial system and purifying the proteins. Respective bacterial codon optimized sequences will be inserted into pQE30 expression vectors that will be used to express the proteins. Analysis of protein expression and solubility profile will be determined by SDS-PAGE and western analyses using appropriate antibodies. Various approaches will be used to solubilise and recover biochemically active proteins and the oligomeric status of PfHsp70-3 will be determined through fast protein liquid chromatography (FPLC).

iii) *In vitro* biochemical characterization of PfHsp70-3

To better understand the biochemical properties of PfHsp70-3, a number of functional assays will be conducted against PfHsp70-3 alone and with PfHep1 and Pfj1. The ability of PfHep1 and Pfj1 to function as co-chaperones of PfHsp70-3 will be analyzed. To achieve this, PfHsp70-3 chaperone activities in suppressing aggregation of model proteins such as MDH and citrate synthase in combination with Pfj1 and PfHep1 will be assessed. The aggregation properties of PfHsp70-3 will be analyzed and the role of PfHep1 in suppressing the aggregation will also be investigated. Furthermore, the ability of PfHsp70-3 to refold β-galactosidase will be determined. The role of Zn^{2+} found in the structure of PfHep1 will be investigated to establish the role it plays in PfHep1's functions in relation to PfHs70-3. The ability of PfHep1 and Pfj1 to stimulate the ATPase activity of PfHsp70-3 will be investigated.

iv) Cell biological characterization of PfHsp70-3 and PfHep1

Intra-erythrocytic properties of PfHsp70-3 and PfHep1 will be investigated in this study. Peptide specific antibodies will be raised against PfHsp70-3 and the cultured parasite lysates will be used to determine the expression profile, heat induciibility, localization and solubility profile of the protein in *P. falciparum*. Constructs of PfHep1 will be made where the genomic sequence for PfHep1 will be inserted into GFP-pARL2 plasmid for transfection of *P. falciparum* parasites. GFP-tagged PfHep1 will be used for localization studies.

CHAPTER TWO

Bioinformatics analysis of PfHsp70-3 and PfHep1

2.0 Introduction

The current sequencing technology has generated a colossal amount of biological data organized in various databases that can be easily accessible (Soon et al. 2013) making it possible to characterize organisms and their biological components (Hugenholtz et al. 2002). Bioinformatics makes the vast, diverse, and complex life sciences data easy to acquire, store, archive, analyze and visualize (Manichaikul et al. 2010; Zhang et al. 2010). A number of databases with biologically related information on Plasmodium species have been developed which include NCBI malaria genetics and genomics site, Tropical Disease Research (TDR), Malaria Research and Reference Reagents Resource center (MR4) and PlasmoDB (Aurrecoechea et al. 2008). These databases make it possible to analyze and determine orthologues between different species facilitating the discovery of shared biological features between lineages (Chen et al. 2006). Furthermore, the information contained in such databases can be used for predictions of subcellular localization of proteins using signal peptides and transmembrane domains (Krogh et al. 2001; Hiller et al. 2004).

An understanding of the nature and biology of *P. falciparum* has been advanced through the completion of sequencing of its entire genome (Gardner et al. 2002; Kissinger et al. 2002)as documented in the database PlasmoDB (Bahl et al. 2002). The database contains extensive genome, proteome and metabalome information related to *P. falciparum* and other malaria parasites (Aurrecoechea et al. 2009). It provides access to the entire genome sequence of the 3D7 reference strain of *P. falciparum*, together with computationally predicted and manually curated genes and gene models, protein feature predictions and functional annotations (Aurrecoechea et al. 2009). PlasmoDB database facilitates the mining of functional genomic datasets integrated with genomic sequences from different Plasmodium species such as *P. vivax, P. yoelii* and *P. knowlesi* that have been sequenced (Carlton et al. 2008; Pain et al. 2008). Information generated

from sequencing centers such as Wellcome Trust Sanger Institute and The Institute for Genomic Research (TIGR) integrated with functional genomics datasets generated by the malaria research communities (Stoeckert et al. 2006) make up the PlasmoDB database.

Broadly, information in PlasmoDB includes automated gene model predictions, predicted proteins and protein motifs, DNA sequence data and curated annotations, information on population polymorphisms, expression data generated by a variety of complementary strategies and proteomics data (Aurrecoechea et al. 2009). This information is applied in areas of research such as indentifying gene families in the genome that are responsible for pathogenesis and proteins expressed in blood stream parasites that are different from the human ones for drug discovery (Cai et al. 2012; Doolan et al. 2003). Furthermore, the information in the database can be used to analyze all genes for which clear orthologues are known from other species for evolutionary relatedness and many other specialized features (Eisen, 1998; Sharman and Gerloff, 2013). PlasmoDB integrates data and allows the user to carry out complex queries adapted to specific tasks and interest (Aurrecoechea et al. 2009).

TDR (http://tdrtargets.org) database contains specific genomic information that is aimed at identifying possible molecular targets for drug discovery (Agüero et al. 2008). It integrates information from three dimensional structures (Berman et al. 2007), metabolic pathway classifications, expression and essentiality of specific proteins (Agüero et al. 2008). It also contains resources and published studies, organism-specific functional information such as information on orthologues (Chen et al. 2007). Information curated from literature on chemical and genetic validation status of targets, precedence for druggability and assayability are also supplemented in TDR database to fast track drug discovery efforts and identification of potential vaccine targets (Magariños et al. 2012; Ludin et al. 2012). The database prioritizes tropical disease pathogens that include *M. tuberculosis*, *T. brucei* and *P. falciparum* (Magariños et al. 2012).

MR4 (https://www.mr4.org) is another database that provides a central resource for malaria reagents, protocols and technical support for the research community. MR4 acquires, authenticates, preserves and distributes parasites, mosquito vectors and associated biological and

molecular reagents. It is currently supported by the National Institute of Allergy and Infectious Diseases (NIAID) and is part of NIAID BEI (https://www.beiresources.org/MR4Home.aspx). PlasmoDB provides direct links to external data sources and sites that include MR4 and Malaria Parasite Metabolic Pathways database (http://sites.huji.ac.il/malaria/) (Limviphuvadh et al. 2003; Salinas et al. 2014).

Genes coding for putative *P. falciparum* mtHsp70 (PfHsp70-3) and Hep1 orthologue (PfHep1) proteins are among the fully sequenced and documented gene sequences in PlasmoDB. Besides being predicted to reside in the parasite mitochondria, PfHsp70-3 and PfHep1 have not been fully characterized. Mitochondrial Hsp70s have been found to aggregate due to interdomain communication between the ATPase and linker domains. The Hep1 protein is required to interact with both the ATPase and linker domains of mtHsp70s in the absence of a nucleotide to prevent aggregation and maintain their functional conformations (Sichting et al. 2005). Studies of the three yeast mtHsp70 paralogues have shown that they possess different properties and only Ssq1 and Ssc1 manifest aggregation propensities that require Hep1. The third member, Ssc3 remains partially insoluble even in the presence of Hep1 despite sharing more than 80% sequence homology with Ssc1. Further analysis revealed that the propensity to aggregate, and reliance on Hep1 for functional activities, is a feature specific to mtHsp70s rather than all proteins targeted to the mitochondrial matrix (Blamowska et al. 2012). Indeed non-mitochondrial Hsp70 homologues containing mitochondrial targeting sequences remain soluble in the mitochondrial matrix lacking Hep1 (Blamowska et al. 2012). Specific amino acid residues in the ATPase domain of Ssc1 are responsible for its aggregation properties. Mutational studies involving an exchange of amino acid residues (236–243) on the ATPase domain of Ssc1 for those from bacterial Hsp70 (212-215) increased the solubility of the chimera in the absence of Hep1 and abrogated interaction with Hep1 (Blamowska et al. 2010). This indicated that these residues could be responsible for ATPase-linker interdomain communications that lead to eventual aggregation of Ssc1 and interactions with Hep1.

It has also been further pointed out that minor amino acid residue variations in the ATPase domain of Ssc3 contributes to its insolubility in the presence of Hep1 as opposed to Ssc1 and Ssq1 (Pareek et al. 2011). Ssc3 mutants carrying corresponding residues of Ssc1 at position 157

and 159 (A157G, S159P) became soluble in the presence of Hep1.This implies that amino acid residues 157 and 159 in the ATPase domain of Ssc3 play a crucial role in its insolubility and aggregation, even in the presence of Hep1 (Blamowska et al. 2010; Pareek et al. 2011). Mitochondrial Hsp70s interact with Hep1 at the convex face of its L shaped structure and residue D111 located at the convex has been shown to play an important role in the interaction of the two proteins. Mutations of D111, R106 and H107 on the Hep1 protein abrogate its functions against mtHsp70. The three residues, D111, R106 and H107 are located in the zinc finger domain that is responsible for the overall structure and function of Hep1. Furthermore, the zinc finger domain has been found to be highly conserved across Hep1 orthologues.

An understanding of the properties of an unknown protein can be enhanced by analyzing and comparing it to well known and characterized orthologues using bioinformatics tools (Sali et al. 1995; Geourjon et al. 2001). For instance, homology modeling allows the construction of a three dimensional protein model of unknown target protein from either its amino acid sequence or from a three dimensional structure of its known homologue that is referred to as a template (Fiser, 2010). During modeling, one or more protein structures that are likely to resemble the query sequence are identified during alignments where residues from query sequence are matched with those on the template sequences for identity (Forrest et al. 2006). Proteins sharing more than 20% sequence identity from the alignment of their sequences are likely to have similar structure since protein structures are more conserved than protein sequences amongst homologues (Chothia and Lesk, 1986; Pearson. 2013). Higher percentage similarities among sequences indicate significant structural similarities (Marti –Renom et al. 2000). Bioinformatics analysis of PfHsp70-3 and PfHep1 would identify whether the two proteins possess the features of their respective characterized orthologues in yeast and other organisms. This would further indicate whether PfHsp70-3 and PfHep1 function as typical mtHsp70 and Hep1 proteins respectively and whether PfHsp70-3 would rely on PfHep1 for structural and functional properties.

2.1. Objectives

The aim of this study was to utilize bioinformatics tools to infer the properties and possible interactions of PfHsp70-3 and PfHep1.

Specific objectives were to:

1) Conduct comparative protein sequence and phylogenetic analyses of PfHsp70-3 and PfHep1 along with their characterized orthologues from other eukaryotic species.

2) Identify domains and residues that could be involved in the interaction of PfHsp70-3 with PfHep1.

3) Carry out homology modeling of PfHep1 and PfHsp70-3.

2.2. Experimental procedure

2.2.1. Sequence searches and comparison

The primary amino acid sequence of plasmodial mitochondrial Hsp70 and Hep1 orthologues were obtained from PlasmoDB v4.4 (http:// plasmodb.org/plasmo/; Aurrecoechea et al. 2009). The rest of the sequences from other species were obtained from the national Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) based on a BLAST search using PfHsp70-3 and PfHep1 sequences as queries. Sequence alignment and percentage identity was done using Clastaw Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) (*Fabian et al. 2011*) and MUSCLE (Edgar et al. 2004). Annotations on the sequences were carried out manually. Shading of conserved and identical residues was performed using the BOXSHADE server by K. Hofmann and M. Baron (http://www.ch.embnet.org/software/BOX_form.htm1).

2.2.2. Phylogenetic analysis

Phylogenetic analysis was carried out on PfHsp70-3 and PfHep1. Molecular Evolutionary Genetics Analysis 6 (mega 6) (Tamura et al. 2013) and Clastaw omega programs were used to

generate the cladogram/phylogenetic tree for analysis of PfHep1 and its orthologues. The primary amino acid sequences of proteins were used as inputs to mega 6, followed by multiple sequence alignment (MSA) using the inbuilt alignment program MUSCLE. The MSA was subsequently used to construct a phylogenetic tree based on the best evolutionary model/substitution method which is calculated based on the Bayesian information criterion (BIC) score. Phylogenetic tree construction was carried out using maximum likelihood (Tamura et al. 2011) with the following parameters: bootstrap consensus tree surmised from 1000 replicates for standard representation and any tree partitions replicated in less 75% of the bootstrap analyzes were collapsed to maintain species boundaries. Based on the calculations, the unrooted tree was generated based on the Poisson substitution model (Fletcher & Yang, 2009). Phylogenetic cladogram of neighbor joining unrooted tree without distance corrections for PfHsp70-3 and PfHep1 along with their orthologues were also generated using Clastaw omega.

2.2.3. Homology modeling

The protein domain mapping for PfHep1 was conducted using the online programs SMART 7 (Simple Modular Architecture Research Tool; http://smart.embl-heidelberg.de/; Letunic et al. 2012) and Prosite (http://prosite.expasy.org/; (Sigrist et al 2009). In order to predict the subcellular localization of PfHep1 and PfHsp70-3, a number of online programs that included NucPred (http://www.sbc.su.se/~ maccallr/nucpred/cgi-bin/single.cgi; (Brameier et al. 2009) MitoPROT (http://ihg.gsf.de/ihg/mitoprot.html; (Claros and Vincens, 1996) MultiLoc (http://abi.inf.uni-tuebingen.de/Services/MultiLoc; (Höglund et al 2006) SignalP version 4.1 (http://www.cbs.dtu.dk/services/SignalP/; (Petersen et al. 2011) and WoLF PSORT (http://www.genscript.com/wolf-psort.html.; (Horton et al. 2007) were used. Alignment was conducted using MAFFT (http://www.ebi.ac.uk/Tools/msa/mafft/; (Katoh and Toh, 2008). The zinc-binding domain structure of PfHep1 was modeled using the online Swiss Model server (Biasini et al. 2014). The solution structure of Tim15c (yHep1) solved by NMR (PBD accession number 2EZZ) was used as the template (Momose et al. 2007). To predict the model structure of PfHsp70-3 protein, the protein sequence was retrieved from PlasmoDB and was used as a query

sequence that was submitted to Homology detection & structure prediction (HHpred) server to generate 3- dimensional protein structure of PfHsp70-3 using the closest homologues among the resolved models in modeling database. The template that shared greater than 30% sequence identity with the E-values closest to zero was selected for homology modeling of PfHsp70-3. MODELLER 9v12 was used for modeling (Sali et al. 1995) and the modeling process was achieved through slow refinement. Generated models for PfHep1 and PfHsp70-3 were rendered using PyMol (DeLano, 2002).

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2.3 Results and Discussion

2.3.1 Sequence alignment and analysis of PfHsp70-3 and other mitochondrial Hsp70s

Ssc1, Ssq1 and Ssc3/Ecm10 are the Hsp70 proteins found in the mitochondria of yeast (Craig and Marszalek, 2002) and only Ssc1 and Ssq1 produce aggregation-prone conformers which are prevented by the presence of Hep1 protein (Baumann et al. 2000; Blamowska et al. 2010). Besides Ssc1 and Ssq1, other mtHsp70 that have been characterised have shown the same tendency to aggregate and a reliance on Hep1 for solubility and functional activities, these include mtHsp70 from*Homo sapiens* (Zhai et al. 2011), *Chlamydomonas reinhardtii* (Willmund et al. 2008), *Arabidopsis thaliana* (Kluth et al. 2012) and *Leishmania braziliensis* (Dores-silva et al. 2015). The purpose of carrying out a sequence alignment of mtHsp70s was to identify the conserved domain residues that are functionally important. The conservation of the domain residues would be used to infer PfHsp70-3 function, including the tendency to aggregate and the requirement of PfHep1 for solubility and functional activities. Despite the fact that the ATPase domain is generally conserved among the Hsp70 proteins families (Sarkar et al. 2013), particular residues within the domain have been shown to be responsible for specific properties unique to mtHsp70s (Blamowska et al. 2010).

	Pf	Hs	Cr	At	SSC1	SSC3	Lb	SSQ1
Pf	100.00	61.54	64.96	60.97	58.40	55.96	57.03	50.48
Hs	61.54	100.00	68.15	59.79	64.11	62.25	60.65	49.76
Cr	64.96	68.15	100.00	72.98	63.62	61.11	65.23	50.43
At	60.97	59.79	72.98	100.00	60.65	57.77	59.20	48.80
SSC1	58.40	64.11	63.62	60.65	100.00	80.75	56.50	53.00
SSC3	55.96	62.25	61.11	57.77	80.75	100.00	54.72	53.65
Lb	57.03	60.65	65.23	59.20	56.50	54.72	100.00	46.50
SSQ1	50.48	49.76	50.43	48.80	53.00	53.65	46.50	100.00

Figure 2.1 : Sequence percentage identities of PfHs70-3 (Pf) and its orthologues: Hs (*Homo sapiens*, NCBI accession number, P38646.2), Cr (*Chlamydomonas reinhardtii*, NCBI accession number: CAA65356.1), At (*Arabidopsis thaliana*, NCBI accession number: NP_196521.1), Ssc1 (*Saccharomyces cerevisiae* Ssc1, NCBI accession number: P0CS91.1), Ssc3 (*Saccharomyces cerevisiae* Ssc3, NCBI accession number: NP_010884.1), Lb (*Leishmania braziliensis*, NCBI accession number: XP_001566868.1) and Ssq1 (*Saccharomyces cerevisiae* Ssq1, NCBI accession number: NP_013473.1).

The percentage sequence identities of selected mtHsp70 proteins that have been characterised were greater than 50% in comparison to PfHsp70-3 (Figure 2.1). The higher percentage identity among mtHsp70 could be attributed to their similar bacterial evolutionary origin (Schilke et al. 2006). The mtHsp70 from *C. reinhardtii* and *A. thaliana* showed relatively high sequence identity to PfHsp70-3. The close identity of *C. reinhardtii* and *A. thaliana* to *P. falciparum* mtHsp70 could be attributed to the close evolutionary relationship between the two organisms as evidenced by the presence of the apicoplast and chloroplast in *P. falciparum* and the organisms. The two organelles along with the mitochondria are of secondary endosymbiotic origin and the presence of all these organelles in both *P. falciparum* and *C. reinhardtii* is an indication of further the two organisms evolved together. Since the parasite apicoplast does not contain any photosynthetic apparatus, it is often referred to as a relict plastid (Ralph et al. 2004).

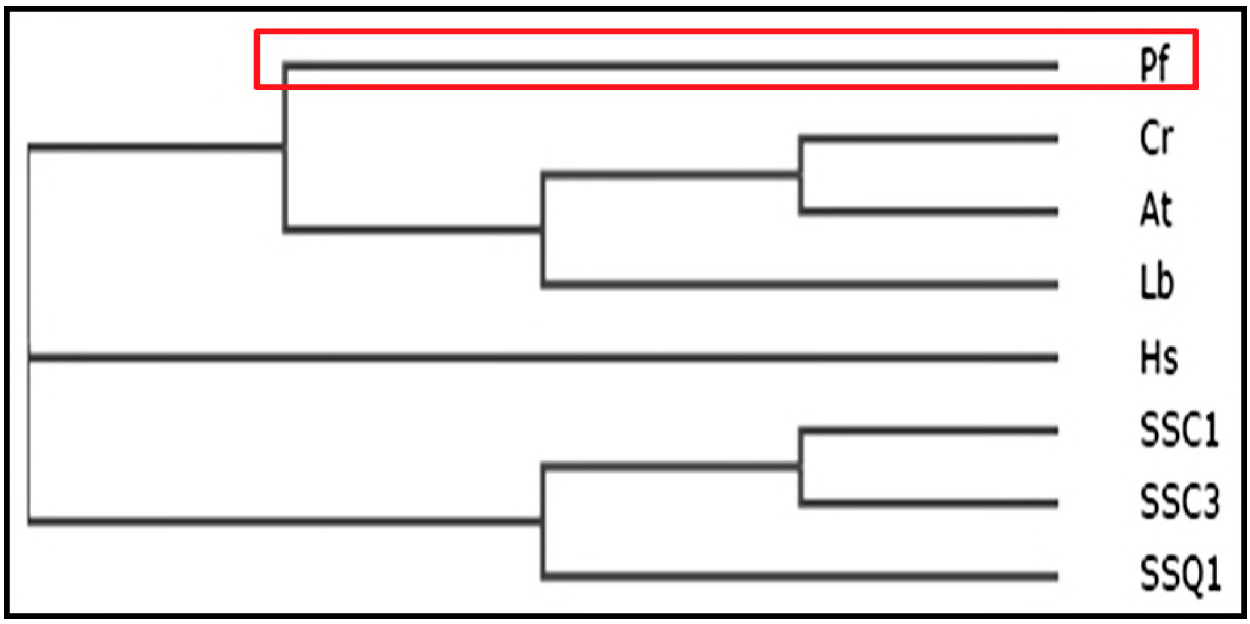


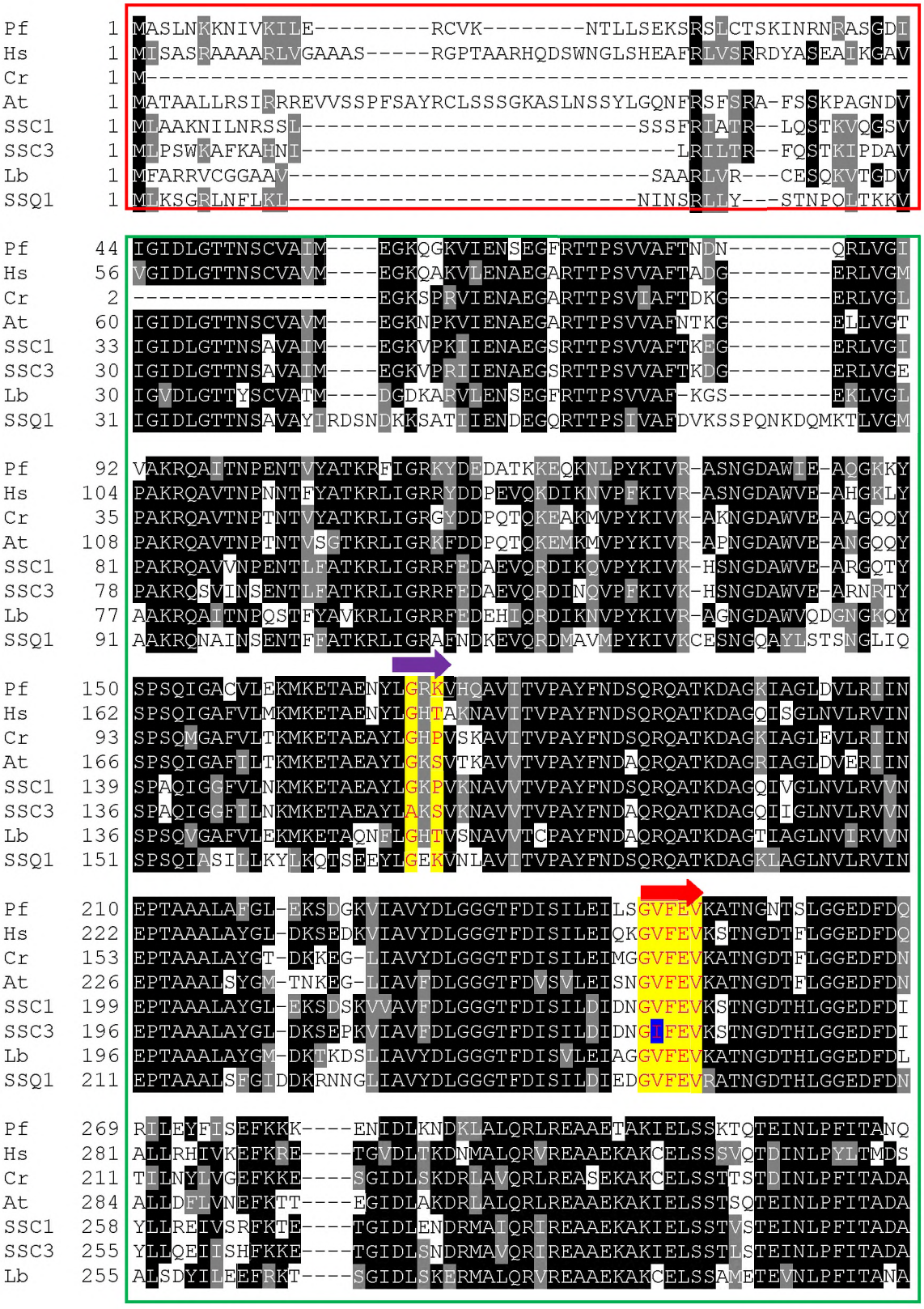
Figure 2.2 : Phylogenetic analysis of PfHs70-3 and its orthologues: The un-rooted phylogenetic tree was generated by Clastaw Omega; Hs (*Homo sapiens*, NCBI accession number, P38646.2), Cr (*Chlamydomonas reinhardtii*, NCBI accession number: CAA65356.1), At (*Arabidopsis thaliana*, NCBI accession number: NP_196521.1), Ssc1 (*Saccharomyces cerevisiae* Ssc1, NCBI accession number: P0CS91.1), Ssc3 (*Saccharomyces cerevisiae* Ssc3, NCBI accession number: NP_010884.1), Lb (*Leishmania braziliensis*, NCBI accession number: XP_001566868.1) and Ssq1 (*Saccharomyces cerevisiae* Ssq1, NCBI accession number: NP_013473.1).

Ssq1 showed the lowest percentage identity to PfHsp70-3 and the other mtHsp70s despite sharing some biochemical properties and overlapping functions with its paralogue Ssc1 (Pareek et al. 2011). Just like many mtHsp70s, Ssq1 is highly specialized for FeS biogenesis and it emerged from the expansion and rearrangement driven by gene duplication (Schilke et al. 2006; Yamada and Bork 2009). Even though Ssq1 functions on FeS biogenesis point to its bacterial origin similar to other mtHsp70s (Schilke et al. 2006; Pukszta et al. 2010), the gene re-arrangements has seen it losing some abilities typical to mtHsp70s such as interaction with other common J-protein co-chaperones present in yeast mitochondrial matrix, Mdj1 and Pam18 (D'Silva et al. 2003; Dutkiewicz et al. 2003). The changes that resulted from gene re-arrangement could attribute to evolutionary differences observed between Ssq1and other mtHsp70s.

Even though all mtHsp70s shared high percentage identity, phylogenetic analysis showed an earlier divergence of PfHsp70-3 from the rest of other Hsp70s (Figure 2.2). This could be attributed to evolutionary changes within *P. falciparum* proteins sequences that contain more hydrophobic residues as compared to other proteins from other organisms including other parasites. Indeed, mtHsp70 from *L. braziliensis* showed distant phylogenetic relationship with PfHsp70-3 (Figure 2.2). Both yeast Ssc1 and Ssc3 shared the same monophyletic clade excluding the third member Ssq1 (Figure 2.2). However, even though both Ssc1 and Ssc3 share greater sequence identity, Ssc3 has been described as an "atypical" yeast mtHsp70 since it does interact with Hep1 proteins. Therefore, unlike its paralogues Ssc1 and Ssq1, Ssc3 often remains insoluble even in the presence of Hep1. This is due to sequences divergence within the ATPase and SBD domains leading to differences in overall biochemical properties and biological functions (Pareek et al. 2011).

Typical to the Hsp70 family, mtHsp70s display a highly conserved ATPase domain compared to the SBD and the C-terminal domains (Figure 2.3). The hydrophobic linker that is mostly involved in allosteric communication between the ATPase and SBD is highly conserved across all mitochondrial Hsp70s. The ATPase domain-linker conformers are aggregation prone and responsible for the aggregation propensities observed among mitochondrial Hsp70s. Mutations of the linker residues abrogate the aggregation properties by disrupting the interdomain communication which is responsible for eventual aggregation of the mtHsp70 proteins (Blamowska et al. 2010). The highly conserved leucine rich hydrophobic linker (**DLLLLDV**) not only mediates allosteric communication between NBD and SBD domains but also participates in the recruitment of co-chaperones (Aprile et al. 2013).

Residues 233-248 of Ssc1 that correspond to 244-258 on PfHsp70-3 have been implicated in mediating ATPase-linker allosteric communication that lead to aggregation of the ATPase domain-linker conformer (Figure 2.3). Mutation of any of the residues found in the GVFEV motif within the ATPase segment abrogates aggregation of ATPase-linker and insolubility of mtHsp70 (Blamowska et al. 2012).


Pf 1 MASLNKKNIVKILE---------RCVK--------NTLLSEKSRSLCTSKINRNRASGDI
Hs 1 MISASRAAAARLVGAAAS-----RGPTAARHQDSWNGLSHEAFRLVSRRDYASEAIKGAV
Cr 1 M---
At 1 MATAALLRSIRRREVVSSPFSAYRCLSSSGKASLNSSYLGQNFRSFSRA-FSSKPAGNDV
SSC1 1 MLAAKNILNRSSL-------------------------SSSFRIATR--LQSTKVQGSV
SSC3 1 MLPSWKAFKAHNI-------------------------LRILTR--FQSTKIPDAV
Lb 1 MFARRVCGGAAV-------------------------SAARLVR---CESQKVTGDV
SSQ1 1 MIKSGRLNFLKL-------------------------NINSRLLY---STNPQLTKKV
Pf 44 IGIDLGTTNSCVAIM----EGKQGKVIENSEGFRTTPSVVAFTNDN--------QRLVGI
Hs 56 VGIDLGTTNSCVAVM----EGKQAKVLENAEGARTTPSVVAFTADG--------ERLVGM
Cr 2 ----------------EGKSPRVIENAEGARTTPSVIAFTDKG--------ERLVGL
At 60 IGIDLGTTNSCVAVM----EGKNPKVIENAEGARTTPSVVAFNTKG--------ELLVGT
SSC1 33 IGIDLGTTNSAVAIM----EGKVPKIIENAEGSRTTPSVVAFTKEG--------ERLVGI
SSC3 30 IGIDLGTTNSAVAIM----EGKVPRIIENAEGSRTTPSVVAFTKDG--------ERLVGE
Lb 30 IGVDLGTTYSCVATM----DGDKARVLENSEGFRTTPSVVAF-KGS--------EKLVGL
SSQ1 31 IGIDLGTTNSAVAYIRDSNDKKSATIIENDEGQRTTPSIVAFDVKSSPQNKDQMKTLVGM
Pf 92 VAKRQAITNPENTVYATKRFIGRKYDEDATKKEQKNLPYKIVR-ASNGDAWIE-AQGKKY
Hs 104 PAKRQAVTNPNNTFYATKRLIGRRYDDPEVQKDIKNVPFKIVR-ASNGDAWVE-AHGKLY
Cr 35 PAKRQAVTNPTNTVYATKRLIGRGYDDPQTQKEAKMVPYKIVK-AKNGDAWVE-AAGQQY
At 108 PAKRQAVTNPTNTVSGTKRLIGRKFDDPQTQKEMKMVPYKIVR-APNGDAWVE-ANGQQY
SSC1 81 PAKRQAVVNPENTLFATKRLIGRRFEDAEVQRDIKQVPYKIVK-HSNGDAWVE-ARGQTY
SSC3 78 PAKRQSVINSENTLFATKRLIGRRFEDAEVQRDINQVPFKIVK-HSNGDAWVE-ARNRTY
Lb 77 AAKRQAITNPQSTFYAVKRLIGRRFEDEHIQRDIKNVPYKIVR-AGNGDAWVQDGNGKQY
SSQ1 91 AAKRQNAINSENTFFATKRLIGRAFNDKEVQRDMAVMPYKIVKCESNGQAYLSTSNGLIQ
Pf 150 SPSQIGACVLEKMKETAENYLGRKVHQAVITVPAYFNDSQRQATKDAGKIAGLDVLRIIN
Hs 162 SPSQIGAFVLMKMKETAENYLGHTAKNAVITVPAYFNDSQRQATKDAGQISGLNVLRVIN
Cr 93 SPSQMGAFVLTKMKETAEAYLGHPVSKAVITVPAYFNDSQRQATKDAGKIAGLEVLRIIN
At 166 SPSQIGAFILTKMKETAEAYLGKSVTKAVVTVPAYFNDAQRQATKDAGRIAGLDVERIIN
SSC1 139 SPAQIGGFVLNKMKETAEAYLGKPVKNAVVTVPAYFNDSQRQATKDAGQIVGLNVLRVVN
SSC3 136 SPAQIGGFILNKMKETAEAYLAKSVKNAVVTVPAYFNDAQRQATKDAGQIIGLNVLRVVN
Lb 136 SPSQVGAFVLEKMKETAQNFLGHTVSNAVVTCPAYFNDAQRQATKDAGTIAGLNVIRVVN
SSQ1 151 SPSQIASILLKYLKQTSEEYLGEKVNLAVITVPAYFNDSQRQATKDAGKLAGLNVLRVIN
Pf 210 EPTAAALAFGL-EKSDGKVIAVYDLGGGTFDISILEILSGVFEVKATNGNTSLGGEDFDQ
Hs 222 EPTAAALAYGL-DKSEDKVIAVYDLGGGTFDISILEIQKGVFEVKSTNGDTFLGGEDFDQ
Cr 153 EPTAAALAYGT-DKKEG-LIAVYDLGGGTFDISILEIMGGVFEVKATNGDTFLGGEDFDN
At 226 EPTAAALSYGM-TNKEG-LIAVFDLGGGTFDVSVLEISNGVFEVKATNGDTFLGGEDFDN
SSC1 199 EPTAAALAYGL-EKSDSKVVAVFDLGGGTFDISILDIDNGVFEVKSTNGDTHLGGEDFDI
SSC3 196 EPTAAALAYGL-DKSEPKVIAVFDLGGGTFDISILDIDNGIFEVKSTNGDTHLGGEDFDI
Lb 196 EPTAAALAYGM-DKTKDSLIAVYDLGGGTFDISVLEIAGGVFEVKATNGDTHLGGEDFDL
SSQ1 211 EPTAAALSFGIDDKRNNGLIAVYDLGGGTFDISILDIEDGVFEVRATNGDTHLGGEDFDN
Pf 269 RILEYFISEFKKK----ENIDLKNDKLALQRLREAAETAKIELSSKTQTEINLPFITANQ
Hs 281 ALLRHIVKEFKRE----TGVDLTKDNMALQRVREAAEKAKCELSSSVQTDINLPYLTMDS
Cr 211 TILNYLVGEFKKE----SGIDLSKDRLAVQRLREASEKAKCELSSTTSTDINLPFITADA
At 284 ALLDFLVNEFKTT----EGIDLAKDRLALQRLREAAEKAKIELSSTSQTEINLPFITADA
SSC1 258 YLLREIVSRFKTE----TGIDLENDRMAIQRIREAAEKAKIELSSTVSTEINLPFITADA
SSC3 255 YLLQEIISHFKKE----TGIDLSNDRMAVQRIREAAEKAKIELSSTLSTEINLPFITADA
Lb 255 ALSDYILEEFRKT----SGIDLSKERMALQRVREAAEKAKCELSSAMETEVNLPFITANA

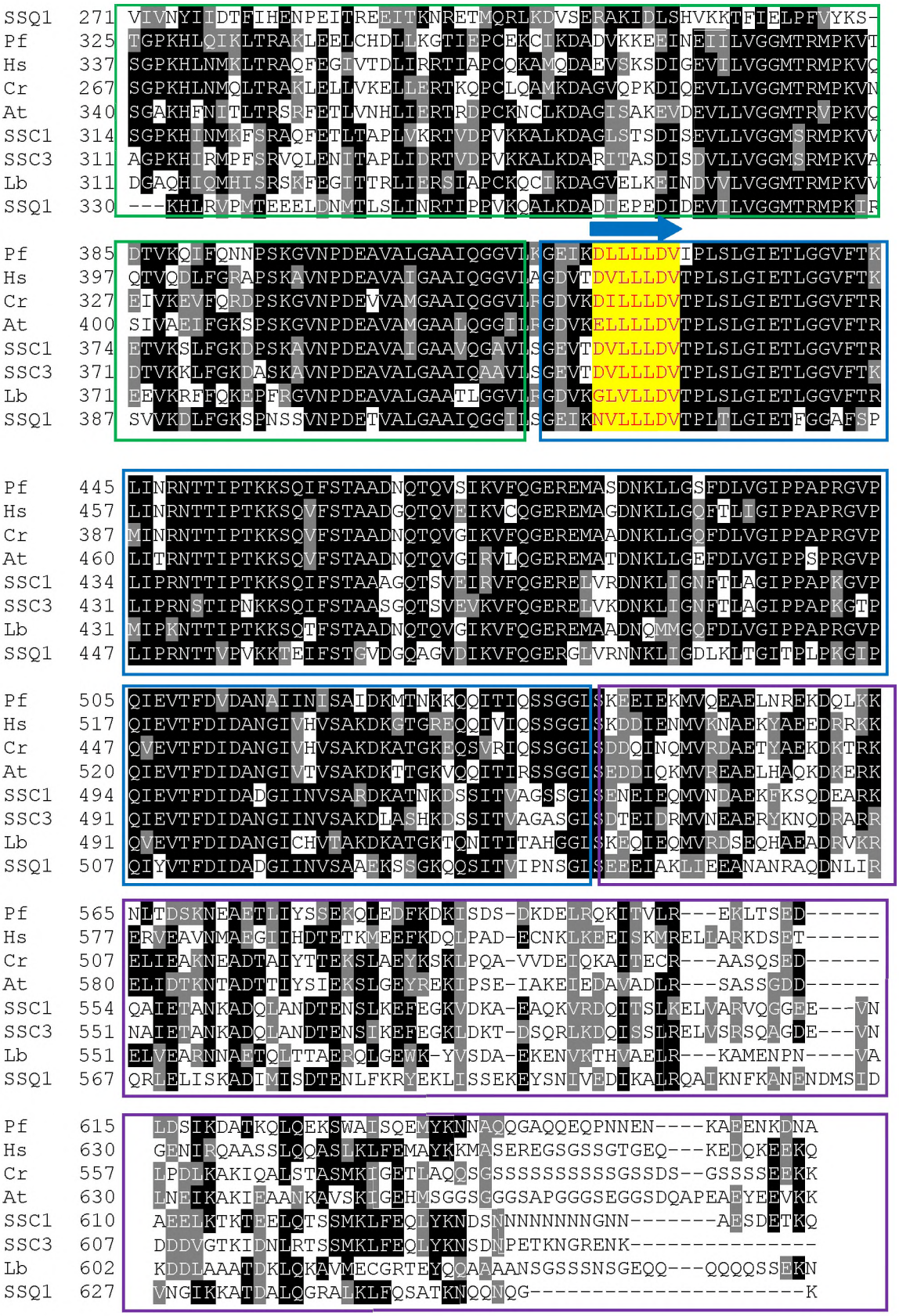

SSQ1 271 VIVNYIIDTFIHENPEITREEITKNRETMQRLKDVSERAKIDLSHVKKTFIELPFVYKS-
Pf 325 TGPKHLQIKLTRAKLEELCHDLIKGTIEPCEKCIKDADVKKEEINEIILVGGMTRMPKVT
Hs 337 SGPKHLNMKLTRAQFEGIVTDLIRRTIAPCQKAMQDAEVSKSDIGEVILVGGMTRMPKVQ
Cr 267 SGPKHLNMQLTRAKLELLVKELLERTKQPCLQAMKDAGVQPKDIQEVLLVGGMTRMPKVN
At 340 SGAKHFNITLTRSRFETLVNHLIERTRDPCKNCLKDAGISAKEVDEVLLVGGMTRVPKVQ
SSC1 314 SGPKHINMKFSRAQFETLTAPLVKRTVDPVKKALKDAGLSTSDISEVLLVGGMSRMPKVV
SSC3 311 AGPKHIRMPFSRVQLENITAPLIDRTVDPVKKALKDARITASDISDVLLVGGMSRMPKVA
Lb 311 DGAQHIQMHISRSKFEGITTRLIERSIAPCKQCIKDAGVELKEINDVVLVGGMTRMPKVV
SSQ1 330 ---KHLRVPMTEEELDNMTLSLINRTIPPVKQALKDADIEPEDIDEVILVGGMTRMPKIR
Pf 385 DTVKQIFQNNPSKGVNPDEAVALGAAIQGGVLKGEIKDLLLLDVIPLSLGIETLGGVFTK
Hs 397 QTVQDLFGRAPSKAVNPDEAVAIGAAIQGGVLAGDVTDVLLLDVTPLSLGIETLGGVFTK
Cr 327 EIVKEVFQRDPSKGVNPDEVVAMGAAIQGGVLRGDVKDILLLDVTPLSLGIETLGGVFTR
At 400 SIVAEIFGKSPSKGVNPDEAVAMGAALQGGILRGDVKELLLLDVTPLSLGIETLGGVFTR
SSC1 374 ETVKSLFGKDPSKAVNPDEAVAIGAAVQGAVLSGEVTDVLLLDVTPLSLGIETLGGVFTR
SSC3 371 DTVKKLFGKDASKAVNPDEAVALGAAIQAAVLSGEVTDVLLLDVTPLSLGIETLGGVFTK
Lb 371 EEVKRFFQKEPFRGVNPDEAVALGAATLGGVLRGDVKGLVLLDVTPLSLGIETLGGVFTR
SSQ1 387 SVVKDLFGKSPNSSVNPDETVALGAAIQGGILSGEIKNVLLLDVTPLTLGIETFGGAFSP
Pf 445 LINRNTTIPTKKSQIFSTAADNQTQVSIKVFQGEREMASDNKLLGSFDLVGIPPAPRGVP
Hs 457 LINRNTTIPTKKSQVFSTAADGQTQVEIKVCQGEREMAGDNKLLGQFTLIGIPPAPRGVP
Cr 387 MINRNTTIPTKKSQVFSTAADNQTQVGIKVFQGEREMAADNKLLGQFDLVGIPPAPRGVP
At 460 LITRNTTIPTKKSQVFSTAADNQTQVGIRVLQGEREMATDNKLLGEFDLVGIPPSPRGVP
SSC1 434 LIPRNTTIPTKKSQIFSTAAAGQTSVEIRVFQGERELVRDNKLIGNFTLAGIPPAPKGVP
SSC3 431 LIPRNSTIPNKKSQIFSTAASGQTSVEVKVFQGERELVKDNKLIGNFTLAGIPPAPKGTP
Lb 431 MIPKNTTIPTKKSQTFSTAADNQTQVGIKVFQGEREMAADNQMMGQFDLVGIPPAPRGVP
SSQ1 447 LIPRNTTVPVKKTEIFSTGVDGQAGVDIKVFQGERGLVRNNKLIGDLKLTGITPLPKGIP
Pf 505 QIEVTFDVDANAIINISAIDKMTNKKQQITIQSSGGLSKEEIEKMVQEAELNREKDQLKK
Hs 517 QIEVTFDIDANGIVHVSAKDKGTGREQQIVIQSSGGLSKDDIENMVKNAEKYAEEDRRKK
Cr 447 QVEVTFDIDANGIVHVSAKDKATGKEQSVRIQSSGGLSDDQINQMVRDAETYAEKDKTRK
At 520 QIEVTFDIDANGIVTVSAKDKTTGKVQQITIRSSGGLSEDDIQKMVREAELHAQKDKERK
SSC1 494 QIEVTFDIDADGIINVSARDKATNKDSSITVAGSSGLSENEIEQMVNDAEKFKSQDEARK
SSC3 491 QIEVTFDIDANGIINVSAKDLASHKDSSITVAGASGLSDTEIDRMVNEAERYKNQDRARR
Lb 491 QVEVTFDIDANGICHVTAKDKATGKTQNITITAHGGLSKEQIEQMVRDSEQHAEADRVKR
SSQ1 507 QIYVTFDIDADGIINVSAAEKSSGKQQSITVIPNSGLSEEEIAKLIEEANANRAQDNLIR
Pf 565 NLTDSKNEAETLIYSSEKQLEDFKDKISDS-DKDELRQKITVLR---EKLTSED------
Hs 577 ERVEAVNMAEGIIHDTETKMEEFKDQLPAD-ECNKLKEEISKMRELLARKDSET------
Cr 507 ELIEAKNEADTAIYTTEKSLAEYKSKLPQA-VVDEIQKAITECR---AASQSED------
At 580 ELIDTKNTADTTIYSIEKSLGEYREKIPSE-IAKEIEDAVADLR---SASSGDD------
SSC1 554 QAIETANKADQLANDTENSLKEFEGKVDKA-EAQKVRDQITSLKELVARVQGGEE---VN
SSC3 551 NAIETANKADQLANDTENSIKEFEGKLDKT-DSQRLKDQISSLRELVSRSQAGDE---VN
Lb 551 ELVEARNNAETQLTTAERQLGEWK-YVSDA-EKENVKTHVAELR---KAMENPN----VA
SSQ1 567 QRLELISKADIMISDTENLFKRYEKLISSEKEYSNIVEDIKALRQAIKNFKANENDMSID
Pf 615 LDSIKDATKQLQEKSWAISQEMYKNNAQQGAQQEQPNNEN----KAEENKDNA
Hs 630 GENIRQAASSLQQASLKLFEMAYKKMASEREGSGSSGTGEQ---KEDQKEEKQ
Cr 557 LPDLKAKIQALSTASMKIGETLAQQSGSSSSSSSSSSGSSDS--GSSSSEEKK
At 630 LNEIKAKIEAANKAVSKIGEHMSGGSGGGSAPGGGSEGGSDQAPEAEYEEVKK
SSC1 610 AEELKTKTEELQTSSMKLFEQLYKNDSNNNNNNNNGNN-------AESDETKQ
SSC3 607 DDDVGTKIDNLRTSSMKLFEQLYKNSDNPETKNGRENK----------
Lb 602 KDDLAAATDKLQKAVMECGRTEYQQAAAANSGSSSNSGEQQ---QQQQSSEKN
SSQ1 627 VNGIKKATDALQGRALKLFQSATKNQQNQG----------K

Figure 2.3 : Sequence alignment of PfHsp70-3 (*P.falciparum*, PF3D7_1134000) and other mtHsp70 orthologues: Ssc1, Ssq1, Ssc3 (*Saccharomyces cerevisiae* paralogues, NCBI accession number: P0CS91.1, NP_013473.1 and NP_010884.1 respectively), Cr (*Chlamydomonas reinhardtii*, NCBI accession number: CAA65356.1), Lb (*Leishmania braziliensis*, NCBI accession number: XP_001566868.1), At (*Arabidopsis thaliana*, NCBI accession number: NP_196521.1). Highlighted segments: Purple arrow, residues whose variations on Ssc3 make it "atypical" mtHsp70 that remain relatively insoluble in the presence of Hep1, Red arrow; residues that promote interdomain communication and facilitate ATPase insolubility in the absence of Hep1, Blue arrow; linker region required by ATPase domain for interaction with Hep1. The red, blue and purple box enclosed regions represent ATPase domain, substrate binding domain and the C-terminal sub-domain respectively. The black shade represented 100% residue identity with dark gray representing some identity.

These residues are conserved in PfHsp70-3 and other mtHsp70 orthologues except in Ssc3 with residues GIFE, the valine residue is substituted for isoleucine (Figure 2.3). Mutation of residues A157 and S159 of Ssc3 ATPase domain to G160 and P162 from Ssc1 increases the solubility of Ssc3 and its interaction with Hep1 (Pareek et al. 2011). PfHsp70-3 contains the residues G172 and K174, which are identical to Ssq1 (Figure 2.3)

Since there is no empirical crystallographic or NMR structures available for PfHsp70-3, homology modelling was carried out using *E.coli*Hsp70 (2KHO) as a template to generate the3-dimensional protein structure of PfHsp70-3. The generated model had greater than 30% identity with PfHsp70-3 with the E-values of -0.772 (Figure 2.4 A); which represents the random background noise where the lower value represents the significance of the score and the alignments. As indicated on the PfHsp70-3 generated model, residues G171 and K173 (Figure 2.4B) have been shown to play a role in the aggregation of mtHsp70 and in the interaction with Hep1. Yeast Hsp70 paralogue Ssc3 which hardly interacts with Hep1 and remains insoluble, increase its interaction with Hep1 and become soluble when these residues are exchanged with those from Ssc1. Furthermore, the association between the ATPase domain and the linker region lead to the aggregation of mtHsp70 and residues GVFEV (Figure 2.4 C) have been implicated for the communication between the linker and the ATPase domains that lead to aggregation. The conservation of these residues in PfHsp70-3 could most likely make it similar to its orthologues especially in terms of aggregation properties in the absence of an escort protein. This could point further to the possibility of PfHsp70-3 being a typical mitochondrial protein and therefore most

likely relying on PfHep1 to remain soluble and functional within the mitochondrial matrix of *P. falciparum.*

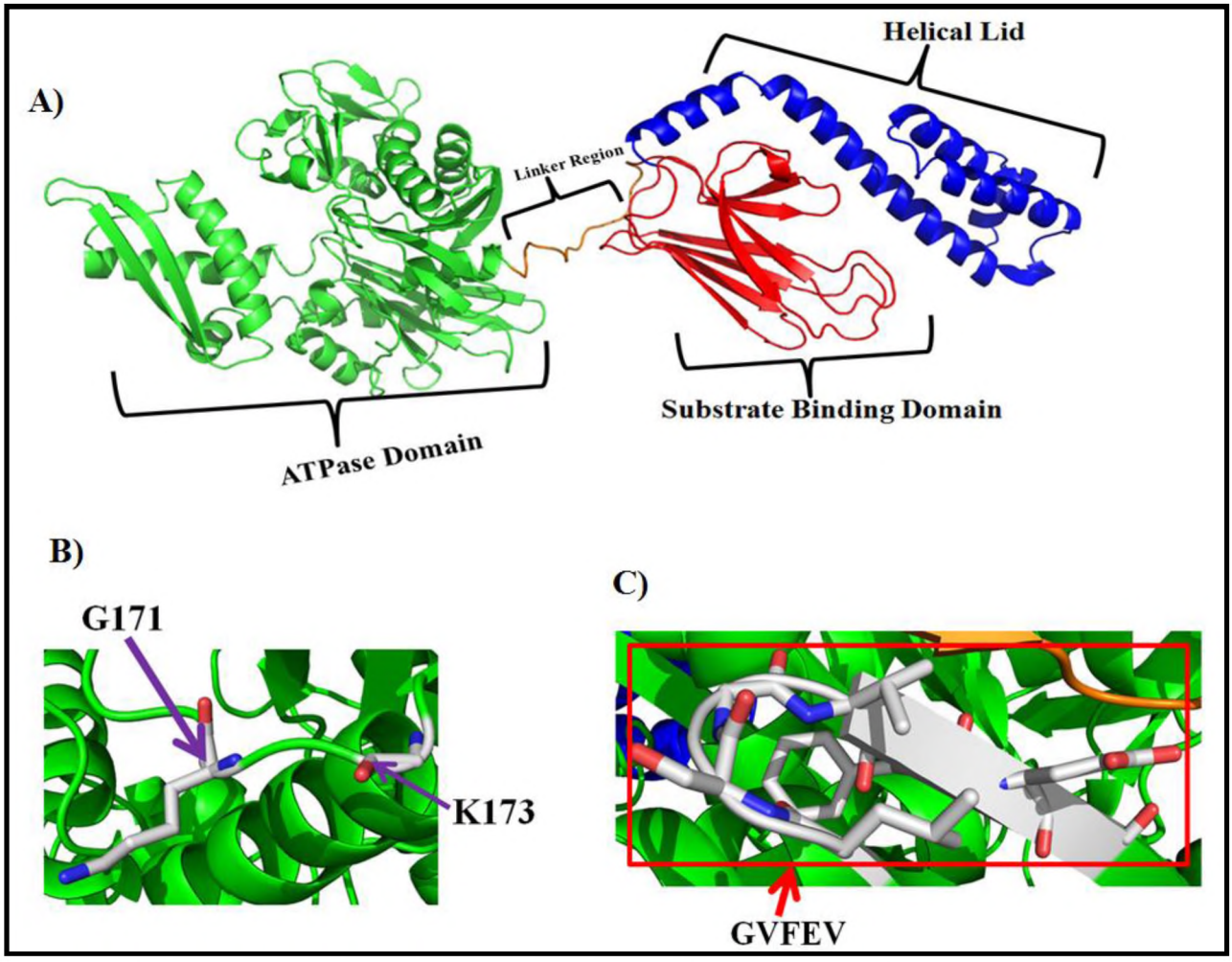


Figure 2.4 : Structural prediction of PfHsp70-3: The model was regenerated by modeller 9 version 12 (Shen and Sali, 2006) using *E.coli*Hsp70 homolog. (A) The whole protein model indicating various domains and regions. (B &C) Highlights of residues proposed to be responsible for mtHsp70 aggregation properties and interaction with Hep1 protein.

2.3.2 PfHep1 comparative sequence analysis and modeling

Based on the observation that mitochondrial members of the plant zinc ribbon (ZR) protein family show sequence similarities to Hep1 from yeast and humans, ZR and Hep proteins have been classified as part of a family consisting of five subgroups: ZR1, ZR2, ZR3, Hep1 and Hep2 (Kluth et al. 2012). The classification is based on sequence identity and sub-cellular localization

of individual proteins within the cell (Kluth et al. 2012). The ZR subfamily refers to plant zinc finger proteins, ZR1 and ZR2 being plastidic and ZR3 residing within the mitochondria. The Hep subfamily refers to non-plant zinc finger proteins with Hep1 being mitochondrial and Hep2 being plastidic (Kluth et al. 2012). In this study a putative mitochondrial targeting sequence and the zinc finger domain (zf-DNL) of the hypothetical zinc finger protein from *P. falciparum* (PF3D7_1420300) were identified and based on its sequence identity and predicted subcellular localization, it was denoted as PfHep1.

Hep and ZR proteins have been observed to play roles in suppressing the self-aggregation of respective mtHsp70 orthologues in humans (Zhai et al. 2008), yeast (Sichting et al. 2005, Momose et al. 2007), Leishmania (Dores-silva et al. 2015), green algae (Willmund et al. 2008) and in *Arabidopsis thaliana* (Kluth et al. 2012). The primary structure of full-length PfHep1 was aligned with its orthologues and the putative mitochondrial or chloroplast signal peptides are highlighted for each sequence (Figure 2.6). The PfHep1 sequence is asparagine-rich, with a continuous stretch of asperagine from residues 128–166 (Figure 2.6). Approximately a quarter of all amino residues found in PfHep1 are asparagines. The asparagines repeats are characteristic of the *P. falciparum* proteome and are often absent from heat shock proteins (Singh et al. 2004). PfHep1 was also found to be larger than its characterized orthologues with 302 amino acid residues.

The highest sequence identity was 38% between PfHep1 and CrHep2 and the two proteins showed relatively close phylogenetic relatedness (Figure 2.5 A&B). This is not surprising as *P. falciparum* possess the apicoplast which is non-photosynthetic plastid that is of aligal origin (Köhler et al. 1998). PfHep1 also shared a closer phylogenetic relationship with Hep1 orthologues from *A. thaliana* that contains photosynthetic plastid; chloroplast (Figure 2.5B). Despite relative sequence identity between PfHep1 and HsHep1 (29%), the two proteins showed distant phylogenetic relationships as compared to PfHep1 and AtZR3 which was at 26% (Figure 2.5B) probably due to functional relatedness between the latter. LbHep1 also showed distant evolutionary relationship with PfHep1 despite both proteins belonging to parasitic organisms (Figure 2.5 B), an indication that the two proteins could possess some functional differences. Generally, there was averagely low sequence identity (26%) between PfHep1 and all other Hep1

protein orthologues considered here (Figure 2.5A). This could be an indication of shared evolutionary functions among Hep1 orthologues regardless of low sequence similarity.

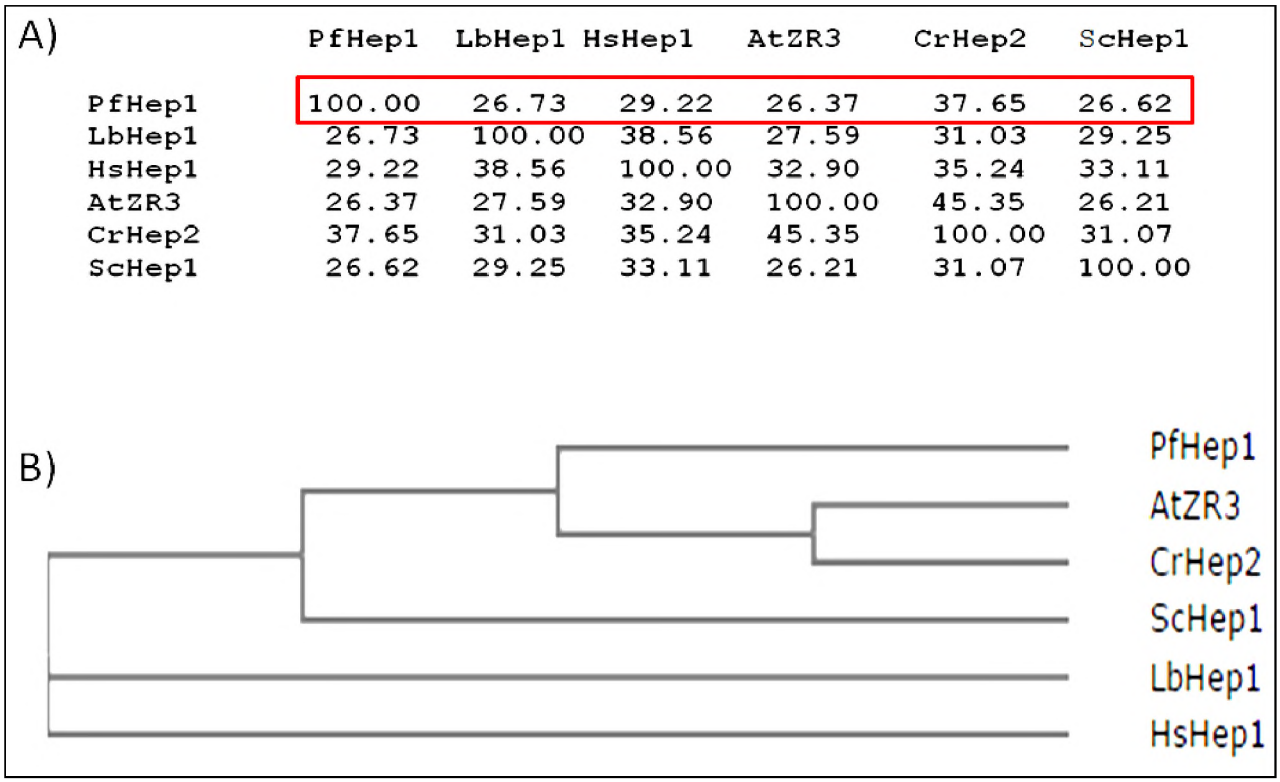
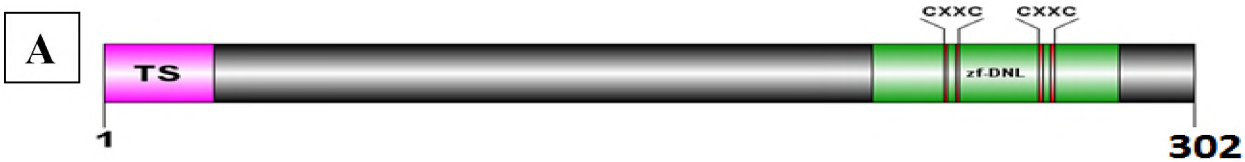


Figure 2.5 : Phylogenetic analysis and percentage sequence identities of PfHep1 and its orthologues: *Leishmania braziliensis* (LbHep1;XP_001565573.1), *Homo sapiens* (HsHep1; NM_001080849), *Arabidopsis thaliana* (AtZR3; AAO64784.1), *Chlamydomonas reinhardtii* (CrHep2; XP_001700157.1) and *Saccharomyces cerevisiae* (yHep1; NP_014089.2). (A) Shows percentage identity of PfHsep1 in reference to its homologues. (B) A cladogram for PfHep1 homologues generated using Clastaw omega.

Whilst the region upstream of the zf-DNL is longer in PfHep1 than its orthologues, the C-terminal region is slightly longer in both the hsHep1 and ScHep1 sequences. There is lack of sequence conservation outside of the zf-DNL domain (Figure 2.6). No functional domains, with the exception of the zf-DNL, have been identified in the primary sequence of Hep1.



B

C/MSP

```
PfHep1     1 MFQKIGRKLIERRTFSRIILTKDINFKKEKKNFSSLFQRYNIN-------NLSSKNDNHF
LbHep1     1 M--------------------PRGSHV--GTRIFSALDQR------------LQPALRTRT
HsHep1     1 ---------------------MLRTALRGAPRLLSRVQPR-------------APCLRR--
AtZR3      1 M-------------------AARLLALRRALSLFSNQQHRFPLSQVSTEQLSLSNSLFSRS
CrHep2     1 ------------------------------------------------------------
ScHep1     1 M------------------IPRTRTLLQSKIPITRYFAR-----------CWAPRVRY--

PfHep1    54 CIVPFNSTNNFIISKKSFVTKNERVQNEKIIQDDKFEKSNDKVIYDNKSKHLSDKIICDD
LbHep1    28 CCASFSVSSLVAASRR-WCSTGSLSSNA------------------SNPRHSQAKTPSTD
HsHep1    26 -LWGRGARPEVAGRRRAWAWGWRRSSSE------------------QGP----------G
AtZR3     43 HVYGRLFQRQLSVIREANEASVTNVCNS------------------SNSATESAKVP--S
CrHep2     1 ------------------------------------------------------------
ScHep1    30 ----------------------NVCRT-------------------------------L

PfHep1   114 KIIELNENIITNTINNNNNNNNNNNNNNNNSNNNNNNSNNNNNNSNNNNNNNEEIINTS
LbHep1    69 PSAVASTSLAAHH-----------------------------DATASATSVEEQLKMLS
HsHep1    57 PAAALGRVEAAH------------------------------------------------
AtZR3     83 PATPSEEMMVKYK------------------------------------------------
CrHep2     1 ------------------------------------------------------------
ScHep1    36 PAAALHTNIIAHN-------------------------------------------EVKK--

PfHep1   174 SNILDIENNKIIQINNGEESEILDKNLNEDLVDKEIDQKKKEYLVLMFTCNICEKKSAKK
LbHep1    99 PE----DQEHIIAALNAPENE------KSSVMGGTGIGPANGDMVAAFTCGPCDYRMVKR
HsHep1    69 ------------------------------------------YQLVYTCKVCGTRSSKR
AtZR3     96 ------SQLKI-------------------------NPRHDFMMVFTCKVCDTRSMKM
CrHep2     1 ------------------------------------MMVFTCTKCDTRSTKA
ScHep1    53 ------DDKKV-----------------HLGSFKVDKPK--MMIAFTCKKCNTRSSHT
```

Zf-DNL

```
PfHep1   234 FSKQAYYNGVVIVRCPSCENLHLISDQLGWFQD----GKTNIEKILEEKGEKVVKKF-SY
LbHep1   149 FSKHAYTKGIVIVECPNCRSKHLLADNLGWMED----TATNIEDILKAKGESFVRIGETE
HsHep1    86 ISKLAYHQGVVIVTCPGCQNHHIIADNLGWFSDL--NGKRNIEEILTARGEQVHRVA-GE
AtZR3    123 ASRESYENGVVVVRCGGCDNLHLIADRRGWFGE------PGSVEDFLASQGEEFKKGS--M
CrHep2    17 FSKQSYQNGVVLVRCPGCQKLHLVADHLGWFGEEPFVLHEHVAQ-LAAVAAAVSSDG---
ScHep1    86 MSKQAYEKGTVLISCPHCKVRHLIADHLKIFHD----HHVTVEQLMKANGEQVSQDV---

PfHep1   289 NNLL------EVDDLLNAYK----------------------
LbHep1   205 GDYQV-----VADPAVGASS--------------P--------
HsHep1   143 GALE------LVLEAAGAPT STAAPEAGEDEGPPSPGKTEPS
AtZR3    176 DSLNL-----TPEDLAGGKI STE-------------------
CrHep2    73 GLFELSDES-QVRAALAEAR ELKGQQRQRQQ--PRDESKGKQ
ScHep1   139 GDLEFEDIPDSLKDVLGKYA KNNSENASQL---PHPSQK---
```

Figure 2.6 : Sequence analysis of PfHep1: (A) Schematic representation of the domain organization of PfHep1, highlighting the mitochondrial targeting sequence (TS), and zinc finger domain (zf-DNL) within a tetracysteine motifs (CXXC). (B) Alignment of full-length PfHep1 with selected Hep orthologues from *Leishmania braziliensis* (LbHep1; XP_001565573.1), *Homo sapiens* (HsHep1; NM_001080849), *Arabidopsis thaliana* (AtZR3; AAO64784.1), *Chlamydomonas reinhardtii* (CrHep2; XP_001700157.1) and *Saccharomyces cerevisiae* (yHep1; NP_014089.2). The mitochondrial/chloroplast signaling peptide (M/CSP) for proteins is shown in dark grey, and the zinc binding domain (zf-DNL) is shown in black. Degree of amino acid residue conservation is indicated on black and grey backgrounds, with black indicating absolute conservation and grey indicating some conservation. The conserved cysteine residues and those implicated in facilitating interaction with respective mtHsp70 chaperone partner with the zf-DNL domain are highlighted with red and green boxes respectively. Residues unique to PfHep1 are heighted on yellow background along with the long stretch of asperagine residues (indicated with purple arrow) that is typical to *P. falciparum* proteome. The blue box enclosure shows the extended residues on the c-terminal end of other orthologues absent from PfHep1 sequence.

However, the generation of truncation mutants in LbHep1 had indicated that the region upstream of the zf-DNL contributes to enhancing the solubility of LbmtHsp70 (Dores-silva et al. 2015). Despite the low overall sequence identities between Hep proteins, the zinc-finger motifs (CXXC) that are part of the zf-DNL were found to be conserved in all of the sequences shown here including PfHep1 and are separated by 21 amino acids (Figure 2.6).

In some other organisms, the motif is conserved as zf-DHL, in plants mostly as zf-DRL and in , *Plasmodium* species as zf-DQL, with aspartic acid (D) and leucine (L) residues absolutely conserved across all species with differences in the middle residues that remains hydrophilic (Appendix P). All Hep1 like proteins considered in this study shared more than 80% similarity within the zf-DNL (Appendix P). The functional properties of Hep1 proteins have been shown to be limited within the zf-DNL domain that provides both structural integrity of the entire protein and the site for interaction with mtHsp70s.

Yeast mutants harbouring either a C75S or C100S mutation in the tetracysteine motifs of Hep1 were found to be incapable of rescuing growth defects in cells lacking Hep1 (Yamamoto et al. 2005). It was also observed that mutations of the key residues R81, H107, and D111 in human Hep1 decreased the binding affinities for HSPA9 (Zhai et al. 2011). Furthermore H107 played a critical role in stimulating the ATPase activity of HSPA9 (Zhai et al. 2011). Interestingly two of

these residues are conserved in PfHep1 (H255 and D259), while R81 is replaced with K229 (Figure 2.6). Yeast Hep1/Zim17 residues R106, H107 and D111 have been shown to play a critical role in interactions with mtHsp70 (Momose et al. 2007). These residues are highly conserved among all other Hep1 othologues across various higher organisms analyzed (Appendix P).

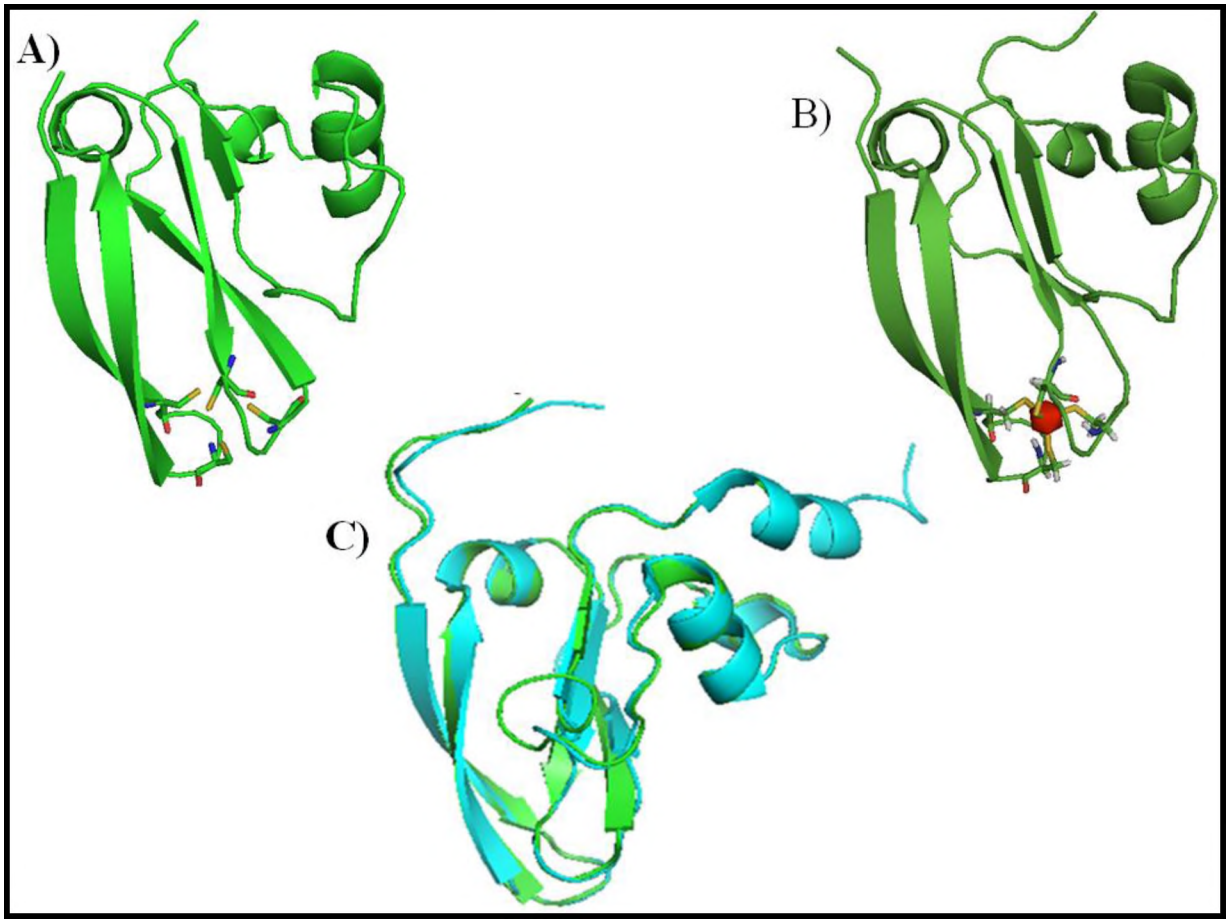


Figure 2.7 : PfHep1 and yeast Hep1 comparative protein modeling: (A) Structure of the zf-DNL of PfHep1 modeled using the yeast Hep1 (Zim17/Tim15) structure (PBD accession no. 2E2Z) as the template and generated using the online Swiss Model program (B) Structure of the zf-DNL of yeast Hep1. The zinc ion is shown in red. (C) Yeast Hep1 and PfHep1 models overlaid in aqua blue and green colors respectively.

Nuclear magnetic resonance (NMR) structural elucidations have shown that Zim17/yeast Hep1 has an L-shape with the two zing-finger motifs located at the end of the L sandwiched by two anti-parallel beta-sheets (Momose et al. 2007). In this study, the zf-DNL of yeast Hep1 was used

as a template to generate a three dimensional structure of PfHep1 (Figure 2.7 A & B). When the PfHep1generated model and its template were overlaid they revealed an overall structural resemblance with the cysteines residues found at the ends of two anti-parallel beta-sheets (Figure 2.7C).

2.4. Conclusion

PfHsp70-3 and PfHep1 are likely to be typical mtHsp70 and Hep1 homologues in *P. falciparum* respectively. Similar to other characterized mtHsp70 homologues from *S. cerevisiae, C. reinhardtii, L. braziliensis, H. sapiens* and *A. thaliana*, PfHsp70-3 is likely to be prone to aggregation and require interaction with PfHep1 for structural and functional activities. The ATPase domain of mtHsp70s is highly conserved and some residues that have been implicated in mediating the ATPase-linker communication leading to aggregation of the conformer are also conserved in PfHsp70-3. Furthermore, the conservation of the key residues that have been shown to play a crucial role in the structure and functions of Hep1 proteins are equally conserved in PfHep1. This could point to the likelihood of PfHep1 being a typical Hep1 protein relying on the same residues for its functional interactions with PfHsp70-3. Besides the conservation of residues implicated for functional activities of Hep1 proteins, PfHep1 showed overall topological features similar to those of yeast L-shaped Hep1.Like other Hep1 proteins, PfHep1 displays little sequence similarity outside the zinc finger domain. However, the zinc finger domain and specific residues implicated for functional and structural properties of Hep1 proteins are well conserved among the homologues across a number of species. This indicates the conservation of functions among Hep1 proteins despite minimal sequence similarities. PfHep1 and LbHep1 lacked the extended portion of the sequence observed at the C-terminal of other Hep1 homologues and due to its larger size, PfHep1 contained unique regions that were absent from other homologues.

CHAPTER THREE

Biochemical Characterization of PfHsp70-3, PfHep1 and Pfj1-J

3.0 Introduction

Despite the availability of the completed *P. falciparum* genome sequence (Gardner et al. 2002), the majority of proteins in the genome have not been expressed and purified using conventional cell-based expression systems (Mudeppa and Rathod, 2013). Traditional *E.coli* cell-based expression systems that over-express recombinant proteins from humans and other parasitic sources have only managed to express a few proteins of *P. falciparum* origin (Mehlin et al. 2006). There are a number of factors that contribute to the challenges of expressing *P. falciparum* proteins which include codon bias, the presence of internal start and stop sites, nonspecific nucleic acid binding regions and the presence of high AT content of the Plasmodium genome (Mudeppa and Rathod, 2013; Caro et al. 2014). With the challenges of expressing recombinant Plasmodium proteins notwithstanding, there is an urgent need for producing these proteins in functional forms to be used in screen of new drugs to fast track discovery of new therapeutics and identification of novel drug targets. To circumvent the setbacks inherent with use of conventional cell-based expression systems, codon optimization and harmonization and alteration of host expression systems have been used with occasional success (Mehlin et al. 2006).

Successful production of recombinant protein requires the target protein to be over-expressed in a soluble form and purified using an appropriate combination of gene, vector and expression host to optimize the quality and amount of the protein (Bernaudat et al. 2011). The choice of the host to be used depends on the protein of interest (Demain and Vaishnav, 2009), with *E.coli* being the most widely used host for recombinant protein production (Sahdev et al. 2008). It is characterized by fast growth, well known genetics, easy genetic manipulation and culturing on inexpensive media components (Baneyx, 1999; Shiloach and Fass, 2005). However, the inability *E. coli* cell system to perform post translational modifications may affect the production of

properly folded and biologically active target recombinant proteins of eukaryotic origin (Francis and Dana, 2010). Furthermore, some eukaryotic membrane-bound proteins are difficult to express in *E. coli* and form insoluble aggregates referred to as inclusion bodies (Wingfield et al. 2014), often due to improper disulfide bond formation (Kadokura et al. 2003). Moreover, sequences of proteins that contain highly hydrophobic amino acids are also likely to form insoluble aggregates (Singh and Panda, 2005). Indeed, insolubility has remained the major bottleneck in recombinant protein production using *E. coli* host cells as the expression system (Jia and Jeon, 2016; Gopal and Kumar 2013).

Several *E. coli* strains have been identified and modified to improve the levels of expression and eventual production of recombinant proteins (Miroux and Walker, 1996). There are strains that are protease deficient that can used to minimize degradation of target protein (Lefebvre et al. 2009; Zerb et al. 2014). Whenever mRNA of target genes is over-expressed in *E. coli*, there is a likelihood of hindered translation due to lack of tRNAs that may be rare in the expression host which result in differences in codon usage (Bass and Yansura, 2000). A replacement of rare codons with those that are optimized for *E. coli* expression machinery can improve the expression levels of recombinant proteins in *E. coli* cells (Makino et al. 2011). This can also be achieved through codon harmonization where the codons with usage frequencies in the expression host that most closely match the usage frequencies in the native target host are selected (Angov et al. 2008). There are commercially available plasmids that encode rare tRNAs that prevent early termination (Miroux and Walker, 1996; Zerb et al. 2014). However, due to diversities in protein properties, it becomes almost impossible to predict whether the intended protein of interest will be expressed in a soluble form, purified and recovered in an active form within any given experimental setup (Hartley, 2006; Bernaudat et al. 2011).

Insoluble aggregates have been traditionally solubilized using high concentrations of chaotropic reagents or denaturants. The denatured peptides are then restored into native conformations through refolding processes (Fink, 1998; Burgess, 2009). However, the use of chaotropes such as urea and guanidine hydrochloride (GdnHCl) can denature target proteins and destroy existing secondary structures leading to further aggregation and irreversible inactivation of the protein during refolding process (Dill and Shortle, 1991; Panda, 2003; Singh et al. 2012).

Other approaches that are employed to produce biologically active soluble proteins include growth at low temperatures and reduced IPTG concentrations that promote proper folding and solubility (Studier, 2005; Vera et al. 2007). Low temperatures lead to slow rates of protein production resulting in reduced cellular protein concentrations at a given time which favors proper folding and production of bioactive protein (Vera et al. 2007). Furthermore, increased use of IPTG inducer increases cell mass and target protein yields (Grabski et al. 2005). Expression of proteins can be regulated at different levels with the adjustments of IPTG concentrations and lower level expression increases the solubility and activity of some target proteins (Dormiani et al. 2007).

There is evidence to indicate that proteins contained in insoluble aggregates do have native secondary structures (Ami, et. al. 2006; Ventura and Villaverde, 2006) and any mild solubilization conditions would preserve these structures during refolding and facilitate recovery of bioactive forms of the proteins (Singh and Panda, 2005; Khan et al. 1998). Alcohols and detergents such as N-lauroylsarcosine in combination with low concentrations of urea are often used for mild solubilization of protein aggregates (Upadhyay et al. 2014). Organic solvents, such as alcohols, interact with proteins and affect their basic structures (Kudou et al. 2011), however, many others have a stabilizing effect on protein secondary structures and are known to induce helicity even in non-structured peptides (Singh et al. 2012). Indeed, solubilization of insoluble aggregates using organic solvents provides a practical alternative to conventional urea/guanidine hydrochloride based solubilization that often lead to irreversible denaturation and inactive solubilised target proteins (Singh et al. 2015).

Co-expression of recombinant proteins with molecular chaperones, co-factors and folding modulators or foldases promote proper disulfide bond formation and increase yield of soluble and active proteins (de Marco et al. 2005; de Marco, 2007; Kolaj et al. 2009). Co-expression can be achieved through the use of two or more plasmids each carrying one gene and a different selection marker with compatible replicons (Kholod and, Mustelin, 2001). The use of vectors with multiple cloning sites and different sets of restriction enzymes allow co-expression of more than one gene at one time where genes are transcribed either from individual promoters or from a single promoter, leading to a long polycistronic mRNA (Scheich et al. 2007).

P. falciparum mitochondrial Hsp70-3 chaperone properties have not been investigated neither have any mitochondrial J-proteins been identified and characterized as Hsp70-3 co-chaperones. Pfj1 is a predicted mitochondria J protein and a possible PfHsp70-3 co-chaperone but no *in vitro* study that has been conducted to characterize and verify their interactions. A number of characterized mtHsp70 have shown the propensity to aggregate and require an Hsp70 escort protein (Hep) for prevention of aggregation. However, it has not been established whether PfHsp70-3 will have similar properties and require PfHep1 for its structural and functional activities. The Zn^{2+} ions play an important role in both structural and functional properties of Hep1 proteins (Fraga et al. 2013; Dores-silva et al. 2015). Moreover, human and *L. braziliensis* Hep1 have been reported to have the ability of stimulating ATPase activities of their cognate mtHsp70 (Zhai et al. 2011; Dores-silva et al. 2016). It would be important to investigate whether PfHep1 will have the same properties of other characterized Hep1 orthologues and whether it influences the activity of PfHsp70-3. Therefore this study sought to express, purify and evaluate the biochemical properties of PfHsp70-3 and the influence of Pfj1 and PfHep1 on its activity.

3.1 Specific objectives

The specific objectives of this study included:

i) Heterologous expression of PfHsp70-3, Pfj1, Pfj1-J and PfHep1 in *E. coli* cells.

ii) Co-expression studies of PfHsp70-3 and PfHep1.

iii) Purification of PfHsp70-3, Pfj1 and PfHep1and determination of the effects of PfHep1 on the oligomeric status of PfHsp70-3.

iv) Investigation of the ability of PfHep1 to prevent the aggregation of PfHsp70-3.

v) Determination of the ability of PfHsp70-3 and PfHep1 to suppress thermally induced aggregation of MDH and citrate synthase.

vi) Analysis of the importance of Zn^{2+} ions on the functional activities of PfHep1

vii) Investigation of PfHsp70-3 ATPase activities and the possible role of PfHep1 and Pfj1-J as co-chaperones.

viii) Determination of the abilities of PfHsp70-3, PfHep1 and Pfj1-J to refold denatured β-galactosidase.

3.2. Materials and Method

3.2.1. Materials

The genomic and codon optimized sequences of PfHsp70-3, PfJ1, PfJ1-J and PfHep1 that were used are given in Appendix A. Expression plasmid vectors pQE30 (Qiagen, Germany) and pACYCDuet™-1 (Novagen, USA) were used. *Escherichia coli* (*E. coli*) cells used were M15 [p*REP4*], BL21 (DE3) and DH5α™ and XL1-Blue (Stratagene, USA). Antibiotics ampicillin (Sigma-Aldrich, Germany), kanamycin (Sigma-Aldrich, Germany) and chloramphenicol (calbiochem, Germany) were also used. Restriction endonucleases enzymes *BamHI* and *HindIII* (Thermo Scientific, USA), λ*Pst*I DNA marker (BioLabs, New England) and precision plus protein marker (BioRad, USA) were also used. Ultracentrifuge (Beckman coulter life sciences, USA), Micro centrifuge (BOECO, Germany), 7315 UV-Vis spectrophotometer (JENWAY, UK) and Snakeskin® dialysis tubing (Pierce – MWCO 10,000: Thermo Scientific, USA) were also used. ECL™ Western blotting kit (GE Healthcare, UK), Chemidoc chemiluminescence imaging system (Bio-Rad, USA), 96 well microplate (Greiner bio-one, Germany), UV cuvvets (BrandTech, USA), Single use filters (GVS, USA), β-galactosidase (Sigma-Aldrich, Germany), ÄKTAbasic FPLC system (GE Healthcare, Biosciences, UK), Microtitre plate reader (Powerwavex, Biotek Instruments Inc. USA) and nanodrop spectophometer (Thermo Scientific, USA) were used in the study. Malate dehydrogenase (Roche diagnostic, Germany), citrate synthase (Sigma-Aldrich, USA), Glycylglycine (Santa Cruz Biotechnology, USA), BSA (Roche diagnostic, Germany), ATP (Roche diagnostic, Germany), Ortho-Nitrophenyl-β-Galactoside (*ONPG*; Sigma-Aldrich, Germany), isopropyl-β-D-1-thiogalactopyranoside (IPTG; Thermo Scientific, Lithuania) reagents were used. Statistical analysis was done using Microsoft® office excel statistical packages.

3.2.2 Methods

3.2.2.1 Construction of expression plasmids.

E. coli codon-optimized sequences of PfHsp70-3 (PF3D7_1134000) (41–622aa), PfJ1 (PF3D7_0409400) (61-673 aa) and PfJ1-J domain (61-269 aa) lacking the N-terminal sequence, were synthesized and inserted into a pQE30 expression plasmid vector to create pQE30-PfHsp70-3, pQE30-PfJ1 and pQE30-PfJ1-J DNA plasmids respectively. The pQE30-PfHsp70-3 and pQE30-PfJ1 constructs were kind gifts by Dr. James Njunge and Dr. Melisa Botha respectively. PfHep1 coding sequence (PF3D7_1420300) (15–302aa), was also codon optimized, synthesized and inserted into a multiple cloning site of pACYCDuet1 expression vector to create pACYCDuet1-PfHep1 plasmid. Synthesis of coding sequences and insertion into pACYCDuet1 was done using *Bam*HI and *Hind*III restriction endonuclease sites by GenScript Corporation, USA. The pACYCDuet1-PfHep1 was later inserted into pQE30 to create pQE30-PfHep1 plasmid according to procedure in (Appendix B**).** The plasmids were digested with respective restriction enzymes followed by agarose gel electrophoresis (Appendix C) to confirm the size of the coding protein sequence inserts.

3.2.2.2 Expression of PfHsp70-3, PfJ1, PfJ1-J and PfHep1

The pQE30-PfHep1, pQE30-PfHsp70-3, pQE30-PfJ1, and pQE30-PfJ-J plasmid constructs were used to transform competent *E. coli* M15 (pREP4) cells (Appendix D). The *E. coli* M15 (pREP4) plasmid strains are stringent and tightly control protein expression with minimal or no leaky expressions. The cells were grown at 37°C on 2x YT (Appendix E) agar plates supplemented with appropriate antibiotics. One colony was inoculated into 25 mL of YT broth supplemented with respective antibiotics followed by overnight incubation at 37°C. The overnight culture was diluted 10x and incubated at 37°C and grown with shaking to mid-log phase. Protein production was induced by addition of IPTG to a final concentration of 1mM. Cells were harvested by centrifugation (13000g for 2 min) prior to induction and at hourly intervals post induction for 5 hours and overnight. The harvested samples were re-suspended in phosphate buffered saline (PBS) buffer (137mM NaCl, 2.7 mM KCl, 10.3mM Na_2HPO4, 1.8mM KH_2PO4, pH 7.4).

In order to analyze protein expression profiles, the protein samples were treated with SDS-PAGE sample buffer (10% glycerol, 2% SDS, 5% β–mercaptoethanol, 0.05% bromophenol blue,

0.0625 M Tris, pH 6.8) (Laemmli, 1970) and boiled for 10 minutes. The boiled samples were analyzed using 10% and 12% SDS–PAGE and western blotting onto a nitrocellulose membrane (Bio-Rad, Germany) according to proposed protocols (De Blas and Cherwinski, 1983; Gershoni and Palade, 1982) (Appendix F &G). For western analysis, the nitrocellulose membrane was blocked for one hour at room temperature using 5% (w/v) non–fat powder milk in Tris–buffered saline–Tween (TBST: 50mM Tris; pH 7.5, 150mM NaCl, 0.1% (v/v) Tween-20). The membranes were further incubated in 5% (w/v) non–fat powder milk in TBST containing anti-His primary (1:2500) antibodies (Santa Cruz Biotechnology, USA) in an overnight at 4°C with gentle shaking. The membranes were washed three times in 10 minutes interval using TBST followed by incubation in 5% (w/v) non–fat powder milk in TBST containing HRP-conjugated goat anti-mouse IgG secondary (1:3000) antibody (Santa Cruz Biotechnology, USA) for one hour. Washing of the membrane was done as previously described and Chemiluminescence-based protein detection was achieved using Clarity™ Western ECL blotting kit (Bio-Rad, USA) as per the manufacturer's instructions, and captured with a Chemidoc chemiluminescence imaging system (Bio-Rad, USA).

3.2.2.3 Solubilization of PfHsp70-3, Pfj1-J and PfHep1

The solubilities of PfHsp70-3, Pfj1-J and PfHep1 were analyzed and a number of different reagents were used to enhance the solubilization for PfHsp70-3 and PfHep1. Culture samples (250 mL) were harvested by centrifugation (10,000g at 4°C for 5 minutes) at the respective optimum expression times post IPTG induction. The pellets were re-suspended in native lysis buffer (10 mM Tris-HCl, pH 7.5, 300 mM NaCl, 10 mM imidazole, 1 mM PMSF, and 1 mg/mL lysozyme) and frozen at -80°C overnight followed by rapid thawing and sonication at 4°C. The samples were centrifuged (13,000g for 45 minutes, 4°C) to separate the supernatant from the pellet. Both the pellet and supernatant samples were analyzed by SDS-PAGE and western analysis as described earlier.

PfHsp70-3 insoluble pellet was treated with 7.5% (w/v) final concentration N-lauroylsarcosine prepared in sodium Tris -EDTA (STE) buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA pH 7.5) and also with 8 M urea prepared in lysis buffer. Attempts to solubilise the insoluble

pellet were also made using 2 M urea (final concentration) in combination with 10% (v/v) methanol. Further, 1% (w/v) final concentration N-lauroylsarcosine in combination with 10% (v/v) methanol was also used for solubilization of PfHsp70-3. The insoluble pellets were re-suspended in wash buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM EDTA, 1% Triton X-100 and 1 mM PMSF) as described by Singh et al. (2005). The washed pellets were re-suspended in optimized solubilizing buffer (100 mM Tris pH 7.5, 300 mM NaCl, 5 mM DDT, 0.5 mM EDTA, 1 mM PMSF, 10% (w/v) sucrose and 10 mM imidazole) containing either a combination of 10% (v/v) of methanol and 2 M urea or 10% (v/v) methanol and 1% (w/v) N-lauroylsarcosine. Solubilization was allowed to proceed for 30 minutes at room temperature and samples were sonicated and centrifuged as described before. The pellet and supernatants were analyzed by SDS-PAGE and western blot as described earlier. Various percentages and types of alcohols (methanol, propanol, butanol and ethanol) were also used to optimize solubilization of PfHsp70-3 pellets. For solubilization of PfHep1, 3% N-lauroylsarcosine was added to the thawing cells before sonication. Solubilised and sonicated pellet samples were centrifuged and analyzed by SDS-PAGE and Western blot.

3.2.2.4 Co-expression analysis of PfHsp70-3 and PfHep1

Competent *E. coli* BL21 (DE3) cells were co-transformed with pQE30-PfHsp70-3 and pACYCDuet1-PfHep1 plasmid constructs and plated on 2x YT agar plates supplemented with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol and incubated at 37°C. The colonies were inoculated into the broth medium containing the same antibiotics and grown to mid-logarithmic phase. Protein production was induced by adding IPTG to a final concentration of 1mM. Cells were harvested prior to induction and at hourly intervals post induction for 5 hours and overnight. The harvested cells were centrifuged (13000g for 2min) and re-suspended in PBS buffer. Co-transformations of pQE30-PfHsp70-3 and pACYCDuet1 were also included as controls. Protein production profiles of PfHsp70-3 in the presence and absence of PfHep1were evaluated using SDS-PAGE and western blot analysis as described in section 3.3.2.2.

3.2.2.5 Purifications of PfHsp70-3, PfHep1 and Pfj1-J

E. coli cells expressing PfHsp70-3, PfHep1 and PfJ1-J were harvested at the respective optimum expression times post IPTG induction followed by re-suspension in native lysis buffer and freezing at -80°C in an overnight. The cells were thawed, sonicated and centrifuged at 4°C. Thawing and sonication was done in the presence of 3% N-lauroylsarcosine for PfHep1.The supernatant fractions of the samples were mixed with cOmplete His-tag purification resin (Roche, Germany) and allowed to bind overnight at 4°C with gentle rocking. The resins were then centrifuged (4500g for 3 minutes, 4°C) to remove unbound proteins and washed three times using wash buffer (100 mM Tris-HCl, , 300 mM NaCl, 50 mM imidazole, pH 7.5) to remove non-specific contaminants. The bound proteins were eluted by re-suspending the resins in elution buffer (100 mM Tris-HCl, and 300 mM NaCl, and 750 mM imidazole, pH 7.5) followed by extensive dialysis using SnakeSkin dialysis tubing into dialysis buffer (100 mM Tris, 100 mM NaCl, 0.5 mM DTT, 10% (v/v) glycerol, 50 mM KCl, 2 mM $MgCl_2$ pH 7.5,) and concentrated against PEG 20000 (Merck, Germany). The efficiency of the purification process was assessed using SDS-PAGE and western blot analysis using mouse monoclonal anti-His primary antibody and HRP-conjugated goat anti-mouse IgG secondary antibody as described before. Chemiluminescence-based protein detection was achieved using the Clarity™ Western ECL blotting kit as per the manufacturer's instructions, and captured with a Chemidoc chemiluminescence imaging system. Purified proteins were quantified using the Bradford's assay (Sigma-Aldrich, USA) with BSA as the standard.

3.2.2.6 PfHsp70-3 size exclusion chromatography

Size exclusion chromatography was carried out to separate PfHep1 from PfHsp70-3 after co-expression, and in order to determine the oligomeric nature of PfHsp70-3. The dialyzed co-purified PfHsp70-3 and PfHep1 protein samples were filtered through 0.2µm filters and loaded into HiPrep™ 16/60 Sephacryl™ S-200 HR column driven by an ÄKTA fast-protein liquid chromatography system (GE Healthcare, Biosciences, UK) and monitored using the Unicorn

software version 4.11. The column was equilibrated with the dialysis buffer (100 mM Tris, 100 mM NaCl, 50 mM KCl, 2 mM $MgCl_2$, 5% (v/v) glycerol, 1 mM DTT, pH 7.5) which also served as a mobile phase. The proteins were injected at a flow rate of 1 mL/min and retention times were monitored at 280 nm. The molecular mass of PfHsp70-3 was estimated using elution volumes for catalase (250 kDa), BSA (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and myoglobin (17 kDa) as standards. Eluted fractions (1 mL) were collected and analyzed by SDS-PAGE and western blot using anti-His antibody as described previously. The samples containing PfHsp70-3 proteins were combined and concentrated using PEG 20000 where protein in the dialysis tube was covered with PEG 20000 to remove water and concentrate the protein by osmotic ultrafiltration.

3.2.2.7 Aggregation suppression of PfHsp70-3 by PfHep1

An evaluation of the ability of PfHep1 to suppress thermally-induced aggregation of PfHsp70-3 was adapted and modified from Dores-Silva et al. (2015). Temperature control was achieved using peltier integrated UV spectophometer that allowed preliminary assessment of the temperature at which PfHsp70-3 would aggregate in UV cuvette. Aggregation suppression of PfHsp70-3 by PfHep1 and E-PfHep1 (EDTA-treated PfHep1) was monitored by light scattering at 360 nm for 30 min at 50^0C in the assay buffer (50 mM Tris-HCl, 100 mM NaCl; pH 7.5). For this assay, 2 µM PfHsp70-3 was used with (0.25 µM, 0.5 µM, 1 µM and 2 µM). A separate evaluation of PfHep1 and E-PfHep1 to self-aggregate under the assay conditions was also conducted. Each assay was conducted in triplicates and three independent experiments on three independently purified batches of proteins were used. Absorbance was plotted as percentage of PfHsp70-3 aggregation subsequent to normalizing against assay buffer with PfHsp70-3 alone.

3.2.2.8 Suppression of MDH aggregation by PfHsp70-3 and PfHep1.

An evaluation of the ability of PfHsp70-3 and PfHep1 to suppress thermally induced aggregation of MDH was adapted from Burger et al. (2014). Varying concentrations of PfHsp70-3 (0.25 µM

-1μM) and PfHep1 (0.25μM -1μM) proteins in the assay buffer (50 mM Tris-HCl, 100 mM NaCl; pH 7.5) were used separately and in combination against 0.72 μM MDH. Suppression was monitored by light scattering at 360 nm for 30 min at 45^{0}C and temperature control was achieved by peltier integrated UV spectophometer that allowed preliminary assessment of the temperature at which MDH would aggregate in the path length UV cuvette. Absorbance was plotted as percentage MDH aggregation over 30 min subsequent to normalizing against the assays with MDH alone in the same buffer. Each assay was conducted in triplicate and three independently purified batches of proteins were used.

3.2.2.8 Suppression of citrate synthase aggregation by PfHsp70-3 and PfHep1.

To assess the ability of PfHsp70-3 and PfHep1 to suppress the thermally induced aggregation of citrate synthase, different concentrations of PfHsp70-3(0.25 μM and 1μM), PfHep1 (0.25 μM -1 μM) and a combination of the two proteins were mixed in an assay buffer (100 mM HEPES-KOH, pH 7.5) together with 0.15 μM of citrate synthase from porcine heart (Sigma-Aldrich, USA). Aggregation suppression was monitored at 320 nm for 30 minutes at 45^{o}C in a 1cm path length UV cuvette. Temperature control was achieved by peltier integrated UV spectophometer that allowed preliminary assessment of the temperature at which citrate synthase would aggregate. Absorbance was plotted as percentage over citrate synthase aggregation subsequent to normalizing against assays with citrate synthase alone. Each assay was conducted in triplicate and three independently purified batches of proteins were used.

3.2.2.10 Analysis of PfHsp70-3ATPase activity

The ability of PfHep1 and PfJ1-J to stimulate the ATPase activity of PfHsp70-3 was determined by colorimetric assay as previously reported (Chifflet et al. 1988; Chamberlain and Burgoyne, 1997) and modified by Matambo et al. (2004). Proteins were purified as described in section (3.3.2.5.) and dialyzed overnight at 4^{o}C in ATPase buffer (25 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$; 50 mM KCl; 0.5 mM DDT). The final concentration of proteins was adjusted to 0.4 μM

with the final volume (1 mL) made up with ATPase buffer. The reactions were equilibrated at 37°C and the initiation of the reaction was done by addition of 10 μM ATP. Samples (50μl) were removed from the reaction tubes at regular time intervals and transferred into a 96-well flat bottomed microtitre plate which contained 50 μl of 10% SDS to stop the reactions. The samples were taken in triplicate ranging from time 0 to 240 minutes in an interval of 30 minutes. Samples taken immediately after the initiation of the reaction were considered as at zero time point and were used to normalize other samples. For colour development, 1% solution of ammonium molybdate (50 μl) dissolved in 1M HCl together with 50 μl of 6% ascorbic acid prepared in phosphate free water was added to the reaction. The reaction was left to stand for 30 minutes at room temperature and 150 μl of 2% (w/v) sodium citrate in 2% (v/v) acetic acid was added to the microtitre plate wells to complex the excess molybdate and prevent further increase in color formation. The inorganic phosphate generated by the enzymatic reactions and phosphate standards were quantified at an absorbance of 850 nm using a microtitre plate reader. Specific basal ATPase activity was expressed as nmol Pi released/min/mg by Hsp70-3 protein and percent fold increase. Reactions containing PfHsp70-3, PfHep1 and PfJ1-J alone without ATP were incorporated to detect any inorganic phosphate contaminations in the reaction. The reaction containing ATP alone was also used to estimate the amount of inorganic phosphate released through spontaneous ATP hydrolysis which was subsequently subtracted from all time points. The boiled samples of PfHsp70-3 were included as controls. A standard curve was prepared using potassium hydrogen phosphate and colorimetric detection of phosphate in the reaction samples and standard solutions. Assays were conducted in triplicate for each experiment, and at least three independent experiments were performed using separately purified batches of proteins.

3.2.2.11 Refolding assay using denatured β-galactosidase.

An assessment of the ability of PfHsp70-3 to refold denatured β-galactosidase with the help of Pfj1-J and PfHep1 was adapted and modified from Freeman and Morimoto (1996). The assay is based on the refolding of denatured β-galactosidase by Hsp70 in the presence of a co-chaperone and uses ortho-nitrophenyl-β-galactoside (ONPG) as a chromogenic substrate (Rotman, 1957) to

determine the activity of refolded β-galactosidase as a measure of activity of Hsp70. Briefly, 10 μg/mL of β-galactosidase was diluted nine fold into glycylglycine (pH 7.5). The mixture was further diluted 19-fold into glycylglycine (pH 7.5) to make up native β-galactosidase and into denaturing buffer (25 mM HEPES pH7.5, 5 mM $MgCl_2$, 50 mM KCl, 5 mM β-mercaptoethanol, and 6 M guanidine-HCl) to constitute denatured β-galactosidase. Both the native and denatured β-galactosidase samples were incubated for 30 minutes at 30°C. Refolding reactions containing 372 μl refolding buffer (25 mM HEPES pH 7.5, 5 mM $MgCl_2$, 50 mM KCl, 2 mM ATP, and 10 mM DTT), 3 μl denatured β-galactosidase and 1.6 μM of PfHsp70-3 alone or with 3.2 μM PfHep1 and Pfj1-J were set up. Reactions containing 372 μl of refolding buffer together with 3.2 μM of PfHep1 and Pfj1-J alone without PfHsp70-3 were also set up. Further, reactions containing 372 μl of refolding buffer, 3.2 μM BSA and 3 μl denatured β-galactosidase were used as negative controls, while the positive control containing 372 μl of refolding buffer, 3.2 μM BSA and 3 μl native β-galactosidase was also set up. The reaction tubes and controls were incubated at 37°C and samples were taken at designated time points to assay the recovery of β-galactosidase activities.

To assay the recovery of β-galactosidase activity, 40 μl of refolding reaction samples were mixed with 40 μl of refolding buffer supplemented with 0.8 M ONPG in corresponding reactions for the chromogenic reaction. The reaction was incubated at 37°C and stopped after 20 minutes by the addition of 200 μl of 0.5 M sodium carbonate. The absorbance of each sample was determined spectrophotometrically at 412 nm and the percentage activity was calculated relative to the activity of native β-galactosidase in the refolding buffer supplemented with BSA.

3.3 Results

3.3.1 PfHsp70-3 was insoluble.

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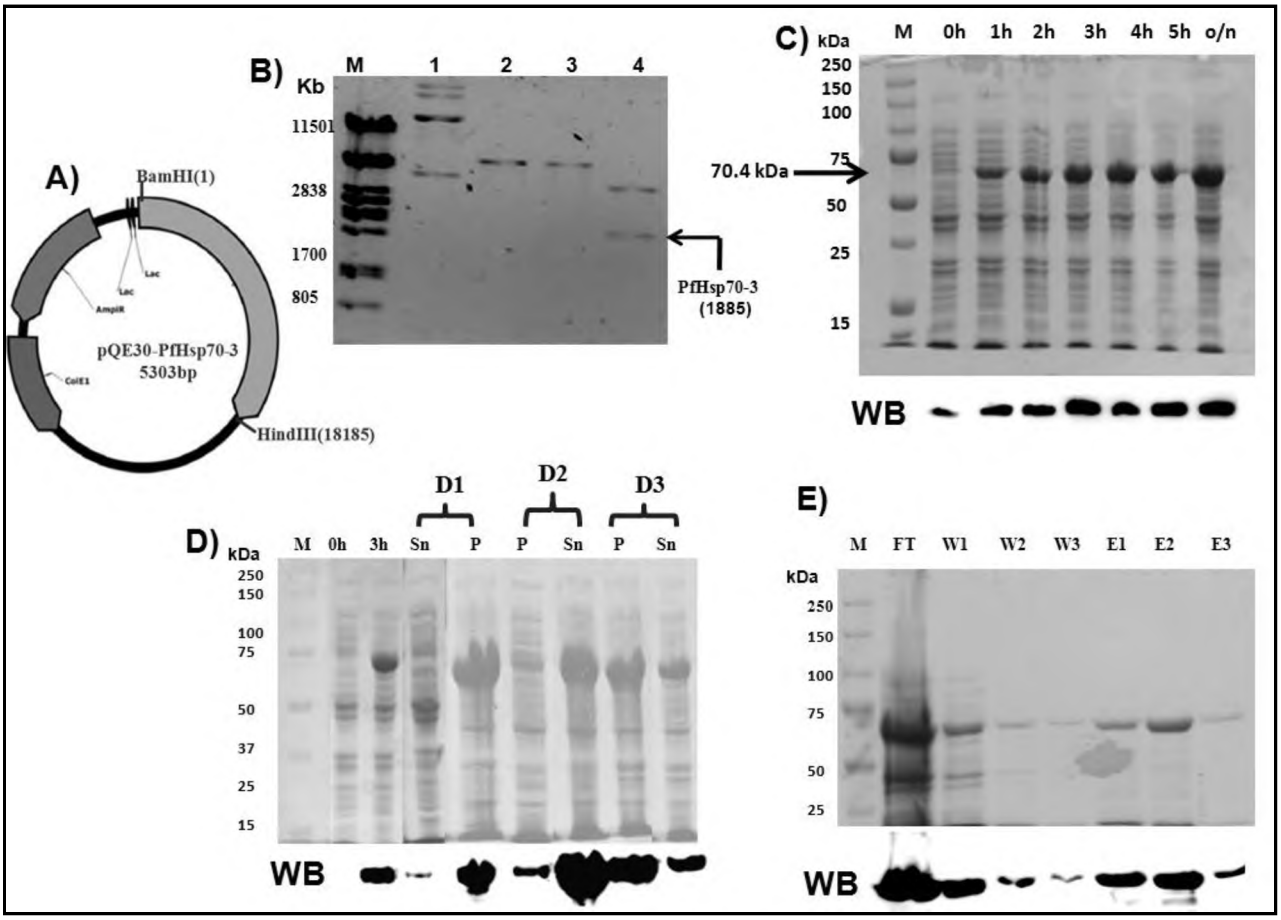


Figure 3.1 : Heterologous expression, solubility and purification of PfHsp70-3: (**A**) Plasmid map of pQE30-PfHsp70-3 designed using PlasmaDNA software (Angers-Loustau, et al. 2007). The 6xHis-tag is upstream of the PfHsp70-3 coding sequence which is inserted between *Bam*HI and *Hind*III restriction sites. (**B**) Restriction digests of pQE30-PfHsp70-3 were run on a 1% agarose gel with lanes M: DNA marker in Kb, lane 1: uncut, lanes 2 &3: single cuts by *Bam*HI and *Hind*III respectively and lane 4: double cut by both enzymes). (**C**) SDS-PAGE (10%) analysis of total protein lysates after induction of *E. coli*M15(pREP4)[pQE30-PfHsp70-3] cells using coomassie stain: Lane M molecular mass maker in kDa; 0h non-induced sample, lanes 1-5h; samples taken hourly for five hours post induction; o/n, overnight induced sample. (**D**) SDS-PAGE (10%) analysis of solubility studies of PfHsp70-3: Lane M, molecular mass marker in kDa; lane 0h, non-induced samples; lane 3h, expression in the third hour post induction, Lanes D1, samples with no solubilizing agent used, lanes D2, 7.5% lauroylsarcosine ; lane D3, 8M urea. Sn: supernatant and P: pellet of the analyzed samples. (**E**) SDS-PAGE (10%) of the fractions collected from the purification of PfHsp70-3 protein after solubilization with 7.5% lauroylsarcosine. Lane FT, flow through; lanes W1-W3 are the three washes; E1-E3, three samples eluted from the beads. (**WB**) The western analysis for the detection of PfHsp70-3 using anti-His antibody.

The aim of the study was to express and purify PfHsp70-3 for further biochemical characterization. SDS-PAGE analysis of whole cell lysates showed expression of PfHsp70-3 (70.4 kDa) after the first hour post induction with IPTG and then a slight increase over the duration of induction (Figure 3.1C). Western analysis using anti-His antibody confirmed that the protein was PfHsp70-3 and was expressed before addition of IPTG, with optimal expression occurring at the third hour post-induction (Figure 3.1C). Solubility analysis indicated that the protein was insoluble (Figure 3.1D Lane D1). The addition of lauroylsarcosine (7.5%) successfully solubilised the protein, with most of it present in the supernatant fraction (Figure 3.1D Lanes D2). The use of 8M urea also solubilised some PfHsp70-3 protein (Figure 3.1D Lanes D3). PfHsp70-3 was purified by nickel affinity chromatography after solubilization with lauroylsarcosine (7.5%) (Figure 3.1 E). However, PfHsp70-3 lacked activity as determined by ATPase assays, and this necessitated the use of different solubilization approaches to recover bioactive protein.

Different alcohols in combination with either urea or N-lauroylsarcosine were used in an attempt to solubilise PfHsp70-3 aggregates and produce biologically active protein. A combination of 2 M urea and 3% methanol led to a higher amount of soluble protein compared to the other alcohols (Figure 3.2A, lanesS1). In order to optimize the solubility of the protein, varying percentages of methanol in combination with 2 M urea were investigated and the highest amount of protein was solubilized with 4% methanol (Figure 3.2 C). The solubility of the protein decreased with the increase of methanol percentage and there was no further solubilised protein beyond 10% methanol (data not shown).

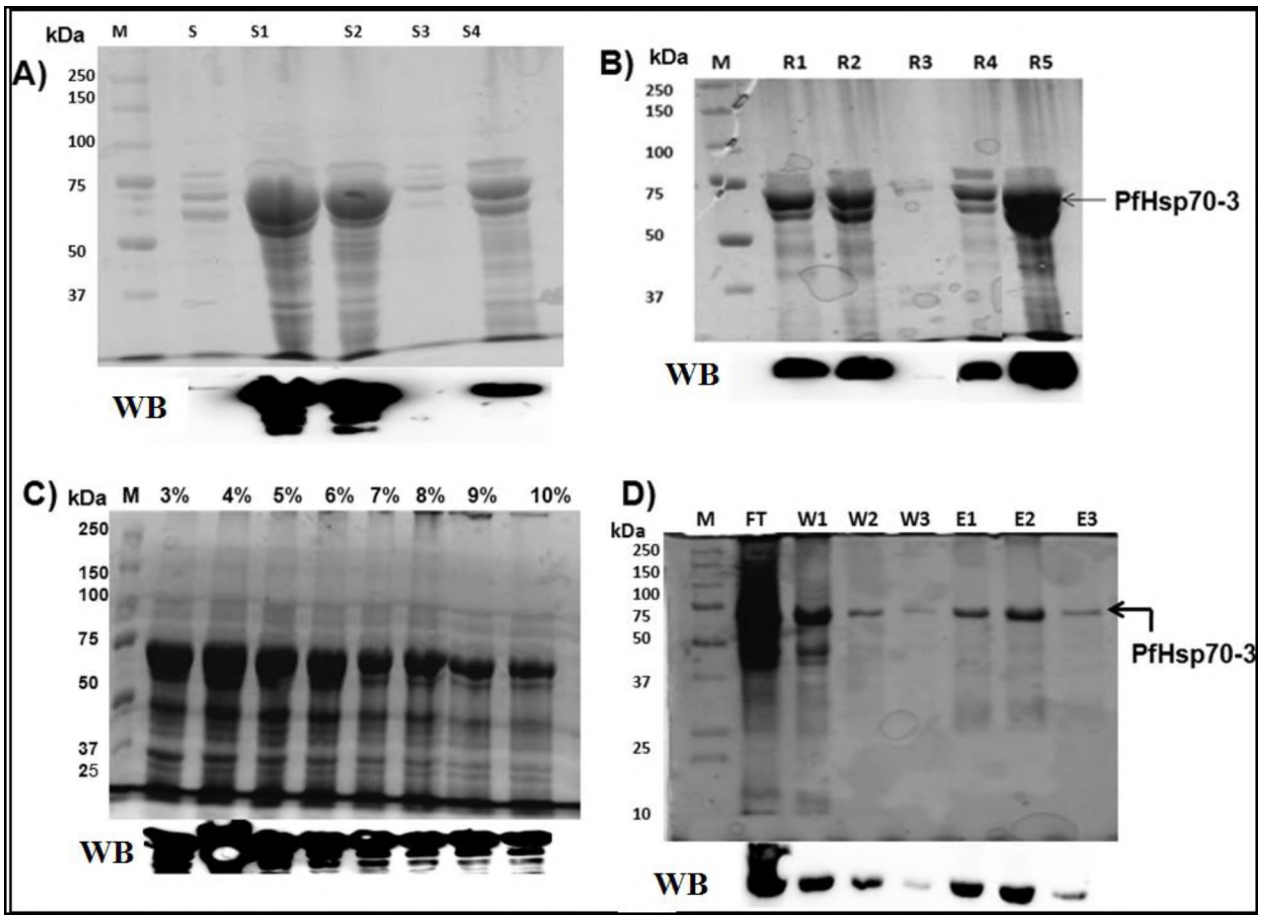


Figure 3.2 : Solubilization and purification of PfHsp70-3 from insoluble aggregates: (**A**) SDS-PAGE (10%) of the PfHsp70-3 protein aggregates solubilised with 2M urea together with 3% alcohols. Lane M, protein molecular maker; lane S butanol; S1 methanol; S2 propanol; S3 ethanol and S4 2M urea alone. (**B**) SDS-PAGE (10%) of the PfHsp70-3 protein aggregate solubilised with 1% N-lauroylsarcosine together with 3% of different alcohols using coomassie stain. Lane M, molecular mass maker, lane R1-R5; methanol, propanol, butanol, ethanol and 1% N-lauroylsarcosine respectively. (**C**) SDS-PAGE (10%) analysis of the solubilised PfHsp70-3 protein aggregates using different percentages of methanol in the presence of 2M urea. Lane M; molecular protein marker, 3% - 10% methanol concentrations (**D**) SDS-PAGE (10%) analysis of protein fractions collected from the purification of PfHsp70-3 protein (after solubilization with 4% methanol and 2M urea). Lane FT, flow through; lanes W1-W3, were three washes and lanes E1-E3 were three elutions. (**WB**) The western analysis for the detection of PfHsp70-3 using anti-His antibody.

A combination of 4% methanol and 2 M urea was used for the solubilization and purification of PfHsp70-3 (Figure3.2D). However, the purified PfHsp70-3 protein showed no ATPase activity (data not shown). Attempts to purify PfHsp70-3 by chemical methods were unsuccessful and the

next approach was to co-express PfHep1 with PfHsp70-3 and therefore produce soluble and functional PfHsp70-3.

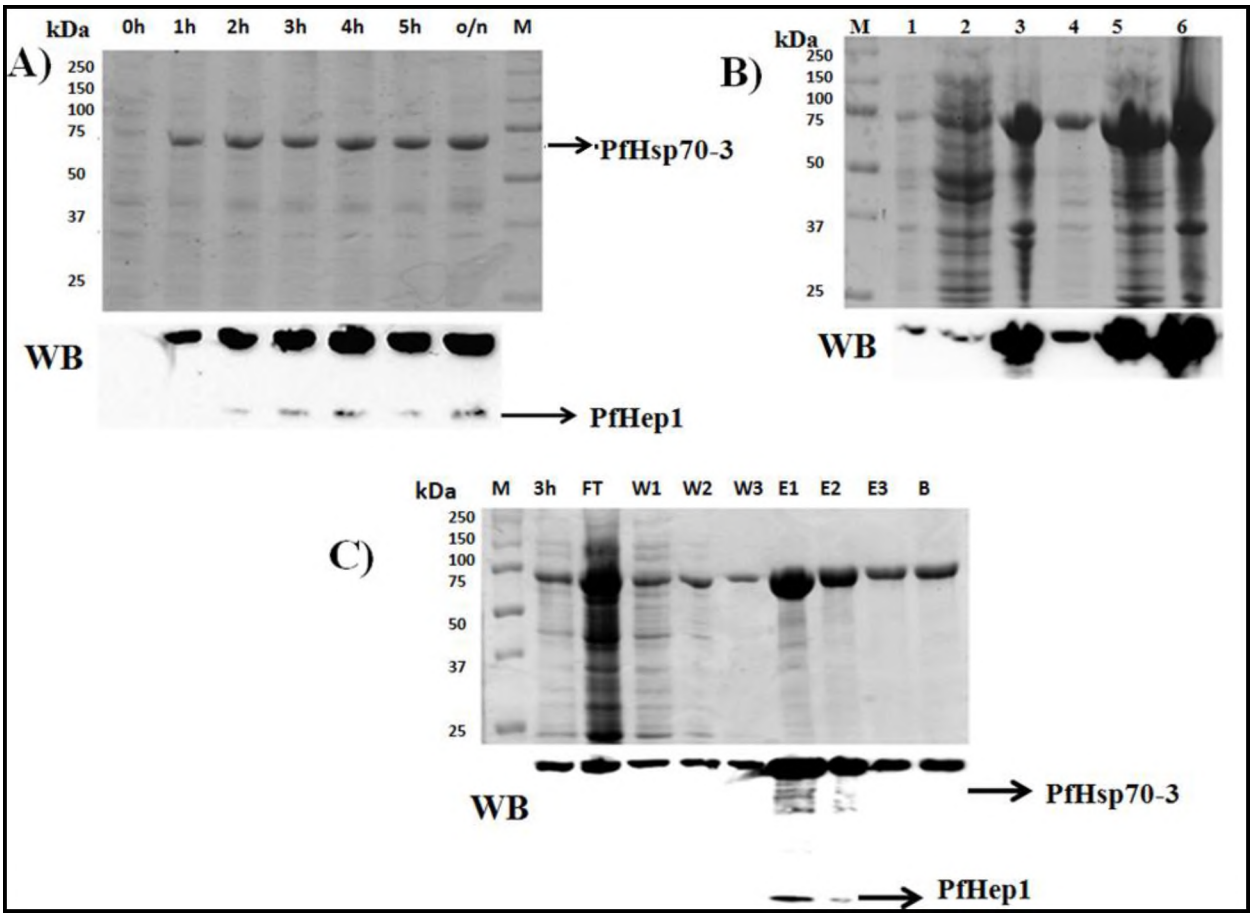


Figure 3.3 : PfHsp70-3 and PfHep1 co-expression and purification: (**A**) SDS-PAGE (10%) analysis of PfHsp70-3 and PfHep1 co-expression in *E. coli* BL21 (DE3) cells. Lane M: protein markers in kDa, lane 0h: *E. coli* total protein sample before IPTG induction, lanes 1h-5h: protein samples taken hourly for five hours post IPTG induction, lane o/n: protein samples taken after overnight post induction. (**B**) SDS-PAGE (10%) analysis of the solubility of PfHsp70-3 in the presence and absence of PfHep1 using coomassie stain. Lane M: protein markers in kDa, lane 1: *E. coli* BL21 (DE3) [pQE30-PfHsp70-3] 4 hrs post IPTG induction (total protein), lanes 2-3: supernatant and pellet fractions of cells harvested and lysed 4 hrs post IPTG induction, lane 4: *E. coli*BL21(DE3) [pQE30-PfHsp70-3; pACYCDuet1-PfHep1] 4 hrs post IPTG induction (total protein), lanes 5-6: supernatant and pellet fractions from lysed cells co-transformed with pQE30-PfHsp70-3 and pACYCDuet-1-PfHep1 4 hrs post IPTG induction. (**C**) SDS-PAGE (10%) analysis of the co-purification of PfHsp70-3 and PfHep1, after co-expression with PfHep1, by nickel affinity chromatography. Lane M: protein markers in kDa, lane 3h: *E. coli* BL21 (DE3) [pQE30-PfHsp70-3; pACYCDuet1-PfHep1] 4hrs post IPTG induction, lane FT: fraction unbound to the cOmplete His-tag purification resin, lanes W1-W3: washes containing 50 mM imidazole, lanes E1-E3: elutions of PfHsp70-3 and PfHep1 using 750 mM imidazole, lane B: bead fraction. (**WB**) Western analysis for detection of PfHsp70-3 and PfHep1 using anti-His antibodies.

PfHsp70-3 and PfHep1 were co-expressed to assess the effect of PfHep1 on the solubility and functionality of PfHsp70-3 (Figure 3.3 A). Both PfHsp70-3 and PfHep1 co-expressed successfully in *E. coli* BL21 (DE3) cells and PfHsp70-3 protein production was observed after induction with IPTG, (Figure 3.3 A). Examining the effect of PfHep1 co-expression with PfHsp70-3 revealed that it substantially enhanced the solubility of PfHsp70-3 (Figure 3.3 B lane 5) when compared to the supernatant fraction obtained without PfHep1 co-expression (Figure 3.3B lane 2). PfHsp70-3 alone was virtually insoluble, as seen by the prominence of the protein in the insoluble pellet compared to the soluble fraction (Figure 3.3B, lanes 2 and 3). The solubility of PfHsp70-3 facilitated its native purification using nickel affinity chromatography without the need for denaturants. Some PfHsp70-3 was removed during the wash steps (Figure 3.3C, lanes W1–W3) and a low concentration of PfHep1 (35 kDa) co-eluted with PfHsp70-3 (70.4 kDa) in elutions E1 and E2, however PfHep1 was not detected in elution 3 (Figure 3.3C, lanes E1–E3). Furthermore, PfHsp70-3 was found to be biologically active and this permitted further biochemical characterization.

3.3.2 PfHsp70-3 that co-purifies with PfHep1 exists as a monomer.

The size exclusion chromatography was carried out to separate PfHsp70-3 from PfHep1 as the two proteins co-eluted during purification and since it was necessary to separate the two proteins for biochemical characterization. Size exclusion chromatography was also used to determine the oligomeric status of PfHsp70-3. PfHsp70-3 eluted as a monomer and western analysis of the eluted fractions confirmed that the observed peak (approximately 70 kDa) was PfHsp70-3 protein (Figure3.4). The concentration of PfHep1 was too low to be detected by size exclusion chromatography.

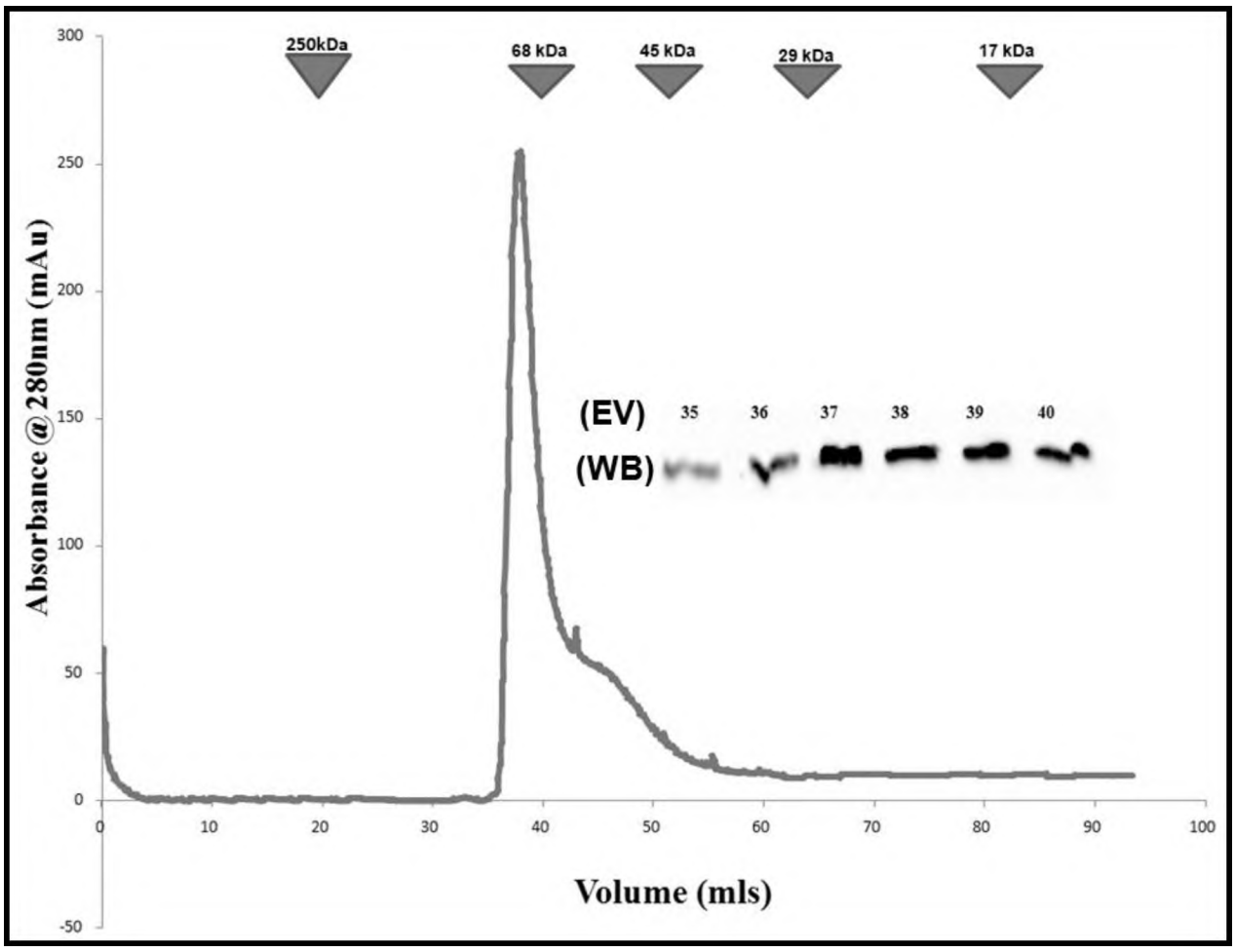


Figure 3.4 : Size exclusion chromatography of PfHsp70-3: PfHsp70-3 (0.85 mg/mL) eluted at 37.9 mLs and its size was approximated from the elutions of standard proteins i.e. catalase (250 kDa) at 18.5 mLs, BSA (68 kDa) at 40 mLs, ovalbumin (45 kDa) at 56.9 mLs, carbonic anhydrase (29 kDa) at 68.6 mls and myoglobin (17 kDa) at 85.2 mLs as shown by the black arrowheads. Western blot (WB) analysis of peak elution fractions using anti-His antibodies with elution volume (EV) indicated above the western analysis.

3.3.3 PfHep1 was insoluble

In order to independently characterize PfHep1, it was necessary for it to be purified separately. Since over-expression was not possible using the pACYC-Duet-1 expression vector (data not shown), the PfHep1 coding region within the pACYC-PfHep1 was double restricted by *Hind*III and *Bam*HI and inserted into a pQE30 expression vector. A double restriction of the created

pQE30-PfHep1 construct showed successful insertion of PfHep1 coding sequence (Figure 3.5B lane 1).

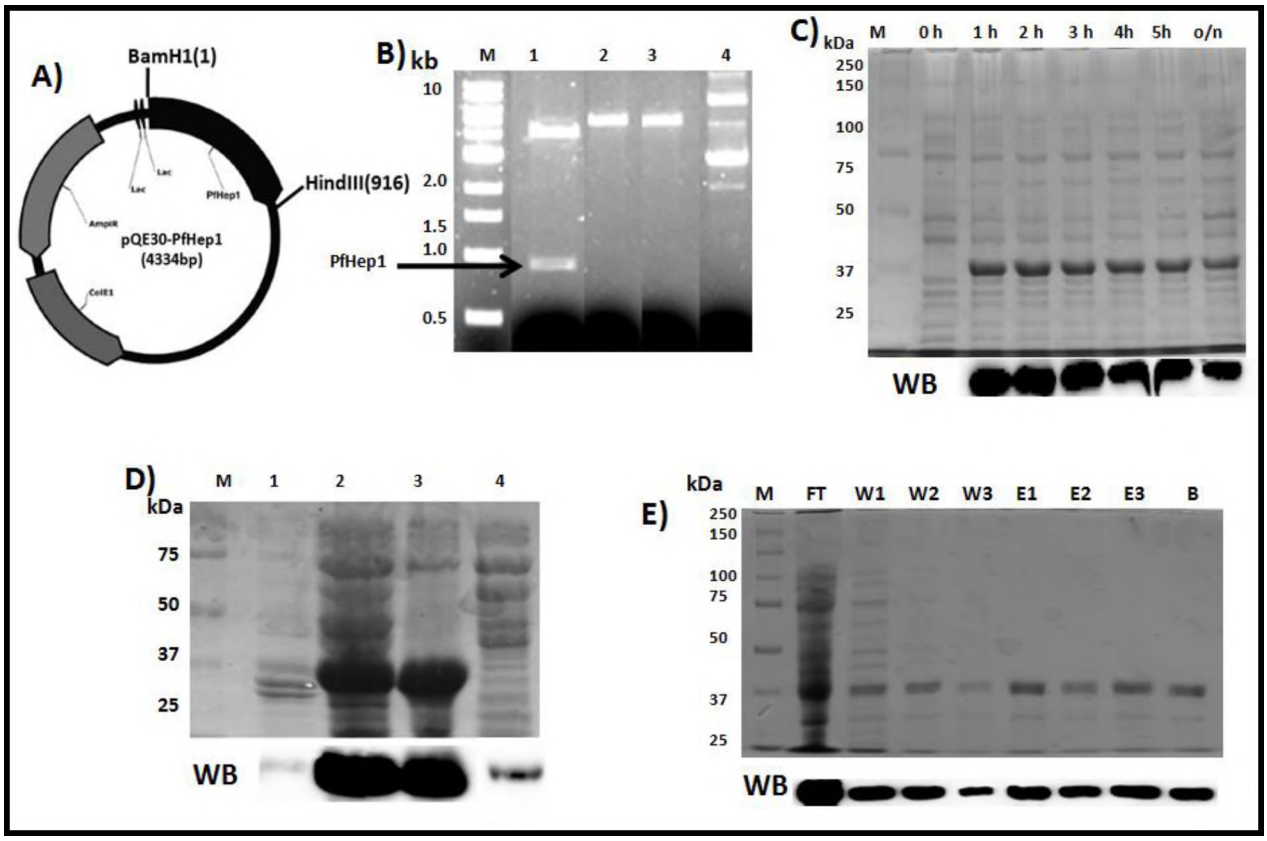


Figure 3.5 : PfHep1 expression, solubility and purification: (**A**) Plasmid map for pQE30-PfHep1 designed using PlasmaDNA software (Angers-Loustau, et al., 2007). The 6xHis-tag segment is upstream of the PfHep1coding sequence which was inserted between *Bam*HI and *Hind*III restriction sites. (**B**) Agarose gel analysis of restriction digest of pQE30-PfHep1with *Hind*III and *Bam*HI restriction digest, lanes M (DNA marker), 1 (double cut), 2&3(single cuts) and 4 (uncut). (**C**) SDS-PAGE (10%) analysis of pQE30/PfHep1 expression in M15 (pREP4) *E. coli* cells using coomassie stain. Lane M: protein marker in kDa, lane 0h: samples taken before IPTG induction, lanes 2h-5h: protein samples taken hourly for five hours post IPTG induction, lane o/n: samples taken after overnight. (**D**) SDS-PAGE (10%) analysis of the solubility of PfHep1 with and without 3% (w/v) lauroylsarcosine. Lane M: protein markers in kDa, lanes 1&2: respective supernatant and pellet fractions of cells without lauroylsarcosine treatment, lanes 3&4: supernatant and pellet fraction of cells treated with 3% lauroylsarcosine respectively. E) SDS-PAGE (10%) analysis of protein fractions collected from the purification of PfHep1protein (after solubilization with 3% lauroylsarcosine). Lane FT, flow through, lanes W1-W3, were the three washes; E1-E3, were the three elutions from the beads, B; beads. (**WB**) Western analysis for the detection of PfHep1 using anti-His antibodies.

PfHep1 was successfully over-expressed using *E. coli* M15 [pREP4] cells with the maximum expression occurring at the third hour post IPTG induction (Figure 3.5 C), there was expression

of the protein throughout the induction but there was no expression observed before induction (Figure 3.5 C).

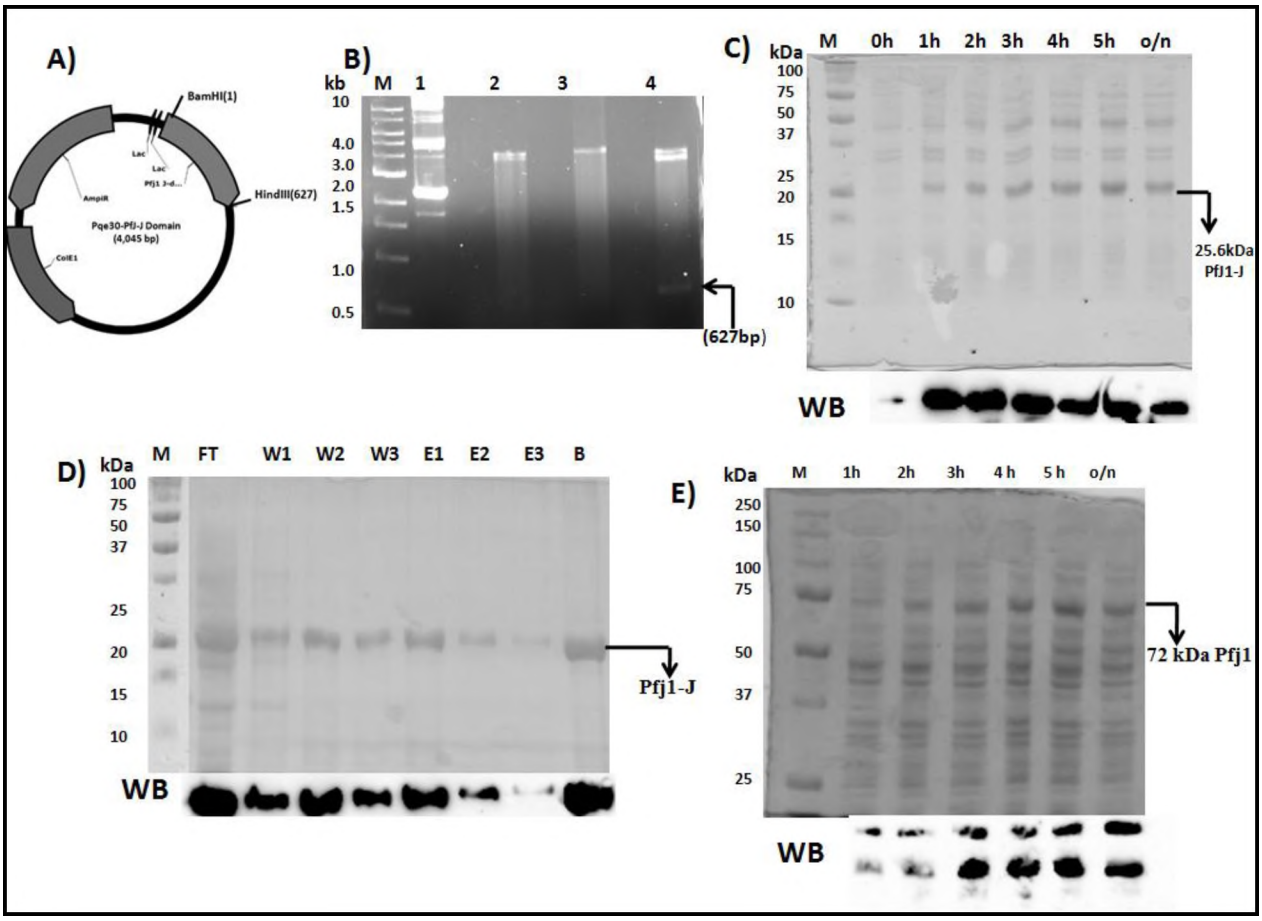


Figure 3.6 : Expression and purification of PfJ1 and PfJ1-J: (A) Plasmid map for pQE30-PfJ1-J domain designed using PlasmaDNA software (Angers-Loustau, et al. 2007). The 6xHis-tag segment is upstream of the Pfj1-Jcoding sequence inserted between *Bam*HI and *Hind*III restriction sites. (B) Agarose gel analysis of restriction digest of pQE30- PfJ1-J using *Hind*III and *BamHI*, lanes,M (DNA marker),1(uncut), 2&3 (single cuts) and 4(double cut) (C) SDS-PAGE (12%) analysis of PfJ1-J domain expression in *E. coli*M15 (pREP4) cells using coomassie stain. Lane M: protein marker in kDa, lane 0h: samples taken before IPTG induction, lanes 2h-5h: protein samples taken hourly for five hours post IPTG induction, lane o/n: samples taken after overnight. (D) SDS-PAGE (12%) analysis of protein fractions collected from the purification of PfJ1-J; Lane FT, flow through, lanes W1-W3, three washes; E1-E3, three elutions of protein from the beads, B, beads. (E) SDS-PAGE (10%) analysis of Pfj1 expression in *E. coli*M15 (pREP4) cells. Lane M: protein marker in kDa, lane 0h: samples taken before IPTG induction, lanes 2h-5h: protein samples taken hourly for five hours post IPTG induction, lane o/n: samples taken after overnight. (**WB**) Western analysis for the detection of respective proteins using anti-His antibodies.

Surprisingly, PfHep1 was insoluble as much of it was observed largely in the pellet of the total cell lysates (Figure 3.5 D lane 2) and was solubilised by use of the ionic detergent N-lauroylsarcosine (Figure 3.5 D lane 3). N-lauroylsarcosine was added to the cell lysate during lysis and the solubilised PfHep1 was purified by nickel-affinity chromatography (Figure.3.5 E). Some PfHep1 was removed during washing steps (Figure 3E, lanes W1-W3) and the protein was successfully eluted (Figure 3E).

3.3.4 Pfj1-J was over-pressed in *E. coli* cells but not Pfj1

The full length PfJ1 could not be over-expressed in *E. coli* cells to allow its purification. SDS-PAGE and western analysis showed that the protein produced degradation products (Figure 3.6E). Other expression conditions, such as lowering expression temperatures and IPTG concentrations, were used in an attempt to abrogate the degradation products observed and produce full-length protein for over-expression and downstream purifications. These approaches were not successful and the protein could not be purified (data not shown). The protein appeared to be unstable and it is possible that the very long C-terminal region of the protein could play a role in its instability. A construct encoding the J-domain, GF region and zinc finger domain (referred in here as Pfj1-J) was constructed in order to express, purify and use it for interaction studies with PfHsp70-3. Pfj1-J was successfully over-expressed at the expected size of the protein (25kDa) (Figure 3.6C). Solubility analysis of Pfj1-J was conducted by analyzing both the pellet and supernatant of whole cell protein sample and the protein was soluble (data not shown) hence permitting native purification of the protein. Pfj1-J was purified by nickel-affinity chromatography after washing and elution from the beads. The eluted protein was observed to be relatively free of contaminating proteins (Figure 3.6D lane E1-E3) even though some of the protein remained bound to the beads (Figure 3.6D lane B). An increased concentration of imidazole may have reduced the amount of protein bound to the beads.

3.3.5 PfHep1 suppressed thermal aggregation of PfHsp70-3 but not EDTA treated PfHep1.

PfHsp70-3 was found to aggregate at 50°C while PfHep1 remained soluble (Figure 3.7) and this facilitated the assessment of PfHep1's ability to suppress the thermal aggregation of PfHsp70-3.

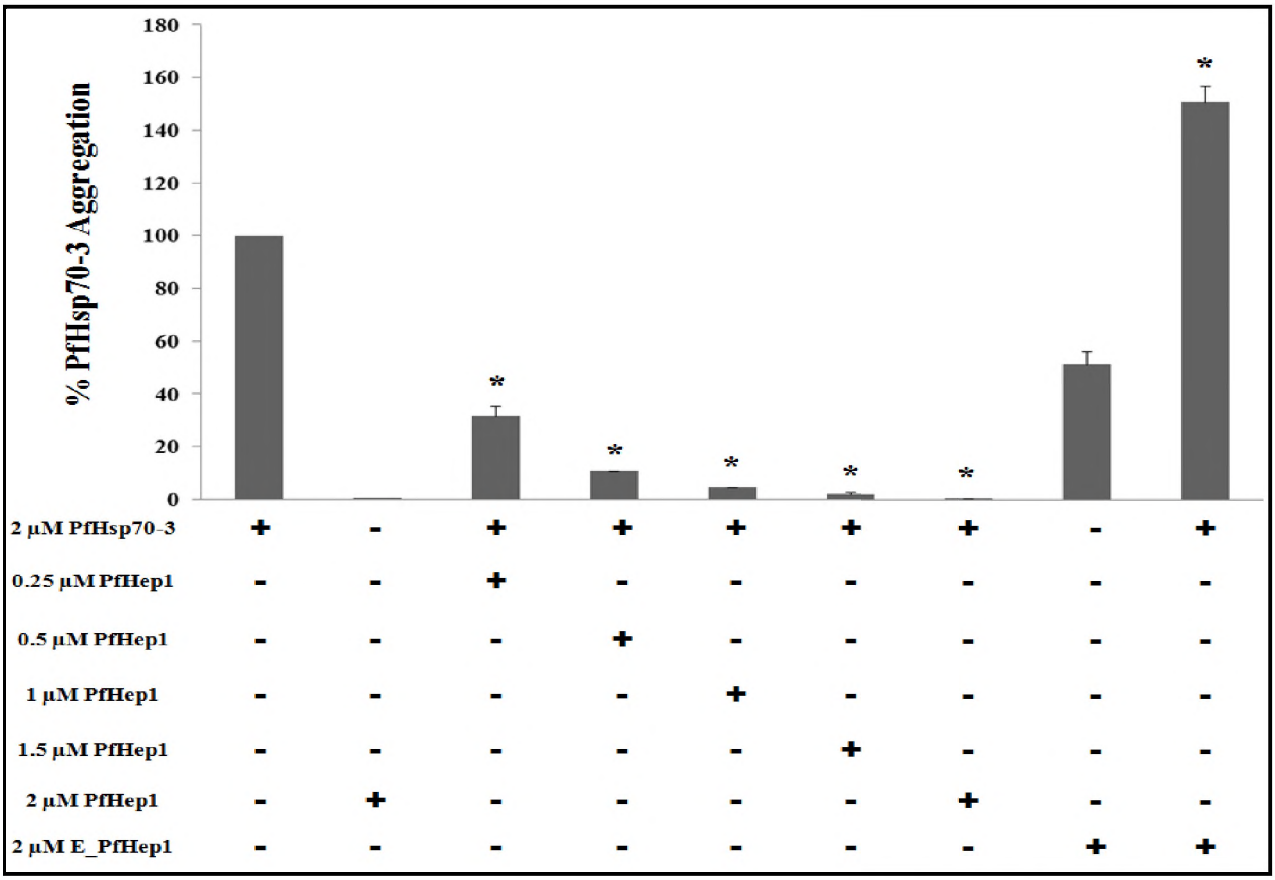


Figure 3.7 : Suppression of thermally induced aggregation of PfHsp70-3 by PfHep1: The bar graphs indicate the suppression of thermally induced aggregation of PfHsp70-3 (2µM) by PfHep1at 50°C for 30 min. Varying concentrations of PfHep1 (0.25µM, 0.5µM, 0.1µM, 1.5µM and 2µM) were added to PfHsp70-3. The ability of EDTA treated PfHep1 (E_PfHep1-2µM) to suppress PfHsp70-3 aggregation was also assessed. Percentage aggregation was calculated relative to PfHsp70-3 alone and expressed as mean ± SEM. The assays were performed in triplicate for at least three independent experiments using at least three independently purified batches of protein for each experiment. The error bars represent standard deviations. A statistically significant difference between a reaction and PfHsp70-3 alone is indicated by * ($p>0.05$) above the reaction using a Student's t-test.

PfHep1 suppressed the aggregation of PfHsp70-3 in a dose dependent manner, with equimolar concentrations (2µM) displaying almost complete suppression of PfHsp70-3 aggregation (Figure 3.7). While PfHep1 alone did not aggregate, EDTA-treated PfHep1 (E-PfHep1), in which zinc ions were removed by EDTA, did aggregate under the assay conditions (Figure 3.7).

Furthermore, E-PfHep1 failed to prevent the thermal aggregation of PfHsp70-3 and showed an additive effect as the percentage aggregation was greater than that of PfHsp70-3 alone and E-PfHep1 alone. Aggregation percentage was calculated by converting the OD values into percentage where the aggregation of PfHsp70-3 was taken as 100%.

3.3.6 PfHsp70-3 suppressed aggregation of malate dehydrogenase

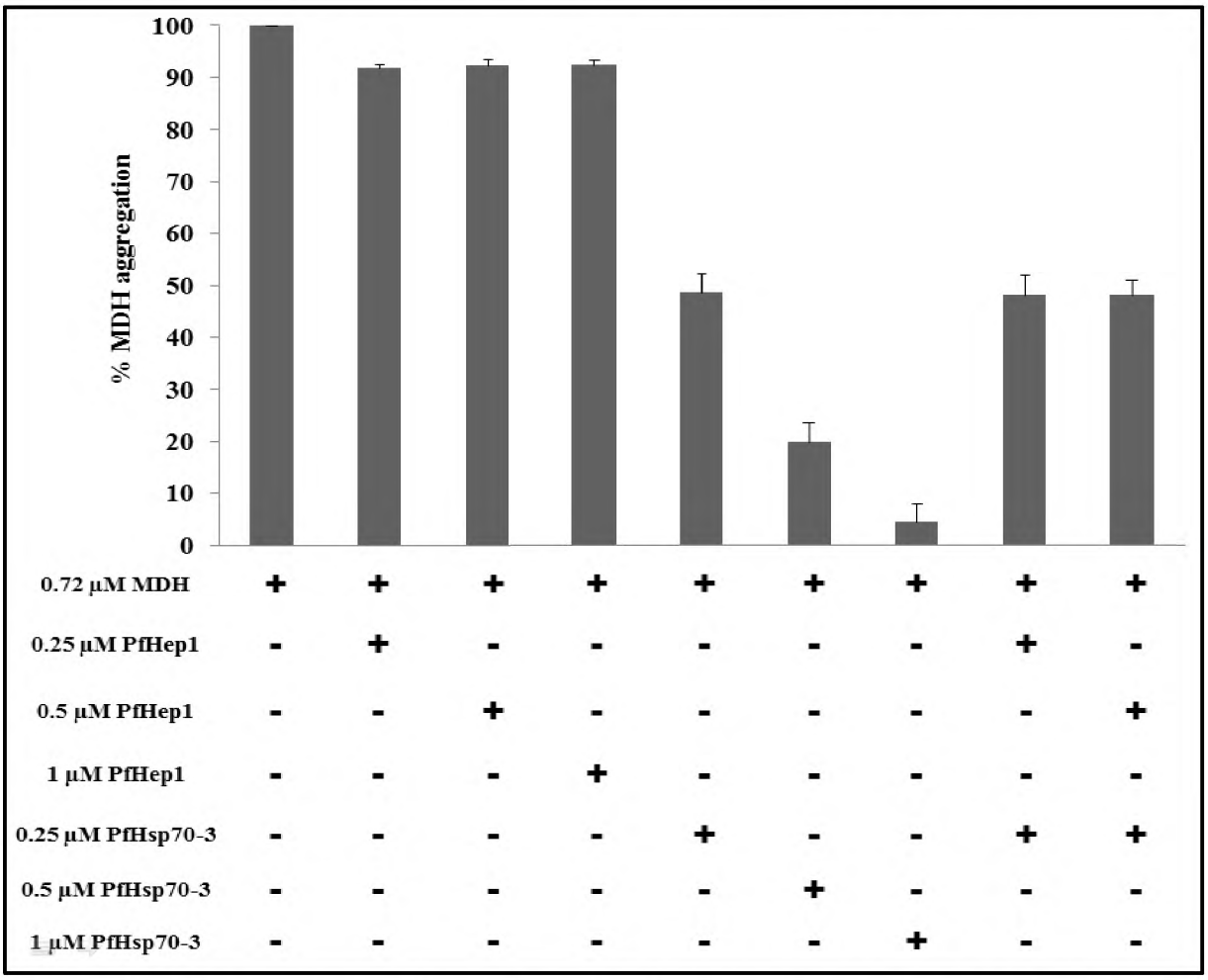


Figure 3.8 : Suppression of thermally induced aggregation of MDH by PfHsp70-3 and PfHep1: The bar graphs indicate the suppression of thermally induced aggregation of MDH (0.72µM) by varying concentrations of PfHep1, PfHsp70-3 (0.25µM, 0.5µM and 0.1µM,) and in combination of the two proteins at 45°C for 30 minutes. Percentage aggregation was calculated relative to MDH alone and expressed as mean ± SEM. The assays were performed in triplicate for at least three independent experiments using at least three independent batches of protein for each experiment. The error bars represent standard deviations. A statistically significant difference between a reaction and MDH alone is indicated by * ($p>0.05$) above the reaction using a Student's t-test.

Chemically or thermally denatured model substrate proteins such as malate dehydrogenase, form large aggregates that scatter light which can be measured *in vitro* (Haslbeck and Buchner, 2015). Malate dehydrogenase (MDH) is a ubiquitous protein found in the tissues of both animals and plants (Noyes et al. 2011) and is commonly used to demonstrate the ability of molecular chaperones to partially bind and prevent formation of protein aggregates (Sanz-Barrio et al. 2011).The abilities of PfHsp70-3 in suppressing the aggregation of proteins was investigated by use of thermally induced aggregation of malate dehydrogenase. MDH was found to aggregate at 45^{o}C (Figure 3.8) the temperature at which PfHsp70-3 and PfHep1 remained stable (data not shown). This permitted assessment of the ability of PfHsp70-3 and PfHep1to suppress the aggregation of MDH at this temperature. The addition of PfHsp70-3 at increasing concentrations (0.25 μM, 0.5 μM and 1 μM suppressed the aggregation of MDH by 55%, 80% and 95% respectively (Figure 3.8).

PfHep1 protein did not have an effect on the aggregation of MDH and the use of increasing concentrations of the protein (resulted in approximately 7% suppression of MDH aggregation Figure 3.8). Furthermore, the addition of PfHep1 did not increase the ability of PfHsp70-3 to suppress the aggregation of MDH. The combination of the two proteins at varying concentrations resulted in no significant change of aggregation suppression by PfHsp70-3 (Figure 3.8). Aggregation percentage was calculated by converting the OD values into percentage where the aggregation of MDH was taken as 100%.

3.3.7. PfHsp70-3 suppressed aggregation of citrate synthase.

The effects of molecular chaperones on aggregation and folding of citrate synthase has been demonstrated (Ehrnsperger et al. 1997; Haslbeck et al. 2005).The mechanism of folding and unfolding of denatured citrate synthase is well understood hence making it a suitable model of studying the mechanisms of chaperone functions (Grallert and Buchner, 1999).

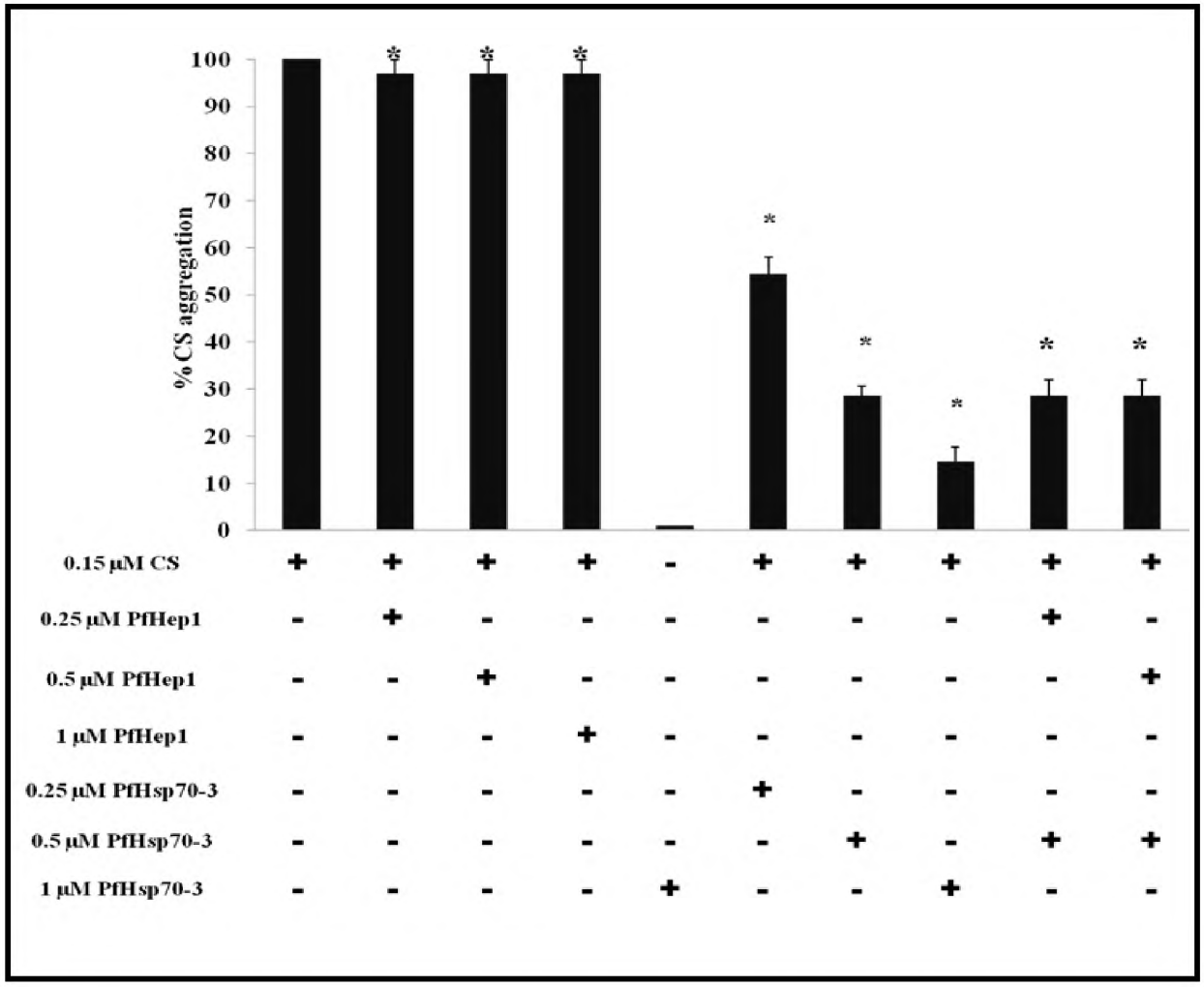


Figure 3.9 : Suppression of thermally induced aggregation of citrate synthase (CS) by PfHsp70-3 and PfHep1: The bar graphs indicate the suppression of thermally induced aggregation of citrate synthase (0.15µM) by varying concentrations of PfHep1, PfHsp70-3 (0.25µM, 0.5µM and 1µM,) and in combination of the two proteins at 45°C for 30 minutes. Percentage aggregation was calculated relative to citrate synthase alone and expressed as mean ± SEM. The assays were performed in triplicate for at least three independent experiments using at least three independent batches of protein for each experiment. The error bars represent standard deviations. A statistically significant difference between a reaction and MDH alone is indicated by * ($p>0.05$) above the reaction using a Student's t-test.

Citrate synthase aggregated at 45°C and this allowed the effect of PfHsp70-3 and PfHep1 on its thermal aggregation to be assessed as both PfHsp70-3 and PfHep1 remained soluble at this temperature. The effect of PfHsp70-3 and PfHep1 on thermally induced aggregation of citrate synthase was investigated. Increasing concentrations of PfHsp70-3 (0.25 µM, 0.5 µM and 1 µM) suppressed aggregation of citrate synthase by 50%, 70% and 85% respectively (Figure 3.9).

PfHep1 alone did not show any suppression effects on citrate synthase aggregation as increasing concentrations (0.15 μM, 0.25 μM and 0.5 μM) resulted in 3% suppression of aggregation (Figure 3.9). Moreover, PfHep1 did not enhance the aggregation suppression activities of PfHsp70-3 (Figure 3.9). Aggregation percentage was calculated by converting the OD values into percentage where the aggregation of citrate synthase was taken as 100%.

3.3.7 The ATPase activity of PfHsp70-3 was stimulated by Pfj1-J and not PfHep1

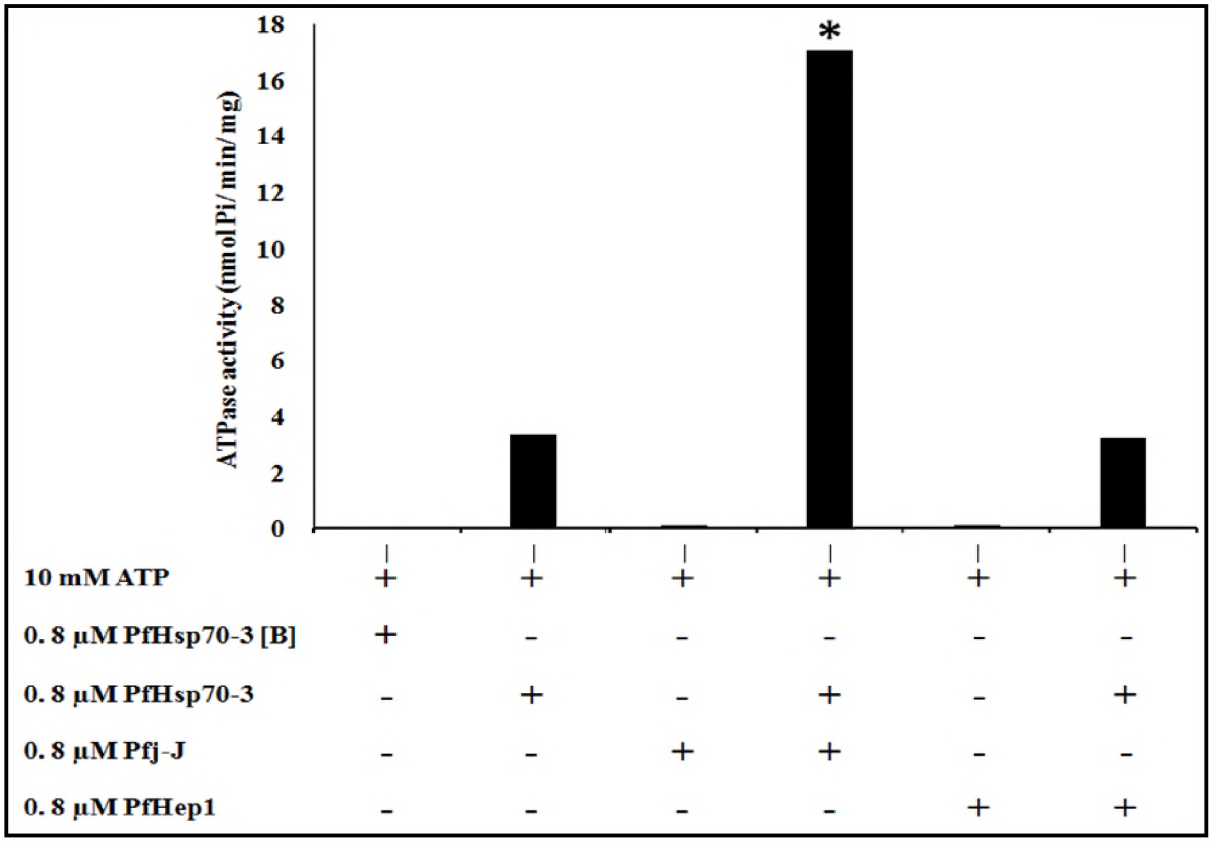


Figure 3.10 : PfHsp70-3 ATPase activity was stimulated by PfJ1-J and not PfHep1: Equimolar concentrations (0.8μM) of Pfj1-J and PfHep1 proteins were used to assess the ability of any co-chaperone activities against PfHsp70-3 in stimulating its basal ATPase activities. Stimulation of ATPase activities of PfHep1and PfJ1-J was also analyzed separately and boiled samples of PfHsp70-3, indicated as [B] were included as negative controls. The averaged data from three independent experiments done in triplicate using three batches of independently purified proteins for each experiment in shown here. Error bars indicated on each bar (SEM) and * indicate statistical significance at $P<0.05$ relative to basal ATPase value for respective chaperone using paired Student T-test.

The conversion of ATP to ADP and inorganic phosphate can be used to assess *in vitro* ATPase activities of molecular chaperones using calorimetric methods (Chifflet et al. 1988; Lanzetta et al. 1979). The ability of Pfj1-J and PfHep1 to stimulate the basal ATPase activity of PfHsp70-3 was examined using a colorimetric assay. Pfj1-J increased the basal ATPase activity of PfHsp70-3 (4 nmol Pi/min/mg) by approximately four-fold (17 nmol Pi/min/mg) (Figure 3.10). PfHep1 did not stimulate the basal ATPase activity of PfHsp70-3. Both Pfj1-J and PfHep1 showed no basal ATPase activities neither did the boiled PfHsp70-3 that was used as a negative control (Figure.3.10). PfHsp70-3 manifested typical Hsp70 chaperone properties which normally possess low basal ATPase activities which are increased by J-protein co-chaperones (Hennessy et al. 2005; Kampinga and Craig, 2010). PfHep1 is unlikely to act as a co-chaperone as it did not stimulate the ATPase activity of PfHsp70-3, while Pfj1 resulted in a four-fold stimulation.

3.3.8 The refoldase activity of PfHsp70-3 was enhanced by PfJ1-J and not by PfHep1

When Hsp70s interact with substrates, they form Hsp70–substrate complexes where the substrate is bound to a conserved nucleotide binding domain (NBD) which exhibits low basal/intrinsic ATPase activity (Kampinga and Craig, 2010). The ADP-bound state of the NBD domain manifests the highest affinity for substrate, while the ATP-bound state possesses intermediate affinity and nucleotide-unbound state demonstrates the least affinity for substrate. The functional cycle of Hsp70 is influenced by stimulation of the intrinsic ATPase activities that are regulated by a number of factors, including nucleotide exchange factors and J-proteins (Ramsey et al. 2009). J- Protein interacts with the NBD and the SBD, promoting both ATPase activity and substrate binding and hence substrate refolding by Hsp70s (Han and Christen, 2003).

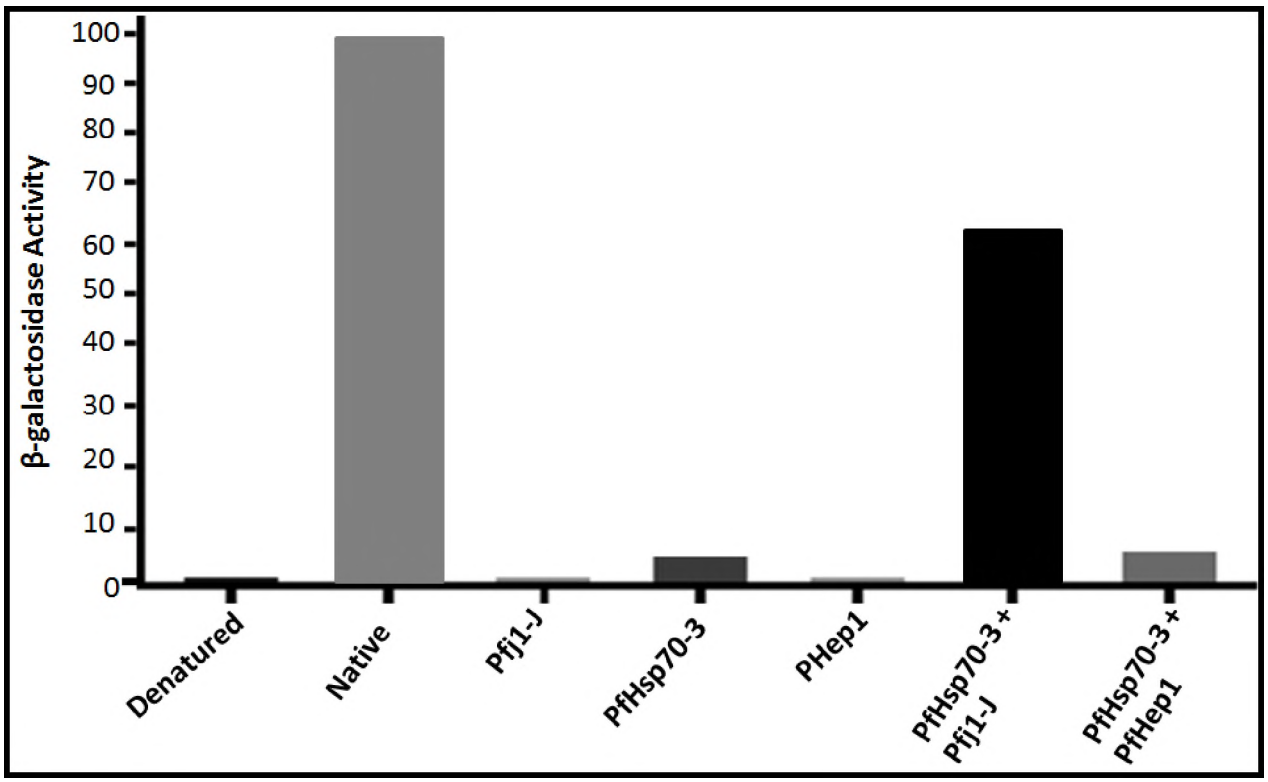


Figure 3.11 : PfHsp70-3 refolded β-galactosidase: PfHsp70-3 (1.6μM) together with Pfj1-J (3.2 μM) refolded chemically denatured β-galactosidase by 66% relative to the refoldase activity of native β-galactosidase in refolding buffer. β-galactosidase activity was measured using ONPG as a chromogenic substrate and the intensity of the yellow color resulting from the hydrolysis of ONPG was measured at 412 nm after 240minutes. Denatured β-galactosidase in refolding buffer was used as a negative control and the assay was repeated thrice using three independently purified batches of proteins. The averaged data from three independent experiments done in triplicate is shown.

The ability of PfHsp70-3 to refold denatured proteins was determined by use of β-galactosidase enzyme. The protein was chemically denatured using guanidine hydrochloride followed by incubation with either PfHsp70-3 alone or in combination with Pfj1-J and PfHep1 for refolding. The activity of refolded β-galactosidase was determined relative to native β-galactosidase using an artificial chromogenic substrate ortho-nitrophenyl-β-D-galactopyranoside (ONPG). The ONPG is a colorless lactose analogue that can act as a substrate for theβ-galactosidase enzyme. Hydrolysis of ONPG by β-galactosidase results in stoichiometric amounts of yellow ortho-nitrophenol and colorless galactose products (Juers et al. 2012).

The produced yellow color by ortho-nitrophenol was measured and determined spectrophotometrically. The activity of refolded active β-galactosidase was thus measured by the intensity of the yellow colour by measuring the OD of the solution at 412nm.The greater the intensity of the color in the solution, the more hydrolyzed ONPG and the higher the absorbance. The addition of sodium carbonate makes the reaction too basic for β-galactosidase activities and hence stops further hydrolysis of ONPG. To determine the amount of activities of refolded β-galactosidase by PfHsp70-3, both native and denatured β-galactosidase samples were included in the experimental set up as positive and negative controls respectively. The activity of refolded β-galactosidase by PfHsp70-3 was determined relative to the activities of native β-galactosidase.

PfHsp70-3 in co-operation with Pfj1-J refolded denatured β-galactosidase resulting in approximately 66% recovery of enzyme activity relative to native β-galactosidase after 240 minutes (Figure 3.11). PfHsp70-3 alone showed minimal refolding effect on β-galactosidase and the enzyme had only 5.1% activity relative to native β-galactosidase (Figure 3.11). Both PfHep1 and Pfj1-J did not show any significant refolding effect on β-galactosidase and PfHep1 did not increase the refoldase activity of PfHsp70-3 hence showing no co-chaperone properties against PfHsp70-3.

3.4 Discussion

PfHsp70-3 was successfully expressed, however the protein was insoluble. Purified PfHsp70-3 produced after solubilization with lauroylsarcosine was found to have no enzymatic activities as it did not manifest any ATPase activities perhaps due to incomplete folding. The use of organic solvents causes destabilization of protein tertiary structures with retention of secondary structures, hence protecting basic existing protein structural organization (Roccatano et al. 2002; Singh et al. 2012). Urea has been shown to reduce hydrophobic interactions that are the major cause of protein aggregation and a combination of low concentrations of urea and organic solvents can functionally solubilize insoluble aggregates of recombinant proteins (Singh et al. 2012). Based on these observations, PfHsp70-3 was solubilized by use of a combination of 4% methanol and 2M urea but it was still found to be inactive.

The co-expression of PfHsp70-3 and PfHep1 resulted in both soluble and biochemically active PfHsp70-3 protein. PfHep1 functioned as a co-factor that ensured proper solubility and folding of PfHsp70-3, leading to a functionally active protein. It was evident that PfHep1 needed to be co-expressed with PfHsp70-3 in order to obtain a biochemically functional PfHsp70-3. PfHsp70-3 behaved like other typical eukaryotic mtHsp70s that depend on their respective Hep1 proteins for prevention of aggregation and biochemical activities. PfHep1 and PfHsp70-3 proteins co-eluted during nickel affinity chromatography since both proteins were His–tagged. The proteins were separated using size exclusion chromatography as the influence of PfHep1 on the functional activities of PfHsp70-3 was going to be determined. Formation of oligomers by Hsp70 protein family occurs under different conditions and varies among the proteins. However, nucleotide free- and ADP-bound forms of the protein are prone to self-oligomerization *in-vitro* in a concentration and temperature dependent manner which is reversed by the addition of ATP, substrate binding, and the presence of some co-chaperones (Benaroudj et al.1996). Oligomerization serves as a regulatory mechanism for the availability of the active monomeric form of the chaperone cycle (Angelidis et al. 1999). Indeed the presence of PfHep1 resulted in an enzymatically active PfHsp70-3 that was in a monomeric form. Previous studies have shown that co-expression of hHep1 resulted in active and monomeric human mortalin (Zhai et al. 2008). Since ATP and co-chaperones are likely to have the same oligomeric effect on Hsp70s, it would be important in the future to investigate the separate roles of ATP and J-proteins on the oligomeric status of PfHsp70-3 in relation to its functional activities. The oligomeric state of PfHep1 itself should also be investigated as hHep1 was found to oligomerise in a concentration dependent manner (Dores-Silva et al. 2013).

Surprisingly PfHep1 was insoluble when over-produced in *E. coli* cells and this may be due to plasmodial proteins being notoriously difficult to express heterologously in a soluble form, even after codon-optimization. This may be due to the hydrophobic nature of the majority of the amino acid residues found in plasmodial protein sequences (Katti et al. 2000). Indeed, sequences of proteins that contain highly hydrophobic amino acids are likely to form insoluble aggregates (Singh and Panda, 2005). The use of 3% N-lauroylsarcosine, which is considered a mild solubilizing agent (Heikar et al. 2010; Singh et al. 2012), was used to solubilise PfHep1 which facilitated its purification for further characterization and interaction studies.

Characterized PfHep1 orthologues in various eukaryotic organisms that include *S. cerevisiae* (Sichting et al. 2005), *H. sapiens* (Zhai et al. 2008), *L. braziliensis* (Dores-silva et al. 2015), *C. reinhardtii* (Willmund et al. 2008) and *A. thaliana* (Kluth et al. 2012) were found to be soluble and could be purified without the addition of denaturants or detergents. It was determined that PfHsp70-3 aggregated at 50°C and this facilitated the development of an aggregation suppression assay as PfHep1 did not aggregate at 50°C. PfHep1 successfully suppressed aggregation of PfHsp70-3 in a dose dependant manner. The functional importance of the Zn was determined by removal of Zn^{2+} ion from PfHep1 by the metal chelating compound EDTA. EDTA was later removed by extensive dialysis and the removal of Zn^{2+} from PfHep1 abrogated its ability to suppress the aggregation of PfHsp70-3. Furthermore, the EDTA treated PfHep1 (E-PfHep1) aggregated at 50°C, while the native PfHep1 remained stable. The loss of function and stability under higher temperature by E-PfHep1could be attributed to disruption of its native structure due to the removal of the zinc ions. Indeed, earlier findings had shown the vital role that Zn^{2+} plays in the overall structure of Hep1 proteins (Fraga et al. 2013; Dores-silva et al. 2015; Yamamoto et al. 2005; vu et al. 2012). PfHsp70-3 independently suppressed the aggregation of both MDH and citrate synthase, while PfHep1 could not suppress the aggregation of these proteins and had no effect on the activity of PfHsp70. Therefore, PfHep1's ability to suppress aggregation of proteins was specific for PfHsp70-3. A similar observation had been made with *Leishmania braziliensis* Hep1 (LbHep1) that could suppress the aggregation of LbmtHsp70 but not MDH and luciferase (Dores-silva et al. 2015).

Pfj1 is one of the proposed co-chaperones of PfHsp70-3 which could be involved in protein folding in the mitochondrial matrix (Nicoll et al. 2007; Botha et al. 2007; Njunge et al. 2013). Pfj1 protein appeared to be degraded in the *E. coli* expression system and it could not be over-expressed to allow purifications. Perhaps this could be attributed to the toxicity of the protein to the *E. coli* cells upon expression which is characteristic of many plasmodial proteins (Seignovert et al. 2004; Vedadi et al. 2007) or the action of protease enzymes of the bacterial cells that might have caused cleavage of the protein (Gottesman et al. 1997). However, the J-domain of Pfj1 protein (Pfj1-J) was successfully expressed and used for interaction studies with PfHsp70-3. The Pfj1-J construct comprised of the J-domain, GF rich regions and the zinc finger of Pfj1 protein (Appendix A).

Pfj1-J stimulated the basal ATPase activities of PfHsp70-3 fourfold, while PfHep1 did not stimulate the ATPase of PfHsp70-3. Human Hep1 (hHep1) and recently LbHep1 have been reported to display the features of a type I J-proteins by stimulating the ATPase activity of their respective mtHsp70s and hHep1functioned as a holdase by binding unfolded proteins (Zhai et al. 2008; Goswami et al. 2010; Dores-Silva et al. 2016). The mechanisms by which hHep1 andLbHep1 stimulate the basal ATPase activities of their cognate mtHsp70 could be different as Hep1 proteins do not possess the HPD motif found in J-proteins that is essential for stimulation of ATPase activities. PfHsp70-3 and PfJ1-J refolded chemically denatured β-galactosidase resulting in 66% recovery of native activity. PfHep1 did not enhance the refoldase activity of PfHsp70-3. However, this result was expected since PfHep1 did not promote ATPase activity of PfHsp70-3 which could facilitate the binding of denatured β-galactosidase and eventual refolding by PfHsp70-3. Based on the findings in this study, PfHep1 does not appear to function as a co-chaperone of PfHsp70-3 but rather plays a specialized role of co-factor that prevents the aggregation and promotes proper folding of PfHsp70-3 into a native, catalytically active form.

CHAPTER FOUR

Biological characterization of PfHsp70-3 and PfHep1

4.1 Introduction

P. falciparum parasites asymptomatically multiply in the liver of the human host before they are released into the bloodstream to invade erythrocytes. Within the erythrocytes, the parasites develop asexually into trophozoites, schizonts and later into invasive merozoite forms. During development, parasites encounter changes in temperature, pH and degrading enzymes upon entry into the human host leading to up-regulation of some of its heat shock proteins (Pallavi et al. 2010). Indeed, some Hsps such as PfHsp70-1 have been reported to be up-regulated during the clinical phase of malaria (Acharya et al. 2011). The heat inducibility of Hsps leads to the cytoprotection of the parasite and the pathology of malaria disease. The parasite depends on heat shock proteins to adapt, survive and grow in the human host especially during temperature stresses associated with febrile episodes (Pavithra et al. 2004; Njunge et al. 2013). Heat inducibility of heat shock proteins and their role in the modification of the structure and functions of host erythrocytes have been demonstrated and hence are considered as possible drug targets (Cooke et al. 2001; Rao et al. 2010; Neckers and Tatu, 2008).

During its infective asexual developmental cycle in the human host, *P. falciparum* maintains one mitochondrion for respiratory transport chain (Torrentino-Madamet et al. 2010; MacRae et al. 2013). PfHsp70-3 could play a role in protecting the parasite against heat shock insults in addition to promoting protein translocation in the mitochondrial matrix (Njunge et al. 2013). The protein has also been predicted to occur in the Maurer's cleft where it possibly participates in exporting antigens to the erythrocyte surface (Vincensini et al. 2005). PfHsp70-3 mitochondrial localization prediction is based on the presence of the mitochondrial targeting signal sequence at the N-terminal of the protein (Shonhai et al. 2007). However, the localization of the protein in the parasite has not been experimentally determined. Similarly, *in silico* analysis revealed that PfHep1 has a mitochondrial targeting signal sequence indicating its mitochondrial localization (Seraphim et al. 2014) but this has not been validated experimentally. Since PfHep1 has been

demonstrated to be essential in maintaining PfHsp70-3 in a soluble and functional form, it is very likely that the two proteins will be present in the parasite mitochondrion.

Furthermore, in situ chaperone properties of PfHsp70-3 have not been investigated. PfHsp70-3 expression is likely to be up-regulated during heat shock in the event of fever that is characteristic of malaria infection. In a previous study by Njunge in 2014, constructs were designed using a pARL vector for parasite transfection of the N-terminus of PfHsp70-3 with the mitochondrial signal peptide only and also full-length PfHsp70-3 both fused to GFP. The mitochondrial signal sequence localized GFP to the mitochondria but GFP-tagged to the full length protein failed to produce transfectants, possibly due to a lethal phenotype since few parasites that could appear few weeks post transfect could die before establishment. In this study an alternative approach to determine localization was to design antibodies against PfHsp70-3 for immunofluorescence microscopy. The coding sequence of PfHep1 was inserted into the pARL vector for parasite transfection and the localization of GFP-tagged PfHep1 was also determined. Further, the study investigated the expression profile and heat inducibility of PfHsp70-3 within the erythrocytic stages of the parasite development.

4.2. Objectives

This study was aimed at establishing the localization of PfHsp70-3 and PfHep1 using GFP-tagged proteins and antibodies. Further, the study sought to determine PfHsp70-3 expression profile and its heat shock properties in the intraerythrocytic stages of *P. falciparum* development.

The specific objectives of the study were to:

i) Design and commercially raise a peptide-directed antibody specific to PfHsp70-3.
ii) Construct a plasmid for GFP-tagged PfHep1 for parasite transfection.
iii) Determine the heat inducibility of PfHsp70-3.
iv) Investigate the expression profile of PfHsp70-3 during various intraerythrocytic developmental stages of *P. falciparum.*

v) Determine the localization of PfHep1 and PfHsp70-3 in *Plasmodium falciparum*-infected erythrocyte by using GFP-tagged proteins and antibodies using indirect immunofluorescence microscopy.

4.3. Material and Methods

4.3.1. Parasite culture

Plasmodium falciparum (strain 3D7) parasites were cultured based on a previously described method (Trager and Jensen, 1976). Briefly, the parasites were propagated in human erythrocytes at the hematocrit level of 4% in Roswell Park Memorial Institute(RPMI) 1640 liquid medium containing 25mM Hepes and L-glutamine (Lonza, USA) and supplemented with 0.5%AlbumaxII (Gibco, USA), 20mM glucose (Sigma-Aldrich, Germany), 0.65mM hypoxanthine (Sigma-Aldrich, Germany) and 60μg/mL gentamicin (Lonza, USA). Details on the preparation of the culture medium are given in (Appendix H).Culturing was done in an incubator at 37°C under an atmosphere containing 5% (v/v) carbon dioxide (CO_2) and 5% (v/v) oxygen (O_2) in sealed T75 culture flasks. The human blood that was used for culturing was drawn by a nurse at Rhodes University health center (Grahamstown, South Africa) following signed consent by the donor and ethical clearance and approval by Rhodes University. The blood was processed by centrifugation (2500g for 5 minutes) followed by the removal of serum and buffy coat. The erythrocytes were then washed several times in RPMI 1640 medium lacking AlbumaxII to generate clean erythrocytes. Parasitemia was assessed by light microscopy with Giemsa-stained thin blood smears and the life cycles of the cultured parasites were regularly synchronized at the ring stage by treatment with 5% sorbitol as previously described (Lambros and Vanderberg, 1979; Biswas et al. 1979). The culture medium was regularly replaced along with erythrocytes on the basis of parasitemia.

4.3.2 Designing of PfHsp70-3 and PfHep1 constructs for transfection

The pARL-2/PfHsp70-3_GFP construct that was used in this study was a kind gift from Dr. James Njunge. The 302 amino acid coding region of PfHep1 (PF3D7_1420300), including the mitochondrial signal peptide, was inserted into the pARL-2-GFP vector.

Both forward (5'- **CTCGAGATGTTTCAAAAAATAGGAAGGA-3'**) and reverse (5'-**GGTACCTTTGTATGCGTTTAAAAGGTCAT**-3') primers were designed with *Kpn1* and *XhoI* restriction sites respectively (underlined). The primers were used to PCR amplify PfHep1 coding sequence from genomic DNA using the following PCR temperature parameters (initial denaturation, 94°C for 2 minutes; cycles of denaturation, annealing and elongation, 25 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 64°C for 1 minute 30 seconds respectively). Successful isolation and amplification of the PCR product was confirmed by agarose gel electrophoresis. The PCR product was recovered by gel purification using GeneJET Gel Extraction Kit (Thermo scientific, Lithuania) according to the manufacturer's instructions and both the PCR products and pARL-2-GFP vector were restricted with *Xho*I and *Kpn1* enzymes. Both digested PCR products and pARL-2-GFP were ligated (Appendix I) to create the pARL-2-PfHep1_GFP for transfection. Diagnostic restriction digests were conducted to confirm the integrity of generated plasmid DNA.

4.3.3 Transfection of malaria parasites

Malaria parasite transfection was achieved by a red blood cell pre-loading electroporation protocol. Briefly, the plasmid DNAs (pARL-2-PfHep1-GFP and pARL-2-PfHsp70-3_GFP) that were used for transfection was isolated and purified using the Qiagen plasmid maxi kit (Qiagen, Germany) according to the manufacturer's instructions (Appendix J). An aliquot of 100µg of the plasmid DNA was constituted according to (Wu et al. 1995) in 200µl of cytomix buffer(240 mM KCl, 0.30mM $CaCl_2$, 4mM EGTA, 10mM $MgCl_2$, 20mM K_2HPO_4, 25 mM HEPES, pH 7.6). 5 mL of cytomix and 300µl of fresh red blood cells were mixed in a 15mL tube followed by centrifugation (2000g, 3 minutes) and the supernatant was discarded. A sample of 200µl of the freshly washed red blood cells were mixed with 100µl 2× cytomix together with 100µl of plasmid prepared above in 0.2 cm electroporation cuvette (Bio-Rad, USA) and placed on ice for

5 minutes. The mixture was thoroughly mixed and electroporated twice with one minute interval on a GenePulser Xcell^{TM} (Bio-Rad, USA) set to a voltage of 310V and a capacitance of 950µF. Electroporated samples were carefully mixed with 5mL complete culture media and red blood cells infected with trophozoite/schizont-stage parasites enriched from a malaria culture. Enrichment of infected erythrocytes was achieved by percoll density centrifugation (Schlichtherle, 2000) that was done as follows: 15mL of infected red blood cell culture was centrifuged and up to 300 µl of the red blood cell pellet was carefully layered on top of 1mL 60% percoll in a sterile microfuge tube, followed by centrifugation (10,000g, 15minutes). A thin brown layer of red blood cells containing trophozoite and schizont infected cells on top of the percoll cushion was carefully removed followed by mixing with 15mL culture medium and centrifugation (2000g, 3 minutes). The pellet of enriched infected red blood cells were recovered by resuspension in culture medium and enrichment was confirmed by light microscopy.

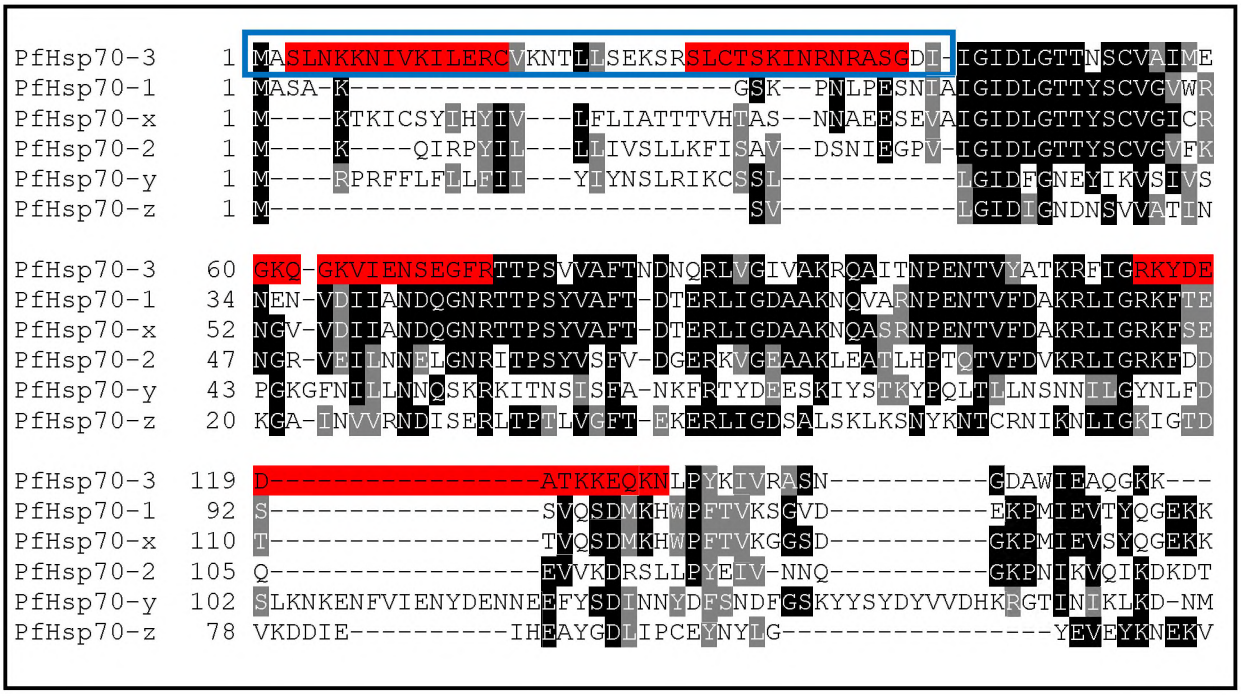
The mixture of transfected red blood cells and enriched infected red blood cells in culture medium was transferred to a 25 cm^2 culture flask, gassed with 5% CO_2/5% $O_2$and incubated at 37^{o}C overnight without shaking/rocking. The medium was replaced with 5mL fresh medium on the second day and on the third day 5mL of fresh medium and 150µl of fresh red blood cells were added and supplemented with2.5nM WR99210 (Sigma-Aldrich, Germany)for selection of positive transformants. Replacement of medium containing 2.5nM WR99210 was thereafter done twice a week along with addition of 50µl of fresh red blood cells once a week until parasites appeared, as detected by Giemsa-stained thin blood smears. Imaging of positively transfected parasites was carried out.

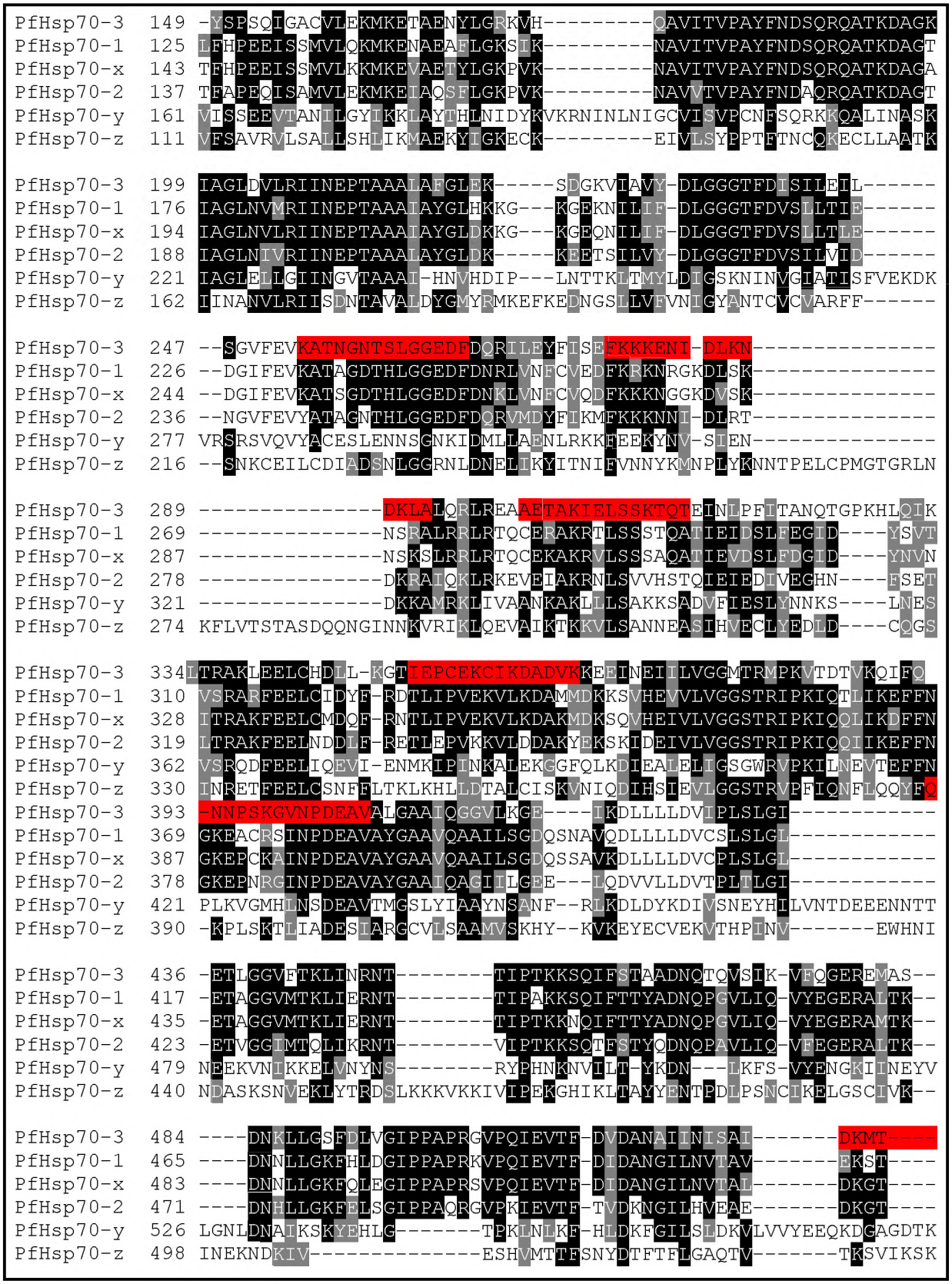
4.3.4 Live cell imaging of transfectants

Red blood cells containing transfected parasites were harvested from culture by centrifugation (12000g, 5 minutes) and washed once with pre-warmed incomplete media. The parasites were transferred into incomplete media containing 10µg/mL Hoechst 33258 (Sigma-Aldrich, Germany) for DNA /nuclei staining and 20nM MitoTracker red/ chloromethyl-X-rosamine (Molecular Probes, USA), a mitochondrion-selective fluorescent probe (Shindo et al. 2011). The

parasites were incubated briefly and centrifuged (12000rpm, 3 minutes) followed by imaging at room temperature (25°C) within 30 min of mounting under a coverslip on a glass slide, using a 100x oil-immersion objective. An Olympus BX60 epifluorescence microscope equipped with a DP72 camera and cellSens Entry 1.9 software (Olympus, Japan) was used to acquire images. Images for trophozoite ring and schizont stages parasites were captured. Live cell imaging was carried out with the kind assistance of Prof. Heinrich Hoppe at the Department of Biochemistry and Microbiology (Rhodes University, South Africa).

The amino acid sequence for PfHsp70-3 (PF3D7_1134000) was obtained from PlasmoDB v4.4 (Aurrecoechea et al. 2009) and analyzed using advanced antigen design algorithim GenScript's Optimum Antigen™ Design Program (GenScript, USA) for the identification of appropriate antigenic peptide sequences. The designing tool helps to establish and specify desired cross-reactivity, unexposed epitopes to be avoided, determination of the strength of antigenicity of chosen peptide, best conjugation and presentation options for desired assays and guaranteed immune response among others.




PfHsp70-3 149 -YSPSQIGACVLEKMKETAENYLGRKVH---------QAVITVPAYFNDSQRQATKDAGK
PfHsp70-1 125 LFHPEEISSMVLQKMKENAEAFLGKSIK---------NAVITVPAYFNDSQRQATKDAGT
PfHsp70-x 143 TFHPEEISSMVLKKMKEVAETYLGKPVK---------NAVITVPAYFNDSQRQATKDAGA
PfHsp70-2 137 TFAPEQISAMVLEKMKEIAQSFLGKPVK---------NAVVTVPAYFNDAQRQATKDAGT
PfHsp70-y 161 VISSEEVTANILGYIKKLAYTHLNIDYKVKRNINLNIGCVISVPCNFSQRKKQALINASK
PfHsp70-z 111 VFSAVRVLSALLSHLIKMAEKYIGKECK---------EIVLSYPPTFTNCQKECLLAATK
PfHsp70-3 199 IAGLDVLRIINEPTAAALAFGLEK-----SDGKVIAVY-DLGGGTFDISILEIL------
PfHsp70-1 176 IAGLNVMRIINEPTAAAIAYGLHKKG---KGEKNILIF-DLGGGTFDVSLLTIE------
PfHsp70-x 194 IAGLNVLRIINEPTAAAIAYGLDKKG---KGEQNILIF-DLGGGTFDVSLLTLE------
PfHsp70-2 188 IAGLNIVRIINEPTAAALAYGLDK-----KEETSILVY-DLGGGTFDVSILVID------
PfHsp70-y 221 IAGLELLGIINGVTAAAI-HNVHDIP---LNTTKLTMYLDIGSKNINVGIATISFVEKDK
PfHsp70-z 162 IINANVLRIISDNTAVALDYGMYRMKEFKEDNGSLLVFVNIGYANTCVCVARFF------
PfHsp70-3 247 --SGVFEVKATNGNTSLGGEDFDQRILEYFISEFKKKENI-DLKN---------------
PfHsp70-1 226 --DGIFEVKATAGDTHLGGEDFDNRLVNFCVEDFKRKNRGKDLSK---------------
PfHsp70-x 244 --DGIFEVKATSGDTHLGGEDFDNKLVNFCVQDFKKKNGGKDVSK---------------
PfHsp70-2 236 --NGVFEVYATAGNTHLGGEDFDQRVMDYFIKMFKKKNNI-DLRT---------------
PfHsp70-y 277 VRSRSVQVYACESLENNSGNKIDMLLAENLRKKFEEKYNV-SIEN---------------
PfHsp70-z 216 --SNKCEILCDIADSNLGGRNLDNELIKYITNIFVNNYKMNPLYKNNTPELCPMGTGRLN
PfHsp70-3 289 ---------------DKLALQRLREAAETAKIELSSKTQTEINLPFITANQTGPKHLQIK
PfHsp70-1 269 ---------------NSRALRRLRTQCERAKRTLSSSTQATIEIDSLFEGID----YSVT
PfHsp70-x 287 ---------------NSKSLRRLRTQCEKAKRVLSSSAQATIEVDSLFDGID----YNVN
PfHsp70-2 278 ---------------DKRAIQKLRKEVEIAKRNLSVVHSTQIEIEDIVEGHN----FSET
PfHsp70-y 321 ---------------DKKAMRKLIVAANKAKLLLSAKKSADVFIESLYNNKS----LNES
PfHsp70-z 274 KFLVTSTASDQQNGINNKVRIKLQEVAIKTKKVLSANNEASIHVECLYEDLD----CQGS
PfHsp70-3 334LTRAKLEELCHDLL-KGTIEPCEKCIKDADVKKEEINEIILVGGMTRMPKVTDTVKQIFQ
PfHsp70-1 310 VSRARFEELCIDYF-RDTLIPVEKVLKDAMMDKKSVHEVVLVGGSTRIPKIQTLIKEFFN
PfHsp70-x 328 ITRAKFEELCMDQF-RNTLIPVEKVLKDAKMDKSQVHEIVLVGGSTRIPKIQQLIKDFFN
PfHsp70-2 319 LTRAKFEELNDDLF-RETLEPVKKVLDDAKYEKSKIDEIVLVGGSTRIPKIQQIIKEFFN
PfHsp70-y 362 VSRQDFEELIQEVI-ENMKIPINKALEKGGFQLKDIEALELIGSGWRVPKILNEVTEFFN
PfHsp70-z 330 INRETFEELCSNFFLTKLKHLLDTALCISKVNIQDIHSIEVLGGSTRVPFIQNFLQQYFQ
PfHsp70-3 393 -NNPSKGVNPDEAVALGAAIQGGVLKGE----IKDLLLLDVIPLSLGI------------
PfHsp70-1 369 GKEACRSINPDEAVAYGAAVQAAILSGDQSNAVQDLLLLDVCSLSLGL------------
PfHsp70-x 387 GKEPCKAINPDEAVAYGAAVQAAILSGDQSSAVKDLLLLDVCPLSLGL------------
PfHsp70-2 378 GKEPNRGINPDEAVAYGAAIQAGIILGEE---LQDVVLLDVTPLTLGI------------
PfHsp70-y 421 PLKVGMHLNSDEAVTMGSLYIAAYNSANF--RLKDLDYKDIVSNEYHILVNTDEEENNTT
PfHsp70-z 390 -KPLSKTLIADESIARGCVLSAAMVSKHY--KVKEYECVEKVTHPINV-------EWHNI
PfHsp70-3 436 -ETLGGVFTKLINRNT--------TIPTKKSQIFSTAADNQTQVSIK-VFQGEREMAS--
PfHsp70-1 417 -ETAGGVMTKLIERNT--------TIPAKKSQIFTTYADNQPGVLIQ-VYEGERALTK--
PfHsp70-x 435 -ETAGGVMTKLIERNT--------TIPTKKNQIFTTYADNQPGVLIQ-VYEGERAMTK--
PfHsp70-2 423 -ETVGGIMTQLIKRNT--------VIPTKKSQTFSTYQDNQPAVLIQ-VFEGERALTK--
PfHsp70-y 479 NEEKVNIKKELVNYNS--------RYPHNKNVILT-YKDN---LKFS-VYENGKIINEYV
PfHsp70-z 440 NDASKSNVEKLYTRDSLKKKVKKIVIPEKGHIKLTAYYENTPDLPSNCIKELGSCIVK--
PfHsp70-3 484 ----DNKLLGSFDLVGIPPAPRGVPQIEVTF-DVDANAIINISAI-------DKMT----
PfHsp70-1 465 ----DNNLLGKFHLDGIPPAPRKVPQIEVTF-DIDANGILNVTAV-------EKST----
PfHsp70-x 483 ----DNNLLGKFQLEGIPPAPRSVPQIEVTF-DIDANGILNVTAL-------DKGT----
PfHsp70-2 471 ----DNHLLGKFELSGIPPAQRGVPKIEVTF-TVDKNGILHVEAE-------DKGT----
PfHsp70-y 526 LGNLDNAIKSKYEHLG-------TPKLNLKF-HLDKFGILSLDKVLVVYEEQKDGAGDTK
PfHsp70-z 498 INEKNDKIV--------------ESHVMTTFSNYDTFTFLGAQTV-------TKSVIKSK

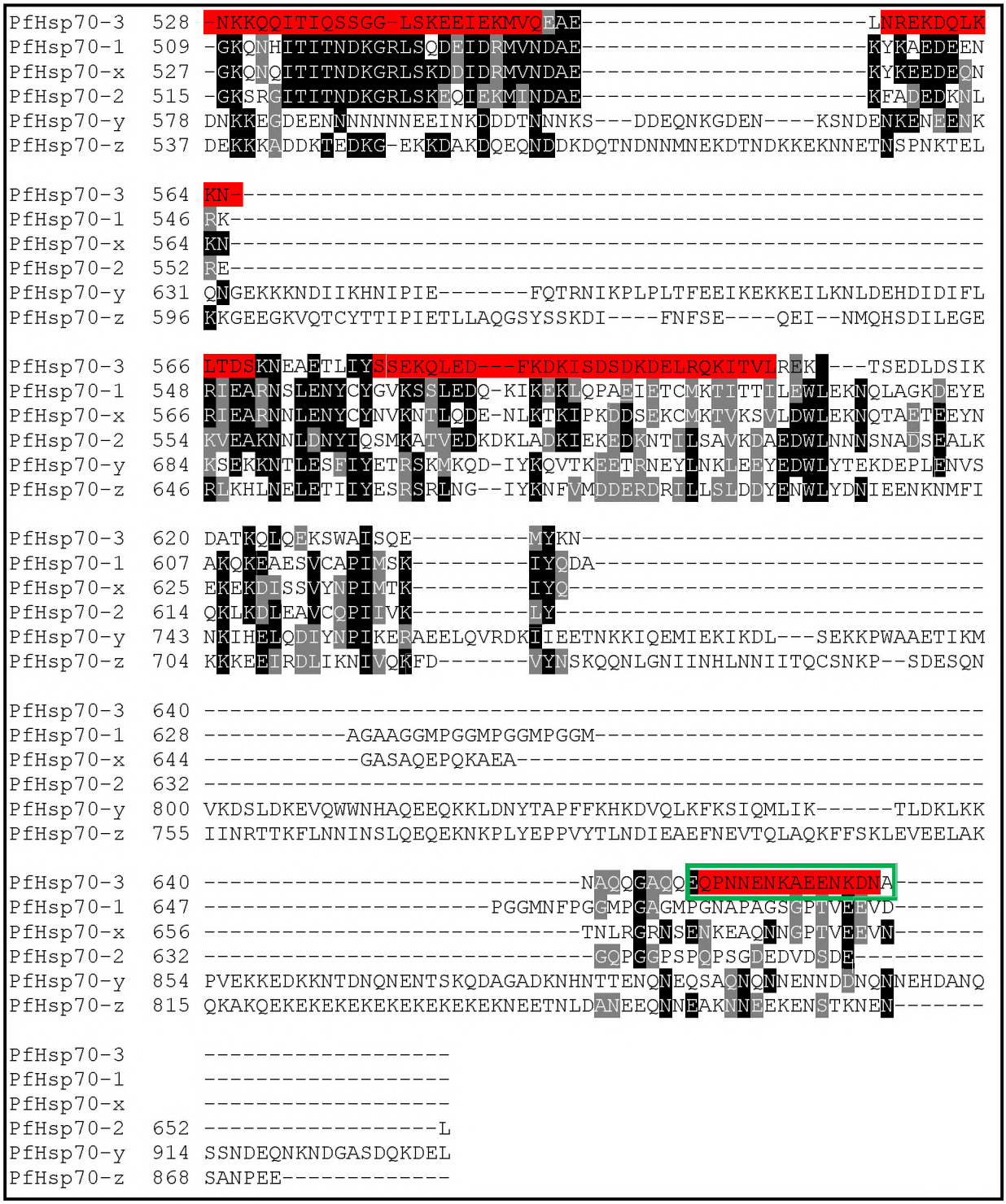


Figure 4.1 : Sequence alignments of *P. falciparum* Hsp70s showing antigenic determinant regions in PfHsp70-3:PfHsp70-3(mitochondrial) aligned with PfHsp70-1 (cytosolic), PfHsp70-2 (ER), PfHsp70-x (exported) and PfHsp70-y and PfHsp70-z using Clustal omega. Red highlight: antigenic determinant regions, blue box: mitochondodrial putative signal pepetide

sequence, green box: peptide used to raise specific antiserum against PfHsp70-3 in rabbit. The black shade represented 100% residue identity with dark gray representing similarity.

Moreover, the designing tool uses a built in tutorial for synthesis and solubility. Sixteen specific unique regions across the entire protein, comprising of 14 amino acids were identified as possible antigenic determinants (appendix R). To select a suitable peptide antigen for antibody development a number of aspects were duly considered that included antigenicity, hydrophilicity, hydrophobicity, surface probability, predicted transmembrane domains, homology, flexible region, helix region, sheet region, signal peptide and modification (Hofmann and Hadge, 1987; Hopp and Woods, 1981; Jameson and Wolf, 1988; Emini et al. 1985). Another important factor to consider was to ensure that the peptide sequence was not similar to any of the other 5 Hsp70 homologues to avoid cross reactivity and unspecificity. The chosen peptide sequence was used as a query in the blast search and the sequence alignment showed that the chosen peptide (Figure 4.2 in green box) present in the C-terminus of PfHsp70-3 is unique to PfHsp70-3. Peptides that were located within the ATPase domain of the protein were avoided due to high conservation of the domain residues among the Hsp70 proteins as compared to the highly variable C-terminal domain (Figure 4.2). Based on these factors, the peptide **CQPNNENKAEENKDN** was selected for antibody development in *Oryctolagus cuniculus* after performing local alignments against the *P. falciparum* proteome. Synthesis of the selected peptide, conjugation to KLH and rabbit immunization was performed by GenScript (Hong Kong), who provided the resultant rabbit antiserum and pre-immune serum.

4.3.5 Parasite lysate preparation and detection of GFP-PfHep1 and PfHsp70-3.

Parasite lysate preparation for the detection of GFP- tagged PfHep1 and PfHsp70-3 with anti-GFP antibodies and peptide raised antibodies respectively was done as previously described (Alleva and Kirk, 2001; Tonkin et al. 2004; Ansorgea et al. 1997). The method is based on the use of saponin, a plant secondary metabolite of glycosidic nature that has biological properties and the ability to lyse erythrocytes and parasite parasitophorous membranes, while leaving

parasite plasma membranes intact (Bruneton, 1995; Rao and Gurfinkel, 2000; Francis et al. 2002). Erythrocytes infected with parasites synchronized to the trophozoite stage and cultured to maximum growth were harvested through centrifugation (12000g, 3min at 4°C). The pellet was resuspended in 0.1% (w/v) saponin (Sigma-Aldrich, Germany)/ phosphate-buffered saline (PBS; 137mM NaCl, 2.7mM KCl, 10.3mM Na_2HPO_4, 1.8mM KH_2PO_4, pH 7.4) to lyse the erythrocytes. The lysis of erythrocyte membranes was allowed to take place for 8 minutes on ice followed by subsequent centrifugation (12000g, 3min at 4°C). The supernatant was discarded and the parasite pellet was washed two times with ice cold PBS to remove residues of hemoglobin and saponin. The washed parasite pellet was re-suspended in SDS-PAGE sample buffer and boiled for 10 minutes followed by centrifugation (12000g for 2 minutes) to remove the cell debris and obtain the soluble supernatant of the samples. Proteins were analyzed by 10% SDS-PAGE and western blotting onto a nitrocellulose membrane (Dassel, Germany) according to standard protocols given (Appendix F) and as described (Gershoni and Palade, 1982; De Blas and Cherwinski, 1983). Further processing of the western membranes was carried out as described in previous sections with 3% (w/v) non–fat powder milk in TBS-T used instead of 5% (w/v). A primary mouse anti-GFP monoclonal antibody (B-2, Santa Cruz Biotechnology) was used for the detection of GFP_PfHep1 (1:4000 dilutions) and the rabbit anti-PfHsp70-3 peptide antiserum (1:4000) for the detection of endogenous PfHsp70-3. HRP-conjugated goat anti-mouse (1:5000 dilution) (GE Healthcare, UK) and goat anti-rabbit (1:4000 dilution) (Cell signaling technology, USA) secondary antibodies were used. Rabbit anti-actin antibodies (Sigma-Aldrich, Germany) generated from rabbit were used as a loading control.

4.3.6 Determination of heat shock induction of PfHsp70-3

To determine the heat inducibility of PfHsp70-3, *P. falciparum* parasites (clone 3D7) were cultured as described in section (4.3.1) and synchronized at the ring stage. Once parasites had developed to trophozoites, the cultures were equally split into three flasks and heat shock was applied by incubating subcultures at 37°C (control), 41°C (heat shock) and 43°C (heat shock) for 2 hours. Parasite pellets collected from the three sub-cultures were prepared by saponin lysis as described earlier and subsequent production of PfHsp70-3 was analyzed by SDS-PAGE and

western blotting as described in the previous sections. Anti-PfHsp70-3 antibody and HRP-conjugated goat anti-rabbit secondary antibodies were used. Rabbit anti-actin antibody and uninfected erythrocytes were used as loading and negative controls respectively.

4.3.7 Analysis of intraerythrocytic time course expression of PfHsp70-3

Infected *P. falciparum* parasites (clone 3D7) were cultured as described in section (4.3.1) and synchronized at the ring stage with 5% sorbitol. The parasites were then split into six flasks and incubated at 37°C for time course expression analysis. Infected erythrocytes were harvested by centrifugation at eight hour intervals over the 48 hour intra-erythrocytic parasite developmental life cycle, parasite pellets obtained by saponin lysis were frozen at -80°C for further analysis. The expression of PfHsp70-3 was analyzed by SDS-PAGE using coomassie stain as described in the previous sections. Immunodetection was carried out on the western blots using Clarity™Western ECL blotting kit (Bio-Rad, USA) as per the manufacturer's instructions, and captured with a Chemidoc chemiluminescence imaging system (Bio-Rad, USA). Anti-PfHsp70-3 antibody and HRP-conjugated goat anti-rabbit secondary antibodies were used. Rabbit anti-actin antibody (1:4000 dilutions) and uninfected erythrocytes were used as loading and negative controls respectively

4.3.8 Indirect Immunofluorescence microscopy

Indirect immunofluorescence microscopy to determine the localization of PfHsp70-3 was carried out as previously described by Tonkin et al. (2004). Infected erythrocytes were cultured as described (section 4.3.1) and harvested at the trophozoite stage by centrifugation (2000g for 3 minutes). The pellet of infected erythrocytes was resuspended in RPMI (without Albumix) medium at room temperature. The mixture was added to the wells of poly-lysine coated chamber slides (Ibidi, Germany) followed by incubation at 37°C for 30 minutes to allow the red blood cells to settle on to the bottom of the slides. The unattached erythrocytes in the wells were gently

washed off with RPMI medium and the formation of an attached erythrocyte layer on the bottom of the wells was confirmed by microscopy. The attached erythrocytes were rinsed twice in PBS containing 0.05% saponin to lyse the cells. Immediately 3% paraformaldehyde and 0.2% glutaraldehyde in PBS was added and incubated for 10 minutes. After incubation, the cells were washed with PBS followed by the addition of 0.2% Triton X-100 contained in PBS and incubation for 2 minutes. 0.15M glycine was added to block free reactive aldehyde groups that could react with antibodies followed by rinsing with PBS after incubation for 10 minutes. Blocking buffer (1% BSA in PBS) was added to the fixed cells in the wells and incubated for 20 minutes. Anti-PfHsp70-3 (primary antibody), diluted 1:200 in blocking buffer, was added and allowed to bind for a minimum of 40 minutes Cells were washed three times using washing buffer (PBS, 0.1% BSA and 0.1% Tween 20) for 10 min each to remove excess primary antibody. FITC-conjugated goat anti-rabbit (secondary antibodies; Sigma-Aldrich) was added at 1:200 dilutions in blocking buffer and allowed to bind for 40 minutes in the dark. The wells were washed thrice by the wash buffer followed by the addition of 4,6-Diamidine-2-phenylindole dihydrochloride (DAPI) and incubated for 1 minute to bind to the DNA. The wells were rinsed with water, mounted under a coverslip in FluorPreserve (Calbiochem) and viewed with an Olympus BX60 epifluorescence microscope using 100 × oil-immersion objectives.

4.4. Results

4.4.1. Specificity of anti-PfHsp70-3 antibody and detection of PfHsp70-3 in *P. falciparum.*

The pARL-2-GFP/PfHsp70-3 construct was used several times for transfection of parasites but these experiments failed to yield a viable population of transfected parasites and therefore the next approach was to produce antibodies against PfHsp70-3 for the determination of localization. The aim was to determine if the antibody could detect both parasite-derived as well as His-tagged PfHsp70-3 purified from *E. coli* cells. The specificity of the PfHsp70-3 antibody was determined by the use of parasite lysate prepared from the trophozoite stage parasites (Figure

4.2, lane 1). A protein band of approximately 70 kDa was detected at almost the same size of purified His-tagged PfHsp70-3 which was used as a positive control (Figure 4.2, lane 3). There was no band detected in the uninfected erythrocytes that was used as a negative control (Figure 4.2, lane 2)

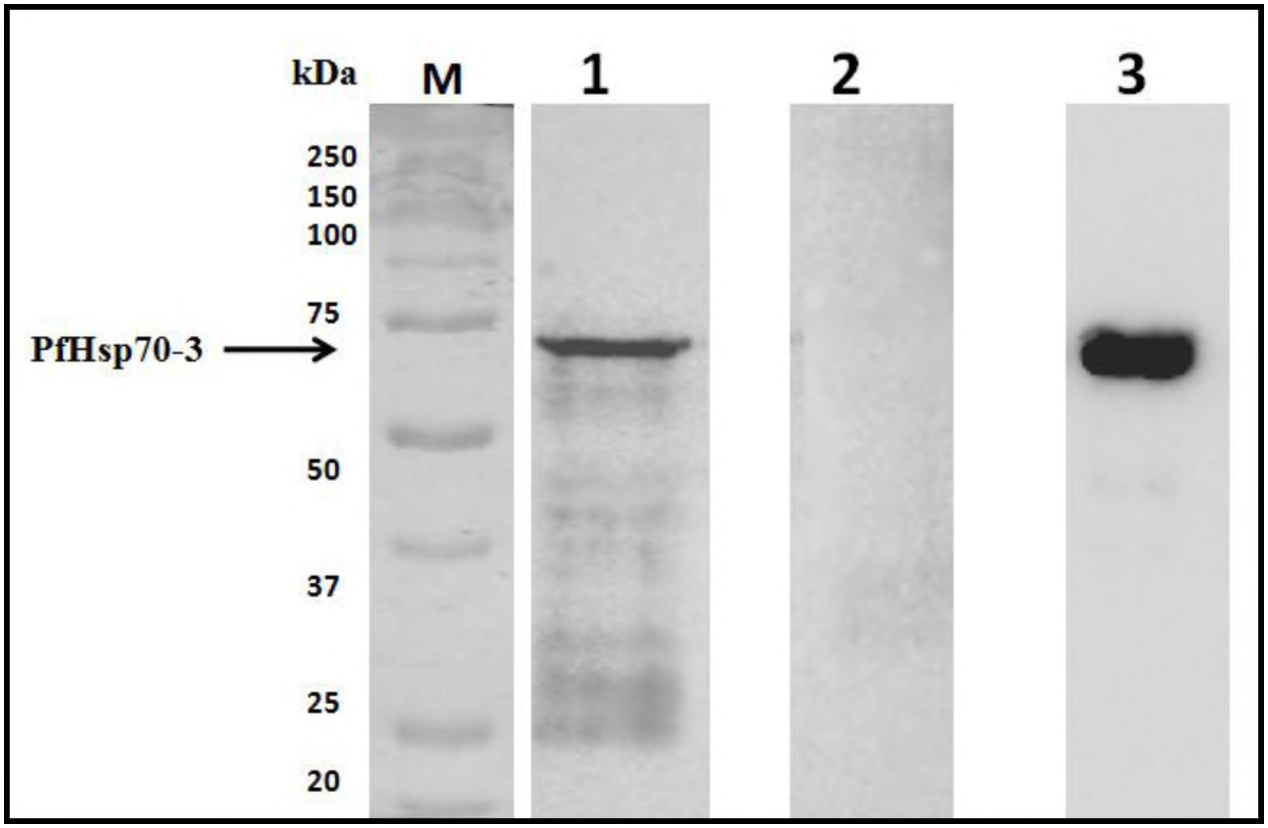


Figure 4.2 : Specificity of anti-PfHsp70-3 peptide directed antibody and detection of PfHsp70-3 in parasite lysate: Western blot analysis of PfHsp70-3 using anti-PfHsp70-3 peptide directed antibody. M, molecular marker, Lane 1, detection of PfHsp70-3 in trophozoite parasite lysate; lane 2, uninfected erythrocytes, lane 3, purified His-tagged PfHsp70-3.

4.4.2 The expression of PfHsp70-3 is induced by heat shock treatment

Protein detection confirmed that PfHsp70-3 was being expressed in the intra-erythrocytic stages of parasite development and this facilitated an analysis of its heat inducibility. A number of

P. falciparum Hsps have been demonstrated to be up-regulated during heat shock. PfHsp70-3 was analyzed to determine whether it would be up-regulated in response to heat shock as an indication of its role in cytoprotection during infectivity.

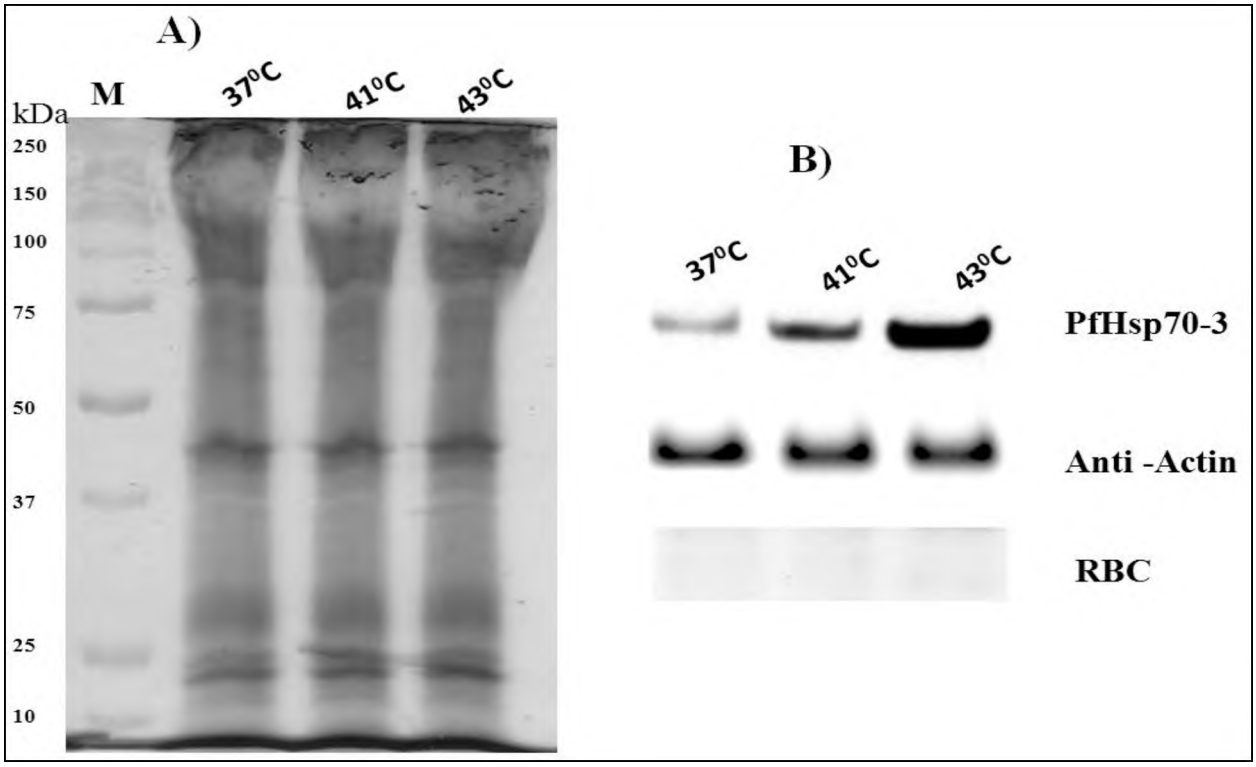


Figure 4.3 : PfHsp70-3 is up-regulated following heat-shock: (**A**) SDS-PAGE (10%) protein profiles of parasites incubated at 37°C, 41°C, and 43°C for 2 hours. (**B**) Western analysis: detection of PfHsp70-3 and actin from saponin-lysed parasite lysates obtained from 3D7 *P. falciparum*-infected erythrocytes using anti-PfHsp70-3 and anti-actin antibodies respectively. Equal amount of infected erythrocytes (RBC) were loaded per lane and three independent experiments were carried out and similar results were obtained.

Parasites in the trophozoite stage were heat shocked at 41°C and 43°C, temperatures higher than the normal human body temperature (37°C). Approximately equal numbers of infected erythrocytes were analyzed using SDS-PAGE (Figure 4.3 A) and western analysis (Figure 4.3 B). An equal amount of uninfected erythrocytes was loaded as a negative control and detection of ubiquitous actin cytoskeletal protein present on cell membranes was used as a loading control (Figure. 4.3 B). PfHsp70-3 was up-regulated after heat treatment at 41°C and 43°C relative to the loading control (Figure 4.3 B).

4.4. 3 Maximum expression of PfHsp70-3 occur during the trophozoite stage

An expression profile of PfHsp70-3 was analyzed over 48 hours of intra-erythrocytic development of *P. falciparum* to determine when the maximum expression of PfHsp70-3 occurred during developmental stages.

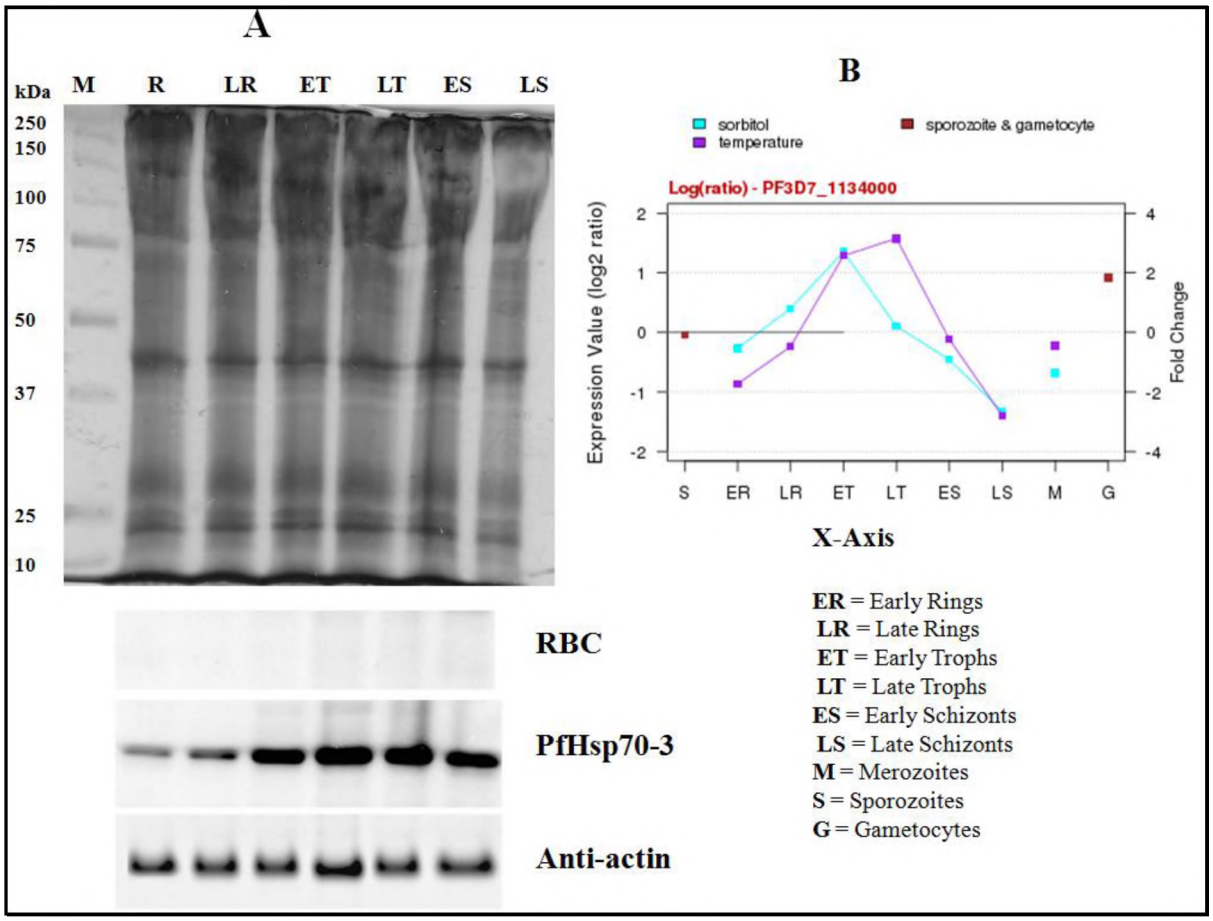


Figure 4.4 : Maximum expression of PfHsp70-3 occurs at trophozoite stage in intraerythrocytic cycle of parasite development: A) SDS-PAGE (10%) stained with coomassie indicating PfHsp70-3 protein expression profile at different time points with corresponding western blot analysis (WB) probed for PfHsp70-3 and actin proteins using respective antibodies. Non-infected erythrocyte extract (RBC) was loaded as a negative control. (B) Messenger RNA expression profile of PfHsp70-3 during different phases of development of the parasite as adapted from PlasmoDB version 4.4 (Aurrecoechea et al. 2009). Equal numbers of infected erythrocytes were loaded per lane and three independent experiments were carried out and similar results were obtained.

The parasite lysates were analyzed at eight hour intervals following sorbitol synchronization at ring stage. The SDS-PAGE and western analyses of equal numbers of infected erythrocytes showed different protein expression profiles at each developmental stage of the parasite (Figure 4.4). Actin was used as a loading control to confirm that loading was equivalent in each lane. The results showed that the protein was being expressed at all stages of *P. falciparum* development. There was least expression of the protein at the ring stage and the expression increased gradually with maximum expression occurring at early and late trophozoite stage (Figure 4.4A). The observed expression profile of PfHsp70-3 appeared to correlate with mRNA expression profiles of the protein as earlier described and documented in PlasmoDB (Aurrecoechea et al. 2009) (Figure 4.4 B).

4.4.4 PfHsp70-3 localizes to the *P. falciparum* mitochondrion

Attempts to localize PfHsp70-3 by transfecting parasites with a PfHsp70-3-GFP fusion construct and analyzing them by live cell microscopy failed. Parasites would grow in the transfected cultures, but failed to multiply further and displayed abnormal morphologies (data not shown). This might be indicative that over-expression of PfHsp70-3 is detrimental to parasite viability. Consequently, localization of endogenous PfHsp70-3 using the antibodies was attempted. The localization of PfHsp70-3 was determined using indirect immunofluorescence microscopy of trophozoite and early schizont-infected erythrocytes immobilized on poly-lysine coated slides. The erythrocytes were briefly lysed with saponin to improve antibody access.

Polyclonal antibodies that were raised in rabbit against a C-terminal peptide "CQPNNENKAEENKDN" of PfHsp70-3 protein coding sequence were used as primary antibodies to investigate the localization of PfHsp70-3. FITC-conjugated goat anti-rabbit secondary antibodies were used. After immobilization and saponin lysis, paraformaldehyde/ glutaraldehyde fixation was used to fix the parasites before antibody binding. Using the secondary green fluorescence signal, an elongated structure was observed at the periphery of the parasite believed to be the parasite mitochondrion (Figure4.5).

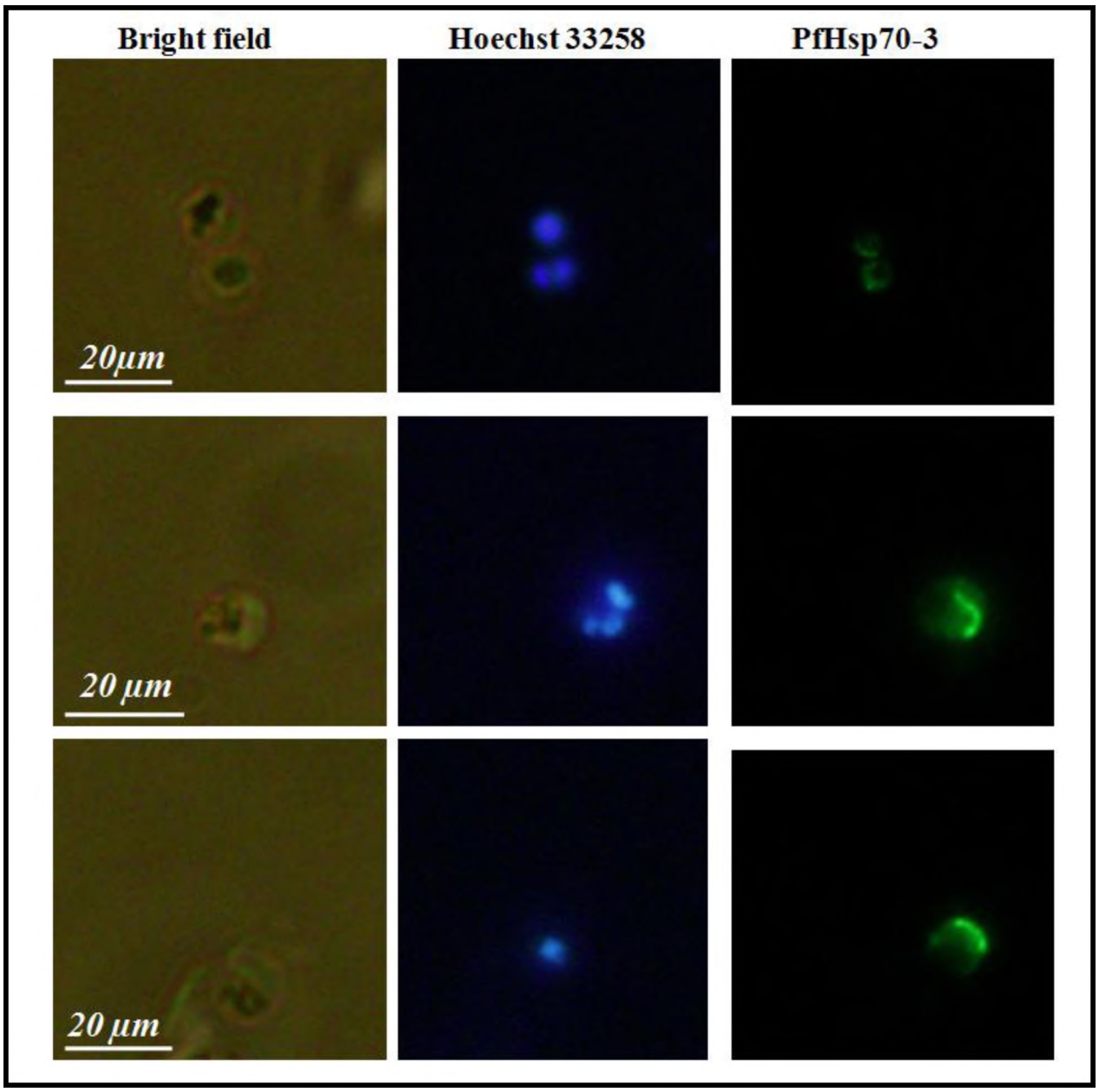


Figure 4.5 : PfHsp70-3 localizes to the mitochondrion in *P. falciparum*: Sample of images obtained from localization study with rabbit anti-PfHsp70-3 peptide antibody on paraformaldehyde/glutaraldehyde-fixed *P. falciparum* trophozoites. Rabbit anti-peptide primary and anti-rabbit FITC-conjugated secondary antibodies was used to detect PfHsp70-3 protein. The parasite nuclei were stained with Hoechst 33258 (in blue) and the bright field (BF) showed the bright field images of the fixed parasites. The experiment was repeated at least three times and representative images are shown here.

These preliminary findings indicated that PfHsp70-3 could indeed localize to the parasite mitochondrial organelle. However, since there was no any signal spotted in the cytoplasm, it could indicate that unlike human mitochondrial Hsp70 that is found in the mitochondria and

cytoplasm, PfHsp70-3 is only located in the parasite mitochondria. This observation would suggest the unique role of PfHsp70-3 compared to its orthologues. Due to the delicate nature of the method of parasite fixation and lysis, most of the images observed showed broken and discontinuous fluorescence signal (data not shown). A fluorescence signal was absent in the negative controls where primary or secondary antibody only was used (data not shown).

4.4.5 Construction of pARL-2-GFP-PfHep1

The aim was to insert the coding region of PfHep1 into the pARL-2-GFP construct to determine the localization of PfHep1 in the parasite.

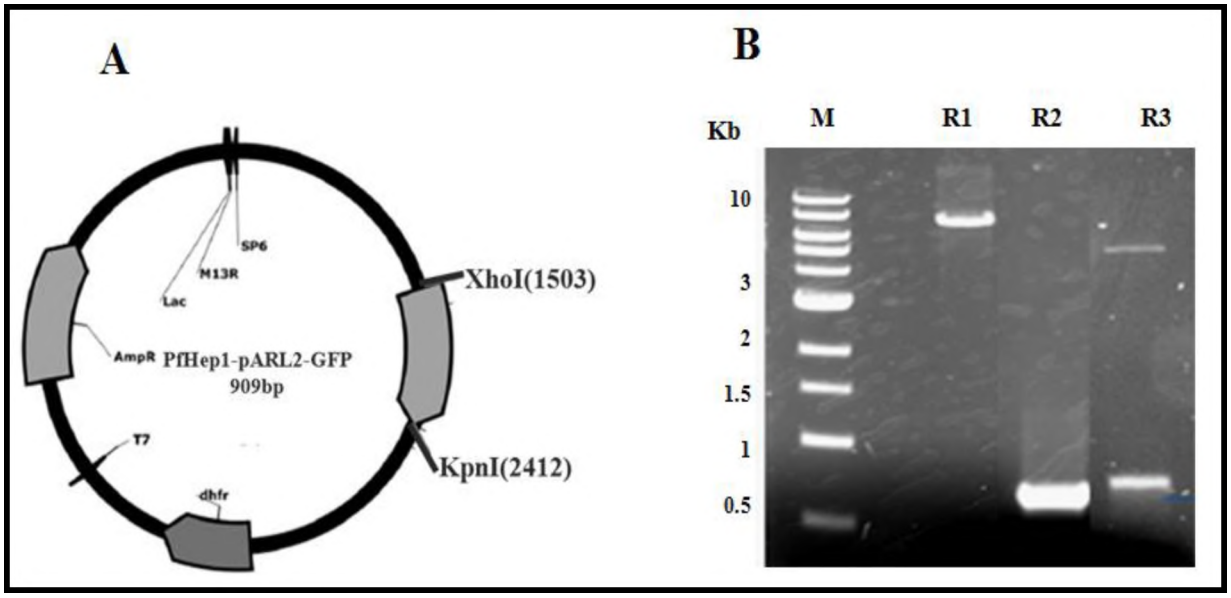


Figure 4.6 : Diagnostic analysis of pARL2-GFP-PfHep1 construct: (A) Constructed plasmid-protein map designed using the PlasmaDNA software (Angers-Loustau et al. 2007). The plasmid confers ampicillin resistance when transformed into *E. coli* as indicated (Amp^R; β-lactamase coding sequence) and confers WR99210 resistance (dhfr; dihydrofolate reductase coding sequence) when transfected into *P. falciparum* parasites. (B) Endonuclease restriction digest of pARL-2-GFP-PfHep1: lane M, DNA molecular makers; lane R1, single digest with *Xho*I , lane R2, PfHep1 PCR product, lane R3, double restriction digest (*Xho*I-*Kpn*I) of PfHep1 -pARL2-GFP ligated products using *Xho*I and *Kpn*I.

The coding sequence of PfHep1 (302 aa) was successfully PCR amplified from the genome of *P. falciparum* 3D7 strain (Figure 4.6 B) and inserted into the pARL2-GFP vector using *Xho*I and *Kpn*1 restriction sites (Figure 4.6 B, lane R2). Restriction digest analysis of the construct using *Xho*I and *Kpn*1 enzymes confirmed the presence of PfHep1 insert (Figure 4.6 R2 and R3).

4.4.6 PfHep1 localized to the parasite mitochondrion

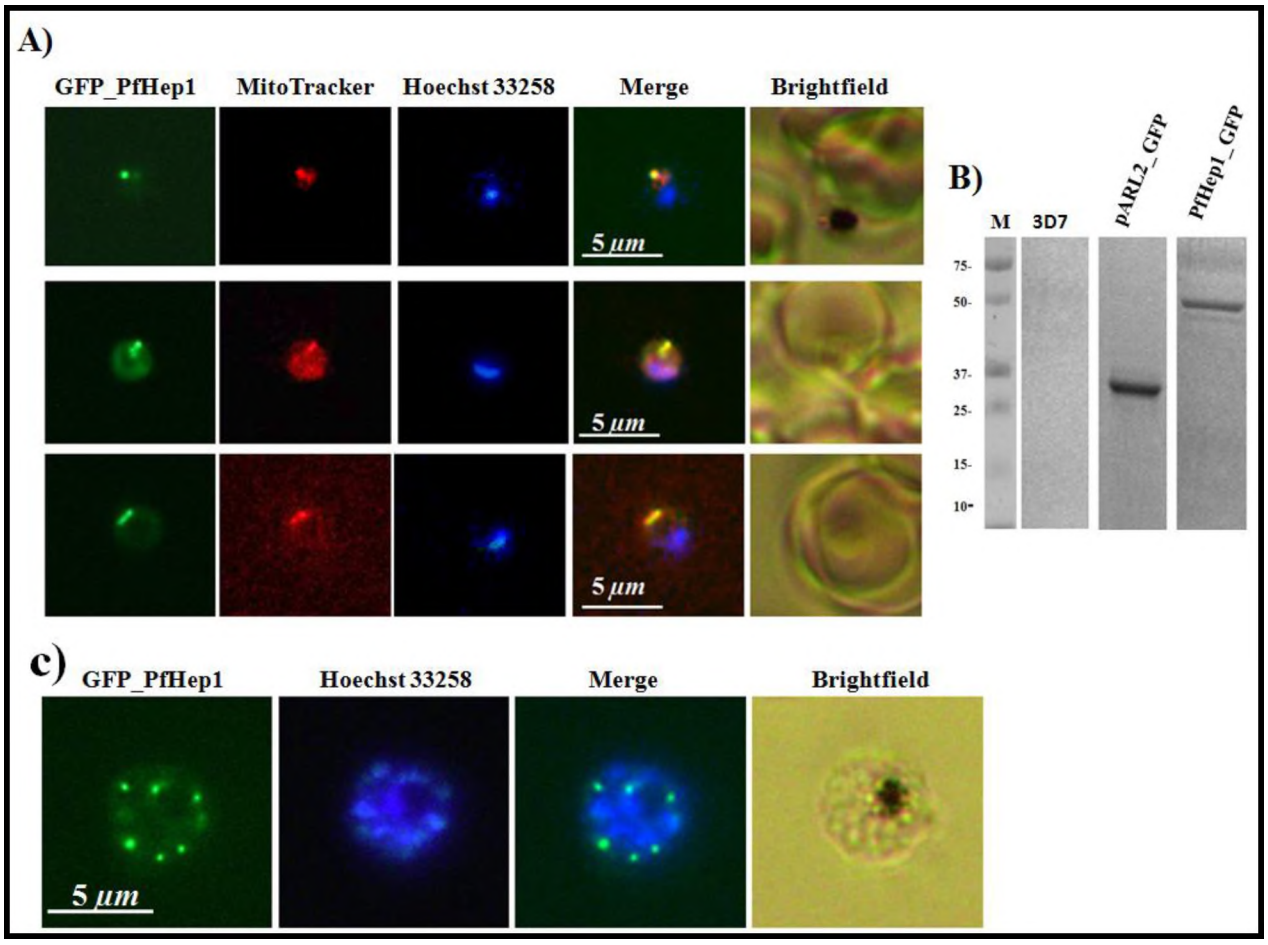


Figure 4.7 : PfHep1 showing mitochondrial localization in the parasites: (A) Localization of the GFP signal through live cell imaging of pARL2-GFP_PfHep1 transfectants in ring and trophozoite stages stained with Hoechst (blue) to visualize the nucleus, MitoTracker (red) to visualize the mitochondrion and Merge (combining GFP with Hoechst and MitoTracker. (B) Western analysis of the PfHep1-GFP transfectants lysate. M, molecular mass marker; 3D7; lysate of *P. falciparum*3D7 used as a negative control; pARL2-GFP; lysate of control parasites transfected with pARL2_GFP plasmid lacking the PfHep1 insert, PfHep1-GFP; lysate of parasites transfected with the pARL-2-PfHep1-GFP construct. (C) Localization of the GFP signal by live cell imaging of pARL2-GFP_Hep1 transfectants in the schizont stage of the parasite.

Hep1 proteins are known to be specifically involved in the aggregation suppression of mitochondrial Hsp70s which indicates the likely presence of PfHep1 in the parasite mitochondrial organelle. The images captured from live transfected parasites of late ring and trophozoite expressing PfHep1-GFP showed that the protein localized in the mitochondrion of the parasite (Figure 4.7A). The images showed the concentration of GFP signal in an elongated organelle consistent with mitochondrial localization (Figure 4.7A).

Live images of schizont stage parasites showed the PfHep1-GFP associated with positive multiple structures of individual forming daughter merozoites. The MitoTracker did not stain mitochondria in schizonts at this stage (Figure. 4.7C). The western blot analysis of the parasite lysate using anti-mouse GFP antibodies detected PfHep1-GFP at the expected size of approximately 63 kDa with PfHep1 of 36 kDa of and GFP protein of 27 kDa (Figure 4.7B). Untransfected 3D7 parasites and those transfected with pARL-2_GFP were used as controls (Figure.4.7C). The merging of the mitochondrial red staining dye (MitoTracker) with green fluorescence confirmed that the elongated structure was indeed the mitochondrion (Figure 4.7).

4.5. Discussion

In silico analysis revealed that both PfHsp70-3 and PfHep1 proteins contain mitochondrial signal peptide sequences which indicate that they localize to the parasite mitochondrion. However, this had not been experimentally validated and this study examined the cellular localization of PfHsp70-3 and its escort protein PfHep1. The late ring and trophozoite stage parasites showed a concentration of green fluorescence from GFP-tagged PfHep1 in transfected parasites within an elongated structure just outside the nucleus that was consistent with mitochondrial localization. The merging of the mitochondrial red staining dye (mitoTracker) with green fluorescence confirmed that the elongated structure was indeed the mitochondrion. Western analysis of the transfected parasites indicated that the size of the expressed protein was that of full-length GFP-tagged PfHep1. Observation of the schizont stage parasite showed a distribution of the GFP signal around the parasite showing six punctate structures that did not stain with mitochondrial

staining dye (mitoTracker). The distribution of the GFP signal was believed to be associated with individual forming merozoites that would soon be released from the erythrocyte to initiate the next phase of parasite developmental cycle by invading new erythrocytes. The presence of the dispersed green signal could represent the distribution of mitochondria into newly formed parasites but since, at this particular stage the parasite mitochondrial electron transport chain (mtETC) is inhibited and the mitochondrial membrane potential is collapsed (Painter et al. 2010; Pasini et al. 2013), the mitoTracker could not stain the young parasite mitochondria.

Indirect immunofluorescence microscopy studies on the localization of PfHsp70-3 indicated that the protein could be localized to the parasite mitochondrion. A fluorescence signal was observed around an elongated structure at the exterior parts of the parasite nucleus that was consistent with mitochondrial morphology. However, since both the parasite mitochondrion and the apicoplast are found lying side by side at this stage of development (Siregar et al. 2015), differential staining using markers for the two organelles would be required in order to be conclusive on the exact localization of PfHsp70-3. An alternative approach that could have been used is the isolation of the mitochondrial fraction from the parasites and subsequent use of the antibodies against PfHsp70-3 to confirm localization. The use of PfCytochrome C as a mitochondrial marker and PfUROD as an apicoplast marker were used to determine the localization of PfHsp60 (Priya et al. 2015) and such as approach could be used for PfHsp70-3 localization studies.

Transcription analysis of PfHsp70-3 has revealed the highest PfHsp70-3 mRNA expression levels during the late stages of the *P. falciparum* life cycle, with maximum expression occurring during the trophozoite stages of development (Aurrecoechea et al. 2009). This study investigated protein expression of PfHsp70-3 to determine whether it correlated with the reported mRNA expression pattern. The analysis of parasite lysates at different stages of parasite development using anti-PfHsp70-3 antibodies revealed PfHsp70-3 proteomic expressions that correlated with the transcriptomic profile. The parasite culture growth was sorbitol synchronized at the ring stage and the samples were collected for 48 hours in eight hour interval. PfHsp70-3 protein expression

progressively increased from the ring stage of the asexual development and attained maximum levels during the late trophozoite stage.

P. falciparum parasites encounter stressful environmental changes during their establishment in the human host. In order for the parasite to adapt to these conditions, they use a repertoire of molecular chaperones whose expression and activities are often increased during stressful conditions to counteract the effect of stress (Neckers and Tatu, 2007). Indeed temperature variations characterize *P. falciparum* life cycle raging from the low temperature in the vector, anopheles mosquito at 25^{o}C, to 37^{o}C in healthy human hosts and 41^{o}C in patients suffering from malaria infection during febrile episodes. Heat shock treatment of *P. falciparum* parasites at 41^{o}C and 43^{o}C for two hours, visibly increased the expression of PfHsp70-3 compared to the expressions at 37^{o}C. Increased expression of PfHsp70-3 due to heat induction could imply the involvement of the protein in the heat-stress response mechanism of the parasite that is aimed at cushioning the parasite proteins against stressful conditions to prevent aggregation and inactivity. In conclusion, this study observed that both PfHsp70-3 and PfHep1 localize in the mitochondria of *P. falciparum*. PfHsp70-3 protein is expressed during all parasite asexual developmental stages with maximum expression occurring at trophozoite stages. The expression pattern of PfHsp70-3 at all infective stages could provide further evidence of the protein's involvement in malaria disease development.

CHAPTER FIVE

Conclusion and future perspective

The fight against malaria requires diverse approaches, which calls for a better understanding of the biology of the causative agent *P. falciparum* (Greenwood et al. 2008). The parasite mitochondrion is essential for viability making it a viable drug target. Furthermore, it is becoming increasingly evident that heat shock proteins occupy a central role in the development of malarial pathogenesis. For this reason Hsps are considered as possible antimalarial chemotherapeutic targets (Przyborski et al. 2015). Plasmodium species possess mitochondrial DNA that encodes for only three proteins, implying that all other mitochondrial proteins have to be encoded in the nucleus, synthesized in the cytosol on the ribosome and transported into the mitochondrial matrix (Vaidya and Mather, 2009).Mitochondrial Hsp70s act as motors that provide the driving force that pull mitochondrial bound pre-proteins into the mitochondrial matrix and promote their proper folding hence ensuring mitochondrial homeostasis (Ran et al. 2000; Bohnert et al. 2007; Custer et al. 2012). PfHsp70-3 (PF3D7_1134000) is one of the six known Hsp70s of *P.falciparum* predicted to reside in the mitochondria (Shonhai et al. 2007; Njunge et al. 2013). The parasite mitochondrial PfHsp70-3 could play a significant role in the translocation and proper folding of mitochondrial bound proteins and hence the biogenesis of the organelle. However, PfHsp70-3 has not been biochemically characterized, and determining its localization is also essential. Indeed, the location of a particular protein molecule can help in predicting its likely partners and metabolic pathways (Rost et al. 2003). This study involved characterization of PfHsp70-3 and included an investigation of PfHep1 (PF3D7_1420300) and Pfj1 (PF3D7_0409400) proteins as possible PfHsp70-3 co-chaperones.

Structural and functional characterization of mtHsp70 proteins has been hampered due to difficulties experienced in heterologous production of the proteins since they are often insoluble. A number of characterized mitochondrial Hsp70 proteins have shown the tendency to aggregate and reliance on Hep1 proteins for structural and functional activities. Hep1 is a small mitochondrial protein that was first described in humans and yeast (Sichting et al. 2005; Sanjuán

Szklarz et al. 2005) where it was shown to promote correct folding and abrogation of aggregation of mtHsp70. Hep proteins are indispensible for the functions of mtHsp70.

Both PfHsp70-3 and PfHep1 showed high levels of conservation of the domains and residues implicated in the functional properties of their orthologues. When the ATPase domain of mtHsp70 is attached to the linker domain, specific residues in the ATPase domain mediate interdomain communication leading to the aggregation of the protein. Sequence analysis indicated that PfHsp70-3 was likely to possess properties typical to mtHsp70s and require the interaction of PfHep1for structural stability and function. PfHep1 was larger and shared no similarity with its orthologues outside the zinc finger domain (zf-DNL). The zinc finger domain has been implicated for functional activities of Hep1 proteins. Furthermore, the residues that determine Hep1 protein structural and functional activities were found to be well conserved in PfHep1, Therefore PfHsp70-3 would likely possess typical properties of mtHsp70s and would rely on PfHep1 for functional activities.

The *in vitro* analysis revealed that similar to many characterized mtHsp70s, PfHsp70-3 was indeed insoluble when heterologously expressed in *E. coli* cells. This study showed for the first time that PfHep1 was required for maintaining the solubility and thereby the activity of PfHsp70-3. Various approaches that could solubilise PfHsp70-3 were attempted but the successfully solubilised and purified PfHsp70-3 displayed no functional activities. Biologically active PfHsp70-3 could only be produced through co-expression with PfHep1, an indication that the latter facilitates the proper folding of PfHsp70-3. However, since some good amount of inactive PfHsp70-3 was produced through various solubilization methods, some future work should involve doping of the inactive protein and use of pure PfHep1 to try and restore the activities of PfHsp70-3. This will further shade more light on the level of PfHsp70-3 dependability on PfHep1; that is to see whether PfHsp70-3 depends on PfHep1 for solubility alone or for functional activities as well. The insolubility of PfHsp70-3 and its reliance on PfHep1 for solubility and activity pointed further to the fact that PfHsp70-3 could indeed be a typical mitochondrial Hsp70 and PfHep1 could be an orthologue of Hep1 in *P. falciparum*. Indeed, sequence analysis of the zinc binding domain of PfHep1 was observed to contain many of the conserved amino acid residues implicated in the interactions of Hep1 and mtHsp70 proteins.

PfHsp70-3 existed as a monomer, an oligomeric state in which Hsp70s exist when bound to nucleotides (Palleros et al. 1992). Hep1 protein bind to their cognate mtHsp70s when they are nucleotide free and are released upon nucleotide binding (Sanjuán Szklarz et. al. 2005; Blamowska et al. 2010; Fraga et al. 2013). The interaction of PfHep1 with PfHsp70-3 made the latter soluble and influenced its oligomeric state that led to functional activities.

PfHep1 suppressed the thermal aggregation of PfHsp70-3. However, PfHep1 did not prevent the aggregation of other aggregation-prone proteins, such as MDH and citrate synthase. Furthermore, PfHep1 did not enhance the aggregation suppression activities of PfHsp70-3.This specific interaction of PfHep1 and PfHsp70-3 would be an attractive druggable avenue. Since the function of PfHsp70-3 proteins depends on its proper folding that relies on PfHep1, inhibition of the interaction of the two proteins offers a promising drug testing prospects. Furthermore, PfHep1sequncce analysis revealed lowest percentage identity against its orthologues which is likely to represent adequate structural variations that would facilitate selective inhibition with minimum effects on its human counterpart, hHep1. Abrogating the specific partnership between PfHep1 and PfHsp70-3 could indeed be a target for new antimalarial drugs and this remains to be explored. Future work should involve testing of compounds such as malonganenone-A, 15-deoxy-spergualin and other antimalarials that have been shown to inhibit the activities of some PfHsp70s (Cockburn et al. 2011; Ramya et al. 2007). It is worth noting that the PfHsp70-3 that was used in subsequent biochemical procedures in this study was co-purified with PfHep1and separated through gel filtration. However, it could be necessary to have more verifiable methods of determining the efficiency of gel filtration in separation of the two proteins. One could consider the use of PfHep1 antibodies to determine whether they could detect PfHep1 in gel filtrated PfHsp70-3. Besides, an additional gel filtration step would be required to be performed on both PfHep1 and FfJ1-1 that were purified through nickel affinity chromatography to ensure that no contaminants produced in the proteins.

The removal of zinc ions from PfHep1 using the chelating compound EDTA abrogated its functions against PfHsp70-3. Furthermore, EDTA-treated PfHep1 was unstable and aggregated at 50°C, whilst untreated PfHep1 remained stable, an indication of disruption of its structure due

to the removal of the zinc ions. However, the supposed structural changes caused by the removal of Zn^{2+} could not be verified in this study and the future work should include structural analysis such as use of circular dichroism (CD) spectroscope to determine the secondary structure and folding properties of Zn^{2+} depleted PfHep1. A comparison between native and Zn^{2+} depleted PfHep1 proteins will shade more light on the changes that might have occurred after removal of the zinc ions in relation to the functions.

Pfj1 could not be purified due to formation of degradation products upon expression in *E.coli*. However, Pfj1-J, which included the J-domain, GF region and zinc finger domain was produced and used for preliminary interaction studies with PfHsp70-3.The toxicity of PfJ1 could probably contribute to its degradation during expression in *E. coli* cells. Indeed, the protein is very unique and large in size compared to other J- protein homologues and does not have an equivalent homologue in the *E.coli* proteomic system. Pfj1 could be degraded by protease enzymes upon expression (Gottesman et al. 1997) that led to degradation products observed. The protein could also be degraded due to inherent instabilities. It is likely that the long C-terminus of Pfj1 played a role in the instability of this protein and consequently affected its *in vitro* production. The use of low copy number plasmids such as pSC101 with stringent controlled replication would be considered in future production of Pfj1. Such plasmids offer advantages especially in those genes whose expression products produce deleterious effects to the host cell (Stoker et al. 1982; Wang and Kushner, 1991). Since the protein appears to have distant properties from any of the proteins in the bacterial genome, the use of alternative expression systems of eukaryotic origin such as baculovirus or insect cell lines, yeast and mammalian cells could be considered. Finally, the use of protease free strains of *E.coli* could also be considered to prevent the protein degradations after expression.

Unlike PfHep1, Pfj1-J stimulated the ATPase and refoldase activities of PfHsp70-3. Taken together, the findings in this study suggest that PfHep1 does not play a co-chaperone role, but is rather a specific co-factor that prevents PfHsp70-3 self-aggregation and maintain it in an active state. Pfj1 is a potential co-chaperone of PfHsp70-3 but its localization needs to be determined. Studies done on human and *L. braziliensis* Hep1 orthologues have shown that these proteins

display features of a J-protein by stimulating the ATPase activities of their respective mtHsp70 proteins (Zhai et al. 2008; Dores-Silva et al. 2016).Majority of known co-chaperones mostly from the J-protein family, possess the HPD motif that is implicated for functions such as stimulation of ATPase activities (Cheetham and Caplan, 1998). Since HPD motif is absent from Hep1 proteins, it could mean an automatic disqualification of their co-chaperone roles especially in the aspect of stimulation ATPase activities. Human Hep1 unique extended C-terminal sequence has been implicated for its ability to stimulate the ATPase activities (Vu et al. 2012). Indeed, the sequences alignment and analysis showed that the human Hep1 orthologue possesses an extended C-terminal that could account for different biochemical properties from those observed in PfHep1. Since the extended C-terminal residues in hHep1 are equally missing in PfHep1, as part of the approach to investigating the mechanism of hHep1's ability of stimulating ATPase activities, future work should consider a construction of PfHep1 chimera containing the extended residues from hHep1 C-terminal to find out whether the exchanged residues could confer co-chaperone properties to PfHep1. However, since the C-terminal residues are also missing from LbHep1 which has been reported to stimulate LbHsp70 ATPase activities, an investigation of specific residue composition and functions among these proteins should be prioritized too.

Since the sequence identity between LbmtHsp70 and mortalin is a little bit lower than that between PfHsp70-3 and mortalin, which is at 61% and 62% (Dores-Silva et al. 2016) respectively, one could expect the interaction of PfHep1 and PfHsp70-3 to be similar to that of their homologues in human at least in ATPase activities. It is possible that a more sensitive ATPase assay could reveal that PfHep1 does influence the ATPase activity of PfHsp70-3 and therefore future work should include a repeat of the assay using sub-stoichiometric amounts of PfHep1.Furthermore, using isothermal titration calorimetry, the thermodynamic signature of mortalin was different to that of LbmtHsp70 (Dores-Silva et al. 2016). It would be useful to dissect the thermodynamic properties of both PfHsp70-3 and PfHep1 to determine if these proteins are similar to their human orthologues.

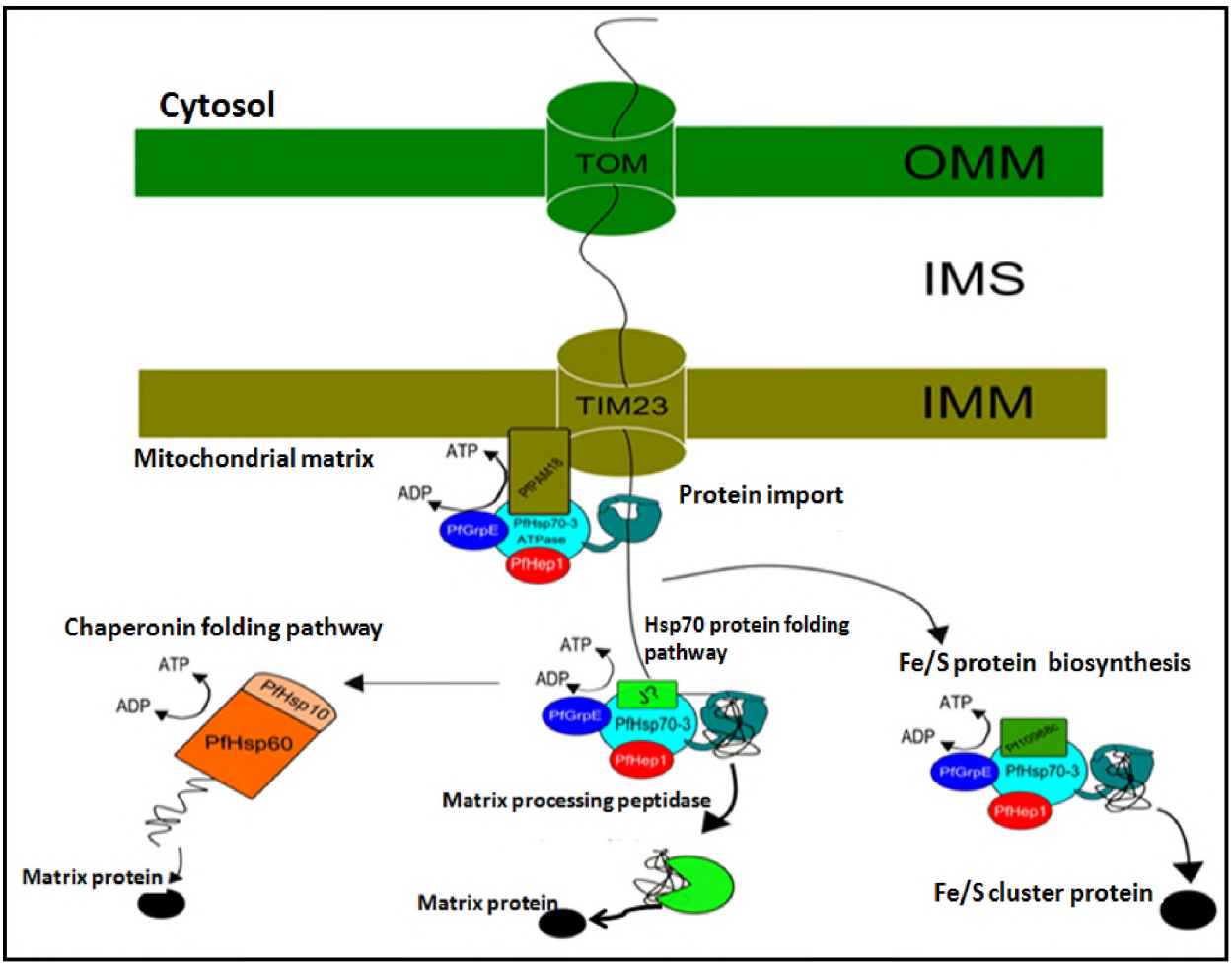


Figure 5.0 : Model of role of PfHep1 against PfHsp70-3: Mitochondrial destined proteins from the cytosol are directed to the matrix through targeting and sorting topogenic signal sequences at their N-terminal. While on transit peptides engage translocator outer membrane (TOM) and either remain in the outer mitochondrial membrane (OMM) or in the inter-membrane space (IMS) or interact with the translocases of the inner membrane (TIM23) to gain entry into the matrix. PfHsp70-3 acts as a molecular clamp to prevent backward movement of pre-proteins through TIM23 and facilitate their entry into the matrix an ATP-dependent manner with PfPam18 stimulating its ATPase activities. PfHep1 holds PfHsp70-3 and prevent it from aggregation and PfGrpE serve as nucleotide exchange factor. With the help of matrix J-protein and Pf10985c, PfHsp70-3 folds and syntheses Fe/S cluster proteins respectively. In both cases, PfHep1 will be required to maintain the functional structure of PfHsp70-3 by preventing it from aggregation. Mitochondrial pre-proteins folding can also be achieved through PfHsp60/ PfHsp10 chaperonin system.

Yeast Hep1/ Zim17 has also been shown to play a role of assisting yeast mtHsp70 in interacting with its client proteins in a J co-chaperone-dependent manner (Lewrenz et al. 2013). There is

also the proposition that Zim17 functions as a "fractured" J-protein that provides a zinc finger domain similar to Type III J-proteins for substrate binding (Burri et al. 2004). Zim17 also possesses an extended C-terminal sequence like hHep1though not similar in terms of residue composition (Figure 2.4). However, the ability of Zim17 to stimulate the ATPase activities has not been reported. Following the observations made in this study relating to the possible roles of PfHep1, a proposed model of interaction of PfHep1 and PfHsp70-3 in the parasite mitochondrion is suggested (Figure. 5.1).

The study had prioritized the use of pARL-2-PfHsp70-3_GFP plasmid for parasite transfection for investigation of PfHsp70-3 localization. However, due to unsuccessful attempts to produce positive parasite transfectants, the study resorted to the use of PfHsp70-3 peptide directed antibodies for indirect immunofluorescence assays. Successful production of peptide specific directed antibodies against PfHsp70-3 and positive pARL-2-PfHep1_GFP parasite transfectants, allowed localization studies for the two proteins to be carried out. Both PfHsp70-3 and PfHep1 localized in the parasite mitochondria and the protein levels of PfHsp70-3 increased under heat shock conditions. The study found out that PfHsp70-3 is expressed during all stages of the intra-erythrocytic phase of parasite development, with maximum expression occurring at trophozoite stages. The heat inducibility of PfHsp70-3 could point to its role in the parasite's response to heat stress. Furthermore, the increased expression of the protein at the infective trophozoite stage of development could further point out to its role in cushioning the parasite against heat stress during infectivity.

Given the fact that parasite apicoplast and mitochondrial organelles are bound to each other and lie side by side during most of the asexual stages of parasite development, co-localization studies of both PfHsp70-3 and PfHep1 would be required to be carried out with differential staining of the two organelles to conclusively determine the proteins' mitochondrial localization. The isolation of parasite mitochondrial fractions for separate investigation of the localization of PfHsp70-3 using antibodies should be considered as well. There is not yet any J-protein that has been characterized as co-chaperone of PfHsp70-3 in the parasite mitochondria. Future work should focus on *in vitro* and *in vivo* characterization of putative parasite mitochondrial matrix and membrane J proteins PfJ1, Pf10985c and PfPAM18.

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APPENDICES

Appendix A: Nucleotide sequences used (Fasta format)

>PfHsp70-3 Codon optimized sequence

ATGTCGGGTGATATTATTGGCATTGACCTGGGCACCACGAACTCCTGCGTCGCTATTATGGAAGGCAAACAAGGCAA
AGTGATTGAAAACAGTGAAGGCTTTCGTACCACGCCGTCCGTGGTTGCGTACCAACGATAATCAGCGTCTGGTCGGT
ATTGTGGCCAAACGCCAAGCAATCACGAACCCGGAAAATACCGTTTATGCTACGAAACGTTTTATTGGCCGCAAATA
CGATGAAGACGCGACCAAAAAAACAGAAAAACCTGCCGTATAAAATTGTGCGTGCCAGCAATGGTGATGCTTGGATC
GAAGCGCAGGGCAAAAAATACAGCCCGTCTCAAATTGGCGCATGCGTTCTGGAAAAAATGAAAGAAACCGCGAAACT
ATCTGGGTCGCAAAGTGCATCAGGCGGTTATTACCGTCCCGGCCTACTTTAATGACAGTCAGCGTCAAGCCACGAAA
GATGCAGGTAAAATCGCTGGCCTGGACGTGCTGCGCATTATCATGACCGACCGCCGCGGCACTGGCATTTGGTCTGG
AAAAATCCGATGGCAAAGTGATTGCAGTTTATGACCTGGGCGGTGGCACCTTTGATATTTCAATCCTGGAAATCCTG
TCGGGTGTCTTCGAATGAAGCAACCAACGGCAATACGTCACTGGGTGGCGAAGATTTTGACCAGCGCATTCTGGAAT
ACTTCATCTCGGAATTCAAGAAAAAAGAAAACATCGACCTGAAAAACGATAAACTGGCTCTGCACGTCTCGCGAAGC
TGCGGAAACCGCGAAAATCGAACTGAGCTCTAAAACCCAAACGGAAATTAACCTGCCGTTCATCACCGCCAATCAGA
CGGGCCCGAAACATCTGCAAATTAAACTGACCCGTGAAAACTGAAGAACTGTGTCACGATCTGCTGAAGGGTACGAT
TGAACCGTGCGAAAAATGTATCAAAGATGCGGACGTCAAAAAAGAAGAAATCAACGAAATCATCCTGGTGGGTGGCA
TGACCCGCTGCCGAAGTTACCGATACGGTCAAACAGATCTTTCAAAACAATCCGAGCAAAGGTGTTAATCCGGATGA
AGCGGTTGCACTGGGTGCAGCAATTCAGGGTGGCGTTCTGAAAGGTGAAATCAAAGACTGCTGCTCTGGATGTCATT
CCGCTGTCACTGGGTATCGAAACCCTGGGTGGCGTGTTCACGAAACTGATTAACCGTAATACCACGATCCCGACCAA
AAAATCACAGATTTTTAGCACCGCTGCGGATAACCAGACGCAAGTTAGTATCAAAGTTTTCCAAGGCGAACGTGAAA
GCTAGCGATAATAAACTGCTGGGTTCTTTTGATCTGGTGGGCATTCCGCCGGCGCCGCGCGGTGTTCCGCAGATCGA
AGTCACCTTCGATGTGGACGCTAACGCGATTATCAATATTAGCGCCATCAAAAATGACCAACAAAAAACAGCAAATC
ACGATCCAGAGTTCCGGTGGCCTGAGCAAAGAAGAAATCGAAAAAATGGTTCAGGAAGCCGAACTGAATCGCGAAAA
AGATCAACTGAAGAAAAACCTACGACTCTAAAAATGAAGCAGAAACGCTGATTTATTCATCGGAAAAACAGCTGGAA
GACTTCAAAGATAAAATCAGTGATTCCGACAAAGATGAACTGCGTCAGAAAATCACCGTGCTGCGCGAAAACTACGA
GTGAAGACCTGGATTCCATTAAAGATGCGACCAAACAGCTGCAAGAAAAAAGCTGGGCCATCTCTCAGGAAATGTAC
AAAAACAATGCACAGCAAGGTGCCCAACAGGAACAACCGAACACGAAACAAAGCCGAAGAAAACAAAGACAACGCAT
AA

>PfHep1 Codon optimized sequence

CATATGGCGCACCTTCAGCCGTATCATCCTGACGAAAGACATCAACTTCAAGAAAGAAAAGAAAAACTTCAGCAGCC
TGTTCCAGCGTTACAACATCAACAACCTGAGCTCTAAAAACGATAACCATTTCTGCATCGTCCCGTTCAATAGCACC
AACAACTTCATCATCAGCAAAAAATCTTTCGTCACGAAAAACGAACGTGTGCAGAACGAAAAAATCATCCAAGATGA
CAAATTCGAAAAATCTAACGATAAAGTTATCTACGACAACAAAAGCAAACACCTGTCTGATAAAATTATCTGTGATG
ACAAAATCATCGAACTGAACGAAAACATCATCACCAACACGATCAATAATAATAATAATAATAACAATAATAATAAT
AACAACAACAACAATAACTCAAATAACAATAACAATAACTCGAACAATAACAACAATAACTCTAACAACAATAACAA
CAACAACGAAGAAATCATCAACACCAGTTCCAACATCCTGGATATCGAAAACAACAAAATCATCCAGATCAACAACG
GCGAAGAAAGCGAAATCCTGGATAAAAACCTGAATGAAGATCTGGTTGACAAAGAAATCGACCAGAAAAAGAAAGAA
TACCTGGTCCTGATGTTTACCTGCAACATCTGTGAAAAGAAAAGCGCGAAAAAATTCTCCAAACAGGCCTATTACAA
CGGCGTGGTTATTGTGCGCTGCCCGTCTTGTGAAAATCTGCATCTGATCAGTGATCAGCTGGGCTGGTTCCAAGACG
GTAAAACGAACATCGAAAAAATCCTGGAAGAAAAAGGTGAAAAAGTTGTGAAAAAATTCTCTTACAATAATCTGCTG
GAAGTGGACGACCTGCTGAACGCATACAAAGGTACC

>Pfj1 optimized coding sequence

ATGAGAGGATCGCATCACCATCACCATCACGGATCCAACCAGGACCCGTACACCGTTCTGGGTCTGTCTCGTAACGC
GACCACCAACGACATCAAAAAACAGTTCAGGCTGCTGGCGAAAAAATACCCCGGACATCAACCCGTCTCCGGACGCG
AAACAGAAAATGGCGTCTATCACCGCGGCGTACGAACTGCTGTCTGACCCGAAAAAGAAAGAATTCTACGACAAAAC
CGGTATGACCGACGACTCTAACACAGAACCACTCTTCTAACTTCGAAGGTGCGTTCTCTGGTTTCGGTGACGCGTCT
TTCATGTTCACCGACTTCGCGGAAATGTTCACCAACATGGCGGGTGGTAACAAAAACACCTCTACCCGTGGGAGACA
TCCAGTCTGAAATCACCCTGAAATTCATGGAAGCGATCAAAGGTTGCGAAAAAAACTCGCGACTGAACGTTAAAGTT
TCTTGCAACAACTGCAACGGTTCTGGTAAAAAACCGGGCACCACCTACCATCTGCAAAGTTTGCAACGGTTCTGGTA

TCCAGCGTATGGAACGTGGTCCGATCATCATCGGTGTTCCGTGCCGTAACTGCTCTGGTAACGGTCAGATCATCAAC
AACCCGTGCAAACACGCTCGGTTCTGGTGTTAAATTCCAGACCAAAAACATCACCCTGGACATCCCGCCCGGGATCA
AAAAAGGTATGCAGATGCGTATCCCGAACCAGGGTCACTGCGGTTACCGTGGTGGTAAATCTGGCACCTTTCGTTAC
CATCAACATCGAACCGCACAAAATCTTCAAATGGGTTGACGACAACATCTACGTTGACGTTCCGCTGACCATCAAAC
AGTGCCTGCTGGGTGGTCTGGTTACCGTTCCGACCCGAACGGGACATGGACCTGCTGATCAAACCGAAAACCTACCC
GAACTCTGAAAAAATCCTGAAAGGTAAAGGTCCGTGCAAAGTTGACTCTCACAACAACGGTGACCTGATCATCAAAT
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GCCGAACCCGCAGACCAACGTTAAACAGAAAAAAAACATCTACGAAACCAAAGGTAAATCAACGAAACATCTTCTCT
ATGAACAACACCTACAACAACATGAAAGGTCCGGAAGGTGAAACCTCTAACACCCAGGCGAAATCTATGAAAAACCA
GAACTGGAACAACGAAAAATCTGTTAACACAAAGGCACATCTCTAAAGACGAAAAAAAACTGAACATGAAAAACAAC
CACATCAACGAAAAATCTAACCTGAAAAACTCTTCTCACATGGACACCAACAAAAACGAAGAAAACATGTCTGACGA
CAAAAAAAAAAATCAAAAAAATCATCCCGGAACCGCCGATGCCGCACACCCACAAAATCGTTAACAACCTGGAATCT
AAAAACTCTTGCAACATCCCGATCCCGCCGCCGCCGCCGAAATCTTCTTCAAACCGATCTCGAAAACCAGAACATCT
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CCGTACAAAAACGTTACCCAGACGACCTGAACAAAACGACAACATCAAAAACAAAATCTACAAAGACAACACCAACA
TCTCTAACCACCACATCTTCAAAAACGACAACATCAACCAGCAGCAGTTCCACTGCGCGGACAACTCTTCTAAAACA
ACAACGATCTGACATGAACACCACCTCTACCTTCTCTTTCGCCAAAAAATGGATCTCTGACAAACTGAAACCGAAAA
ACTAAGGTA

>Pfj1-J optimized coding sequence

ATGGATCCTTATACAGTTTTAGGTTTATCTAGAAATGCTACAACAAATGATATAAAAAAGCAATTTCGCTTACTAGC
CAAAAAATATCATCCAGATATTAATCCATCTCCAGATGCAAAGCAAAAAATGGCTAGTATTACTGCAGCATATGAAT
TATTATCAGATCCTAAAAAGAAAGAGTTTTATGATAAAACAGGAATGACTGATGATTCAAATTATCAAAATCATTCA
TCTAATTTTGAAGGAGCTTTTTCAGGATTTGGAGATGCATCCTTCATGTTTACAGATTTTGCAGAAATGTTTACAAA
TATGGCAGGAGGAAATAAAAATACATCAACAAGAGGTGAAGATATACAAAGTGAAATAACATTAAAATTTATGGAAG
CTATTAAAGGATGTGAAAAAAATATTCGATTAAATGTAAAAGTATCATGTAATAATTGTAATGGATCTGGTAAAAAA
CCAGGTACTAATTTAACCATATGTAAAGTTTGTAACGGTTCAGGTATTCAACGTATGGAAAGGGGACCTATAATTAT
TGGAGTACCATGTAGAAATTGTTCAGGTAATGGACAAATTATAAATAACCCTTGTAAACATTGCTCAGGTAGCGGTG
TCAAATTTCAAACATGA.

Appendix B: Cloning Procedure

The pACYCDuet1-PfHep1 sub-cloning into pQE30 to create pQE30-PfHep1 was done as follows: The PACYCDuet1-PfHep1 chimera and pQE30 expression vector were double digested (*Bam*HI/*HindIII*), the resultant PfHep1coding sequence and digested pQE30 expression vector were subsequently gel purified using GeneJET Gel Extraction Kit (Thermo Scientific, Lithuania) (appendix J) and ligated(appendix I) to each other. Competent *E. coli* DH5α™ cells (appendixH*)* were transformed with ligated products and plated on 2xYT agar plate (1.5% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl and 1.5% (w/v) agar) supplemented with ampicillin to a final concentration of 100µg/mL. Successful transformants were screened as follows; each selected colony was inoculated into 5 mL 2xYT broth (1.5% (w/v) tryptone, 1% (w/v) yeast extract and 0.5% (w/v) NaCl) and incubated at 37^{o}C with shaking overnight. The cells were harvested by centrifugation at 10,000 x g for 2 minutes and the plasmids were

extracted and purified using the alkaline lysis method (Appendix L) using Zyppy^{TM} Plasmid Minprep Kit (ZYMO Reseach Kit, USA). The isolated plasmid was double digested with *BamHI* and *HindIII* and analyzed through agarose gel electrophoresis. The pQE30-PfHep1 plasmid isolates from cells that produced the expected size bands corresponding to the coding sequence insert, were reproduced, propagated and stored in *E. coli* DH5α™ cells.

Appendix C: Agarose Gel Electrophoresis

Agarose gels were prepared as follows; 0.4g agarose was weighed and dissolved in 50mL of 1xTBE buffer (Tris 2.75g , Boric acid 1.375g ,EDTA 0.372g EDTA in 1L water) to make 0.8% gel. The suspension was heated in a microwave until the agarose was completely melted. 10µl of ethidium bromide was added to the suspension after cooling to approximately 30^{o}C and poured into the gel casting tray with combs inserted. The gel was left to set and transferred into a submerging 1x TBE buffer in the gel buffer tank. The sample loading buffer (7mL 1xTBE buffer, Glycerol 30% and Bromophenol blue 0.25%) was added to the DNA samples and 30µl of the sample mixture was loaded on to the wells. The gel was run in the buffer with powerpack at 90v/20 mA.

Appendix D: Preparation of Competent cell (*E. coli* cells)

E. coli cells weregrown in overnight (37°C, 200 rpm) in 5mL of 2x YT (1.6% tryptone, 1% yeast extract, 0.5% NaCl) / LB media (1% tryptone, 0.5% yeast extract, 1% NaCl) supplemented with appropriate antibiotic for strain selection(5µl of the antibiotic was added). The overnight culture was diluted into 100 mL of 2x YT / LB media to an A600 of 0.1 along with addition of 105µl of appropriate antibiotic and allowed to grow until early log phase (A_{600} of 0.6). The cells were harvested by centrifugation (5000 xg, 5 minutes, 4°C) and the pellet was resuspended in 50 mL of ice-cold 0.1 M $MgCl_2$ (4°C). The cells were incubated for two minutes at 4°C and centrifuged as before and resuspended in 25 mL of ice-cold 0.1 M $CaCl_2$ (4°C). The cells were then incubated at 4°C for one hour and harvested by centrifugation as before. The cells were then re-

suspended in 5mL of 0.1 M $CaCl_2$ and 5mL of 30% (v/v) glycerol was added. The cells were divided into aliquots (300µl) and stored at -80°C for further use.

Appendix E: Media preparation Recipes

2×Yeast-Tryptone (2YT) Broth growth medium

Tryptone	16g/L
Yeast Extract	10g/L
NaCl	5g/L

Dissolve in 1L distilled water and autoclave (121°C and 119 kPa for 30 minutes)

2×Yeast-Tryptone (2YT) Agar

The recipe is similar to that of 2YT broth with an addition of 15g bacteriological agar per liter followed by autoclaving (121°C and 119 kPa for 30 minutes).

Luria-Bertani (LB) growth medium

Tryptone	10g/L
Yeast Extract	5g/L
NaCl	10g/L

Dissolve in 1L distilled water and autoclave (121°C and 119 kPa for 30 minutes)

Appendix F: Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE)

The protocol for SDS-PAGE analysis was done as previously described (Shapiro, et al., 1967). Protein samples were treated with 5x SDS-PAGE sample buffer (10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.05% bromophenol blue, 0.0625 M Tris, pH 6.8) in a ratio of 4:1 respectively and loaded onto a polyacrylamide gel constituted by a resolving gel (12% (w/v) acrylamide, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulphate (APS), 0.005% (v/v) N,N,N',N'-tetramethylethylenediamine (TEMED), 0.375 M Tris, pH 8.8) and a stacking gel (4% (w/v) acrylamide, 0.1% (w/v) SDS, 0.05% (w/v) APS, 0.005% (v/v) TEMED, 0.125 M Tris, pH 6.8). The gel was resolved in a Mini ProteanR II system (Bio-Rad) at 150 V for one hour and stained or used for Western analysis (Section F10). Staining of the SDS-PAGE gel was achieved in

Coomassie Blue stain (40% (v/v) methanol, 7% (v/v) acetic acid, 0.25% (w/v) Coomassie Blue R250 in distilled water) for 30 minutes and subsequent destaining was achieved overnight in destain solution (40% (v/v) methanol, 7% (v/v) acetic acid in distilled water).

Appendix G: Protein detection by western analysis

The protocol for the detection of proteins by Western analysis was done aspreviously described (Towbin, et al., 1979). Proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membrane in transfer buffer (20% (v/v) methanol, 192 mM glycine, 25 mM Tris) at 100 V for 90 minutes in a Mini ProteanR III Western trans-blot system (Bio-Rad). Protein transfer was verified with Ponceau S stain (0.5 % (w/v) Ponceau S, 1% (v/v) glacial acetic acid). The membrane was subsequently destained with distilled water and incubated overnight at 4°C in blocking solution comprised of 5% (w/v) fat-free milk powder in Tris Buffered Saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.5). The membrane was incubated with appropriate primary antibody (1:5000 in blocking solution unless otherwise stated in the text) for one hour at room temperature and subsequently washed three times with Tris Buffered Saline-Tween buffer (TBS-T; TBS containing 0.1% (v/v) Tween 20). The membrane was similarly incubated with the appropriate horse-radish peroxidise (HRP)-conjugated secondary antibody (1:5000 in blocking solution unless otherwise stated in the text) for one hour at room temperature and washed with TBS-T as before. Chemiluminescence-based protein detection was achieved using the ECLTM Western blotting kit (GE Healthcare) as per the manufacturer's instructions, and captured with a Chemidoc chemiluminescence imaging system (Bio-Rad).

Appendix H: Preparation of Parasite Medium Culture

Albumax II and 2.5g and 2g glucose were added to a 50mL plastic centrifuge tube. In the laminar flow hood, approximately 50 mL of the RPMI 1640 medium was added into the tube and dissolved in the Albumax and glucose. The 44mg hypoxanthine was weighed out separately into a microfuge tube and dissolved by adding approximately 1mL 0.1N NaOH. The hypoxanthine solution was then added to the Albumax/glucose medium solution in the 50mL tube. 0.3mL of the Gentamicin solution was then added to the 50mL tube. The solution in the 50mL tube was

sterilized by filtration in the laminar flow hood where the solution was pushed through a 0.2μm 50mL syringe filter into a fresh sterile 50mL tube. The filtered solution was added back into the bottle containing the 500mL RPMI 1640 medium. The medium was stored in a fridge at 4°C, and was used within a month.

Appendix I: DNA Ligation Procedure

Ligation reaction was set up in a microfuge tube as follows:

Purified plasmid: 100ng	
PCR product:	6-fold molar excess (= 3x (size of insert/size of plasmid)x100ng)
10x NEB ligase buffer:	1 μL
Water:	to make total volume 10 μL
NEB T4 DNA ligase:	1 μL

The tube containing the reaction mixture was placed on ice/water and incubated in an overnight. The ligation reaction was thereafter stored at 4 degrees

Appendix J: DNA Products Gel Purification

The DNA products were purified according to the Thermo Scientific (Lithuania) DNA gel purification Kit protocol. The gel slices containing the DNA were transferred to a microfuge and weighed to calculate the actual net weight of the gel slices (weight – weight of empty tube). The membrane binding solution was added to the gel slices in the ratio of 10μl : 10mg and heated to 60 degrees in the heating block to dissolve the gel slices. Heating was accompanied with vortexing to ensure complete dissolving. The dissolved gel slice was transferred into the SV mincolumn followed by centrifugation for 1 minute to bind the DNA to the column (the procedure was repeated by adding back the dissolved gel slice into the column). The column was washed twice in with the membrane wash solution (500μl). The DNA was eluted with nuclease-free water (50μl) pre-heated at approximately 50^{o}C.

Appendix K: Plasmid Maxiprep

The procedure was meant to isolate large amount of pure plasmid DNA (200-500µg) for the transfection of malaria parasite. The plasmid was isolated using the QIAfilter^{TM} Plasmid Maxi Kit (Qiagen, Germany). In summary, 200mL of LB broth culture of *E.coli* cells transformed with appropriate plasmid was harvested by centrifugation (5000 rpm, 15 minutes) and the pellet was resuspended in 10mL buffer P1 containing RNase and LyseBlue. 10mL buffer P2 was added to the suspension and mixed thoroughly to lyse the bacteria cells followed by incubation for four minutes and ensuring that the incubation time does not exceed five minutes. After incubation, cold 10mL buffer P3 was added and the bacterial lysate was immediately transferred to a Qiafilter cartridge (nozzle cap attached) followed by incubation for 10 minutes. The bacteria lysate was transferred from the Qiafilter cartridge to a Qiagen tip (equilibrated with 10mL QBT buffer) using a syringe plunger followed by wash of the Qiagen tip with with 3x 15mL QC buffer ensuring complete draining of the wash buffer after each wash. The bound plasmid was eluted into fresh 50mL using 15mL QF buffer. To the eluate, 10.5mL (0.7 vol) isopropanol was added and mixed thoroughly by shaking followed by centrifugation (15000rpm, 30 minutes, 4^{o}C). The supernatant was carefully discarded and the pellet was washed in 3mL of 70% ethanol followed by centrifugation (15000rpm, 5minutes). The washing step was repeated and excess 70% ethanol was removed by air drying. The resultant pellet was dissolved in 1 mL pre-heated (50^{o}C) nuclease free water and stored in -20^{o}C for further analysis.

Appendix L: Isolation of plasmid DNA(Miniprep)

Alkaline lysis method was used for the isolation of plasmid DNA. Competent *E.coli* cells were grown in an overnight (37°C, 180 rpm) in 2x YT culture (1.6% tryptone, 1% yeast extract, 0.5% NaCl) supplemented with relevant antibiotics after transformation with respective plasmids. The cells were harvested in a microcentrifuge (12000rpm, 2minute) and plasmid DNA isolation was performed according to Zyppy^{TM} Plasmid Miniprep Kit (Zymo research, USA). Briefly, the pellet of the harvested cells was resuspended in 600µL of bacterial 2YT culture growth medium in a 1.5mL microcentrifuge tube. To the mixture, 100µL of 7× lysis buffer was added and mixed gently by inverting 6 times and allowed to stand for one and half minutes. This was followed by the addition of 350µL of cold neutralization buffer and mixed thoroughly by inverting until the

mixture sample turned yellow. The mixture was centrifuged (12000rpm, 4minute) and the supernatant was transferred into Zymo-Spin™ IIN column. The columns were placed in the collection tube and centrifuged for one minute. The flow through was discarded and the columns were placed back into the collection tube and to it 200µL of Endo-Wash buffer was added and centrifuged for one minute. To the column, 400µL of Zyppy™ wash buffer was added and centrifuged for one minute and the step was repeated. The column was then transferred into a clean 1.5mL microcentrifuge tube and 30µL of Zyppy™ elution buffer was added directly to the column matrix and left to stand for one minute followed by centrifugation (12000rpm, 1minute). All procedures were conducted at room temperature (25°C).

Appendix M: Plasmid DNA enzyme restriction digest

Appropriate restriction endonuclease(s) were used for plasmid DNA digest by incubating respective DNA and the ezymes for three hours at 37°C. The reaction set up for double digest restriction reaction was set as follows: Water (30µL), plasmid miniprep (6µL), 10x NEB buffer (4µL) and restriction endonuclease enzymes (0.5µL each). The buffers and the respective enzymes used were from BioLabs, New Englands. The 1kb DNA ladder (BioLabs, England) was prepared by mixing 4µl (distilled water), 1µl (DNA Ladder) and 1µl (Gel loading dye, purple 6×) to the total volume of 10µl. The compatibility of the restriction buffers for single and double restriction enzyme digestions were determined as per the supplier's recommendations. The digested DNA was resolved by agarose gel electrophoresis.

Appendix N: Polymerase chain reaction (PCR) protocol

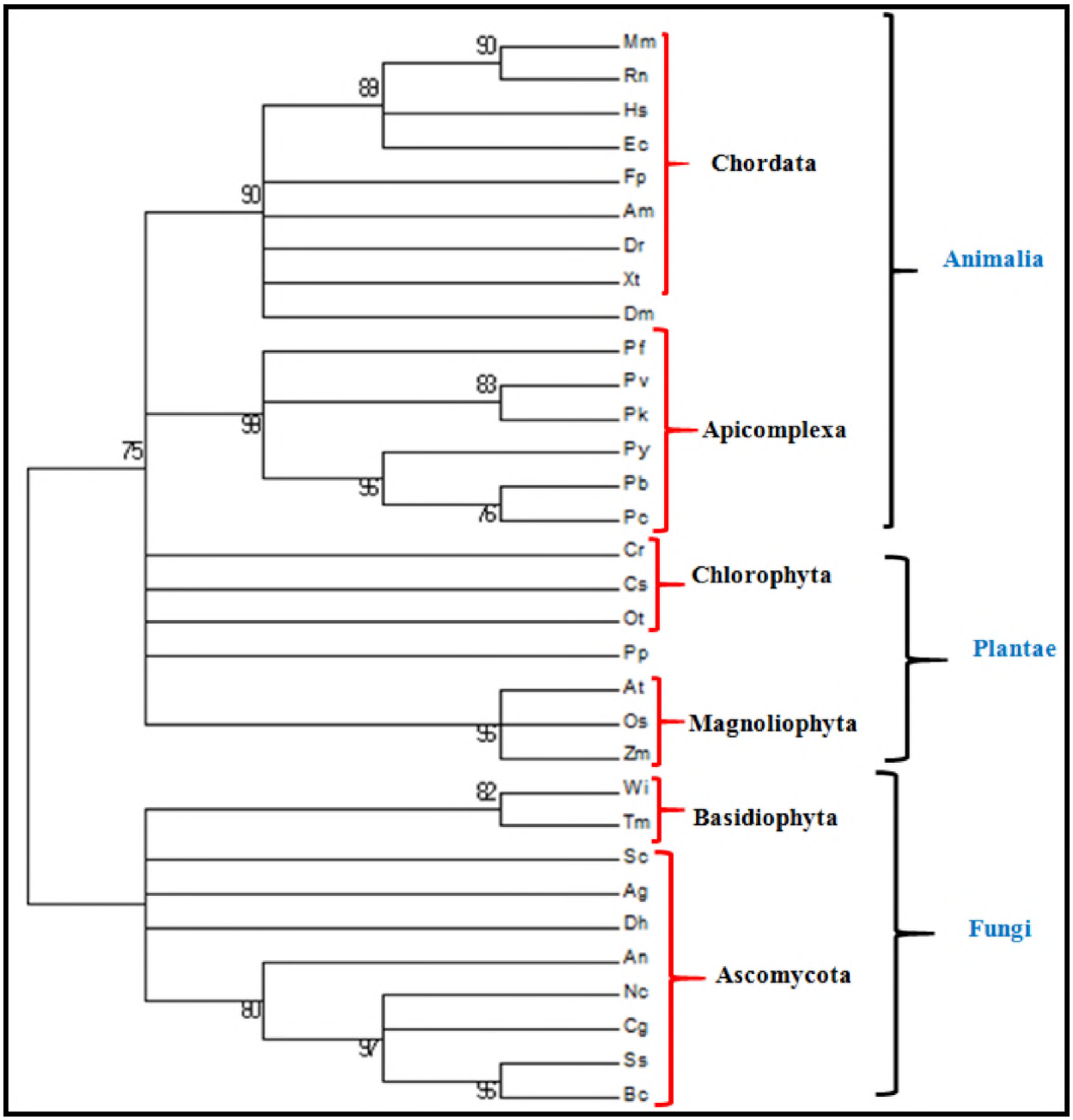
PCR reaction was set up in a 200µl PCR tube as follows; water: 37µl, 5x KAPA HiFi buffer (KAPA Biosystems, USA):10µl, KAPA dNTP mix: 1µl (0.2mM final concentration), DNA template: 2.0µl (for cDNA and genomic DNA and 0.5µl for plasmid) and primers (forward and reverse): 1.5µl of each and the final volume was made to 50µl. The mixture in the PCR reaction tube was inserted into the PCR simpliAmp™ thermal cycler (Biosystems life technologies, Singapore) followed by closing the lid and heating at 94°C for two minutes and then paused. 5µl HiFi DNA polymerase (0.5 units) was added to the reaction tubes as faster as possible to avoid

any excessive cooling and evaporation. The PCR cycle was resumed; 94°C for 0:40 min (denaturation phase), 60-64°C 0:45 minutes (annealing phase) and 70°C for 2:00 min (extension phase).The cycles were repeated 30 times with one final cycle of 5:00 min at 70°C followed by cooling to 4°C. The PCR reaction products were run on the agarose gel to confirm the presence of the target amplified product.

Appendix O: Preparation of PCR product for cloning

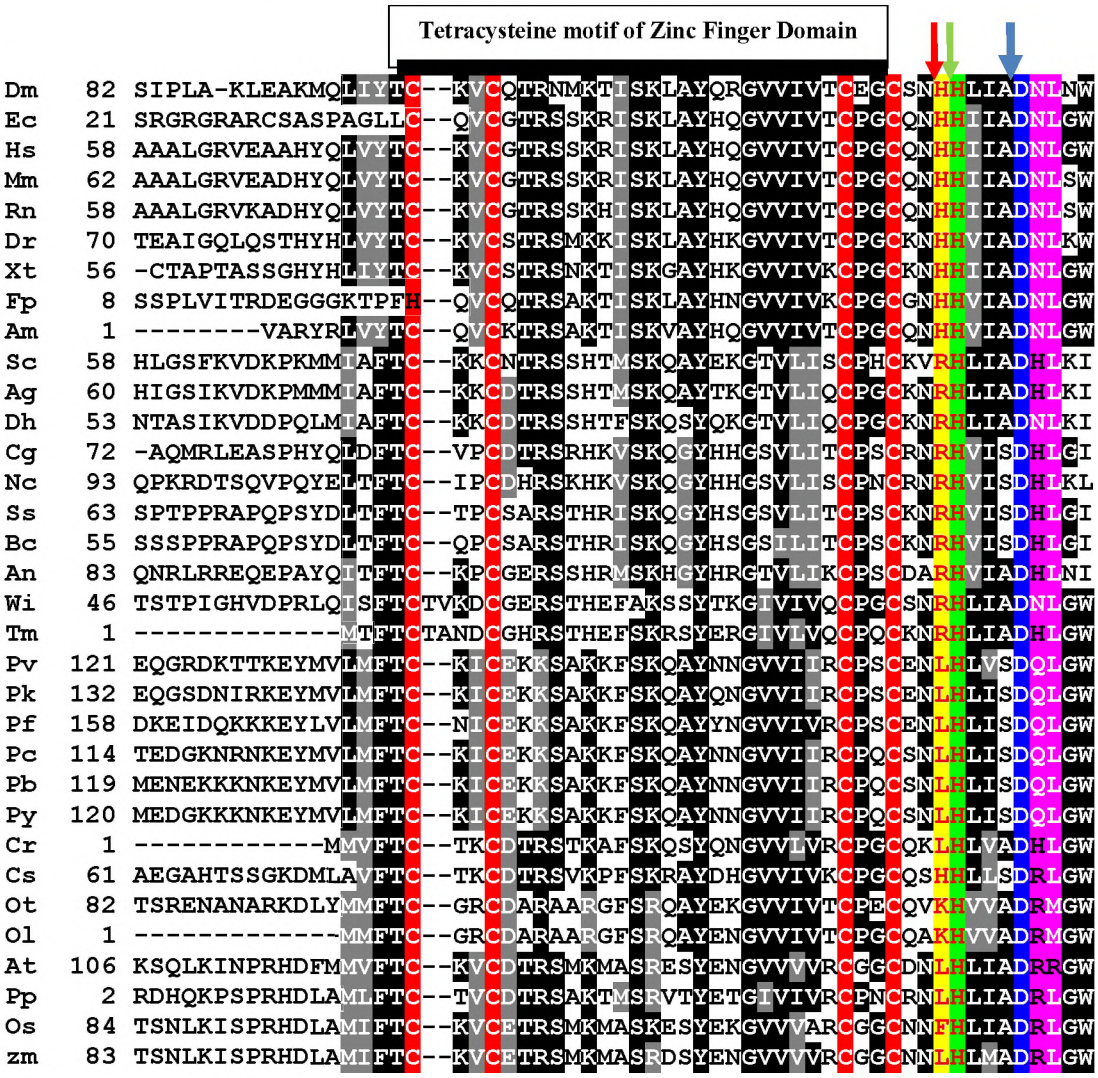
The PCR products were cleaned using GeneJet Gel Extraction Kit (Thermo Scientific, Lithunia) according to the manufacturer's instructions. Briefly, the agarose gel slices containing bands of interest were transferred into a microfuge tube and the net weight of gel slices was determined accordingly (Total weight minus weight of empty tube). Membrane binding solutionwas added to the gel slices in the ratio 10µl:10mg and heated for 10 minutes at 60 degrees followed by maximum vortexing to dissolve the slices completely. The dissolved gel slices containing PCR products was transferred into the column tubes and centrifuged (12000rpm, 1 minute) to bind the DNA to the column followed by washing of the column with 500µL Membrane Wash solution and centrifugation for one minute. The column tubes were centrifuged again to remove all traces of the membrane wash buffer followed by elution of the PCR product with 50µL of nuclease-free water, pre-heated to 50 degrees. Elution was accomplished by centrifugation (12000rpm, 1 minute). The concentration of the eluted samples was determined by a nanodrop instrument. Cleaned PCR products were constituted as follows for restriction digest; 50µL purified PCR product, 4µL nuclease free water, 6µL NEB 10x restriction compatible bufferand1µL of each endonuclease enzyme. The products were double digested as described in appendix.. The restricted PCR products were confirmed by agarose gel electrophoresis as described in appendix C2 with an exception of using TAE buffer (4.84 Tris and 0.37g EDTA and 1.14mL glacial acetic acid). The products were gel purified and concentration was determined by nanodrop.

Appendix P: Evolutionary conservation of Hep1 and functional residues in *P.falciparum* and other species across higher organisms.



Evolutionary conservation of Hep1 in PfHep1 and other species across the kigdom animalia: *Mus musculus* (Mm), *Rattus norvegicus* (Rn), *Homo sapiens* (Hs), *Equus caballus* (Ec), *Peregrine falcon* (Fp), *Alligator mississippiensis* (Am), *Danio rerio* (Dr), *Xenopus tropicalis* (Xt), *Drosophila melanogaster* (Dm), *Plasmodium falciparum* (Pf), *Plasmodium vivax* (Pv), *Plasmodium knowlesi* (Pk), *Plasmodium yoelii* (Py), *Plasmodium berghei* (Pb), *Plasmodium chabaudi*(Pc), *Chlamydomonas reinharditii* (Cr), *Coccomyxa subellipsoidea* (Cs), *Ostreococcus tauri* (Ot), *Physcomitrella patens* (Pp), *Arabidopsis thaliana* (At), *Oryza sativa* (Os), *Zea mays* (Zm), *Wallemia ichthyophaga* (Wi),*Tremella mesenterica* (Tm), *Saccharomyces*

cerevisiae (Sc)*Ashbya gossypii* (Ag), *Debaryomyces hansenii* (Dh), *Aspergillus niger* (An), *Neurospora crassa* (Nc), *Colletotrichum graminicola* (Cg), *Sclerotinia sclerotiorum* (Ss), *Botrytiscinerea* (Bc).



Sequence alignement of the zinc finger domain of PfHep1 and its orthologs. The amino acid sequences for *Plasmodium falciparum* Hsp70 escort protein (PfHep1) and its orthologs from *Mus musculus* (Mm), *Rattus norvegicus* (Rn), *Homo sapiens* (Hs), *Equus caballus* (Ec), *Peregrine falcon* (Fp), *Alligator mississippiensis* (Am), *Danio rerio* (Dr), *Xenopus tropicalis* (Xt), *Drosophila melanogaster* (Dm), *Plasmodium falciparum* (Pf), *Plasmodium vivax* (Pv), *Plasmodium knowlesi* (Pk), *Plasmodium yoelii* (Py), *Plasmodium berghei* (Pb), *Plasmodium chabaudi*(Pc), *Chlamydomonas reinharditii* (Cr), *Coccomyxa subellipsoidea* (Cs), *Ostreococcus tauri* (Ot), *Physcomitrella patens* (Pp), *Arabidopsis thaliana* (At), Oryza sativa (Os), Zea mays (Zm), *Wallemia ichthyophaga* (Wi),*Tremella mesenterica* (Tm), *Saccharomyces cerevisiae* (Sc)*Ashbya gossypii* (Ag), *Debaryomyces hansenii* (Dh), *Aspergillus niger* (An), *Neurospora crassa* (Nc), *Colletotrichum graminicola* (Cg), *Sclerotinia sclerotiorum* (Ss), *Botrytis cinerea* (Bc) were aligned using Clustal Omega (Fabian *et al.* 2011). Highlighted residues and segments are as follows; the tetracysteine residues delimiting the zinc finger domain (Red color), residues

D and H that play critical role on the structure and function of Hep1 protein (Blue and Green respectively) and the zinc finger's c- terminal motif; zf-DNL that is part of residue D (Pink color).

Appendix R: Peptide directed anti-PfHsp70-3 antibody design and production

No	Start	Antigenic Determinant	Length	Antigenicity/Surface/ Hydrophilicity	Disordered Score	Synthesis	*Oryctolagus_cunic ulus* blast % Result
1	649	CQPNNENKAEENKDN	14	3.10/0.93/1.35	0.2119	**Y**	**50%**
2	279	CKKKENIDLKNDKLA	14	2.33/0.71/1.13	0.1136	**N**	**56%**
3	474	CVFQGEREMASDNKL	14	2.22/0.71/0.67	NONE	**Y**	**78%**
4	392	CQNNPSKGVNPDEAV	14	2.20/0.64/0.32	NONE	**Y**	**70%**
5	60	GKQGKVIENSEGFRC	14	2.17/0.79/0.63	0.1049	**Y**	**64%**
6	114	RKYDEDATKKEQKNC	14	2.14/1.00/1.39	0.1533	**Y**	**50%**
7	556	CNREKDQLKKNLTDS	14	2.10/0.86/1.24	0.1249	**Y**	**49%**
8	253	CKATNGNTSLGGEDF	14	1.98/0.86/0.32	0.1271	**Y**	**78%**
9	300	CAETAKIELSSKTQT	14	1.71/0.79/0.60	0.1285	**Y**	**71%**
10	579	CSSEKQLEDFKDKIS	14	1.64/0.79/1.10	0.1169	**Y**	**64%**
11	28	SLCTSKINRNRASGC	14	1.60/0.64/0.55	0.1279	**N**	**42%**
12	538	CSGGLSKEEIEKMVQ	14	1.60/0.71/0.81	0.0974	**Y**	**70%**
13	351	CIEPCEKCIKDADVK	14	1.53/0.64/1.06	0.1200	**N**	**42%**
14	524	CDKMTNKKQQITIQS	14	1.29/0.79/0.34	0.1133	**Y**	**64%**
15	3	SLNKKNIVKILERC	14	1.13/0.57/0.37	0.1308	**Y**	**56%**
16	593	CDSDKDELRQKITVL	14	1.12/0.71/1.16	0.1183	**Y**	**56%**

The table depicting the list of antigenic determinants in the PfHsp70-3 sequence

Antigenicity/ Surface/ Hydrophilicity ratio, disordered score and the blast result against the European rabbit (*Oryctolaguscuniculus*) in which the antibodies were to be raised are shown.An extra "**C**" (high-lighted as green) was added to the C-terminusor N-terminus to facilitate conjugation to a carrier protein keyhole limpet haemocyanin (KLH). Positive charged residues (**K**,**R**,**and H**) are shown in blue. Negative charged residues (**D**,**E**) are in red."N" means the peptide was easy to synthesize and "**Y**" means synthesis could be difficult.