The development of a molecular chaperone-based system to improve the heterologous production of *Plasmodium falciparum* AdoMetDC protein in *E. coli*.

By

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Abstract

S-Adenosylmethionine decarboxylase/ornithine decarboxylase from *Plasmodium falciparum* (PfAdoMetDC/ODC) has been described as an ideal antimalarial drug target. However, the production of this large bifunctional protein to facilitate its biochemical characterization is hampered by the poor yields of recombinant protein. It has previously been proposed that co-expression of target recombinant malaria proteins with molecular chaperones of malarial origin in *Escherichia coli* could improve the yield of the target recombinant proteins. A cytosolic heat shock protein 70 (Hsp70) from *Plasmodium falciparum* (PfHsp70-1), has been previously shown to exhibit chaperone function when heterologously expressed in *E. coli* cells whose endogenous Hsp70 function was compromised. PfHsp70-1 presumably protected the *E. coli* cells against thermal stress by preventing protein aggregation. In the current study, PfAdoMetDC and PfAdoMetDC/ODC were expressed along with PfHsp70-1 in *E. coli* BL21 (DE3) star cells to improve the yield and quality of the PfAdoMetDC/ODC proteins. *E. coli* BL21 (DE3) star cells were transformed with plasmid constructs pMRBAD/PfHsp70-1 (encoding PfHsp70-1) and either pASK-IBA/PfAdoMetDC (encoding PfAdoMetDC, either as wild type or codon harmonized version) or pASK-IBA/PfAdoMetDC/ODC (encoding PfAdoMetDC/ODC, either as wild type or codon harmonized version), followed by induction to facilitate the production of the chaperone and the target proteins, respectively . Protein expression and solubility studies were conducted followed by purification of the PfAdoMetDC and PfAdoMetDC/ODC proteins using strep-tectin column. The co-expression of PfAdoMetDC/ODC with PfHsp70-1 did not necessarily improve the production of the former. Although there is no evidence that PfHsp70-1

improved the solubility of PfAdoMetDC protein, it greatly improved the purity of PfAdoMetDC protein obtained following elution through the strep-tectin column. Whilst PfAdoMetDC were expressed from a codon harmonized coding sequence alone (expressed in the absence of PfHsp70-1) was purified associated with contaminants, the same protein purified after co-expression with PfHsp70-1 was obtained at a much higher purity level. This could have been due to PfHsp70-1 shielding nascent PfAdoMetDC, thus preventing its association with the contaminating species. On the other hand, PfHsp70-1 improved the expression and yield of PfAdoMetDC/ODC (wild type), but it had no effect on the yield of the protein expressed of the plasmid haboring the codon harmonized version. Thus PfHsp70-1 may have facilitated the folding and production of the PfAdoMetDC/ODC (wild type) protein whose folding could have been impeded due to potential delayed translation due to codon mismatch.

Declaration

I declare that this dissertation is my own, unaided work. It has been submitted for degree of Masters of Science at University of Zululand. It has not been submitted before for any degree or examination in any other University. I also state that all the sources that I have used have been duly acknowledged.

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Dedication

This dissertation is dedicated to

My late mother Nomagadi Makhoba and my sister Nokwanda Makhoba

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I would like to thank, my supervisor Dr. A. Shonhai for the support he gave me throughout this work and for introducing me to the field of molecular biology.

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Abbreviations

AHT	Anhydrotetracycline
Ara	Arabinose
DDT	dichlorodiphenyltrichloroethane
LB-Broth	Luria-Bertani liquid medium
Pf	*Plasmodium falciparum*
PfAdoMetDC/ODC	*Plasmodium falciparum* S-adenosylmethionine decarboxylase/ornithine decarboxylase
PfAdoMetDC	*Plasmodium falciparum* S-adenosylmethionine Decarboxylase
PfHsp70	*Plasmodium falciparum* Heat shock protein70
pfu	Plaque forming units
TEMED	N, N, N', N'-Tetramethyl ethylenediamine
%	percent
α	Alpha
β	Betha
µl	Microlitre
mg	milligram
µg	microgram
ng	nanogram

A_{360}	Absorbance at 360 nanometres
A_{260}	Absorbance at 260 nanometres
A_{295}	Absorbance at 295 nanometres
KDa	Kilodalton
μM	micromolar
nmol	nanomoles
pmoles	picomoles
mM	millimolar
nm	nanometers
PBS	Phosphate buffer saline
PAGE	Poly acrylamide gel electrophoresis
SDS	Sodium dodecyl sulphate
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TBS	Tris-Buffered saline
rpm	revolution per minute
^{o}C	Degree Celsius
v/w	volume per weight
v/v	volume per volume

Presentations arising from this work

(1) Poster Presentation: Makhoba. X. H and Shonhai. A. The development of a molecular chaperone-based system to improve the heterologous production of *Plasmodium falciparum* AdoMetDC protein in *E. coli*.

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Presented at: The 5th Annual Faculty of Science and Agriculture Research symposium, 25 November 2010

(3) Oral poster presentation: Xolani H. Makhoba[1], Abraham I. Louw[2], Lyn-Marie Birkholtz[2], Addmore. Shonhai[1]. The use of molecular chaperones system to improve the production of a unique bifunctional malarial drug target in *E. coli*.

Presented at an international conference: 2nd International Conference on the Role of Polyamines and their Analogs in Cancer and other Diseases. Polyamines meeting Rome-Tivoli, Italy, 1st - 6th December 2010.

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Chapter 1

Literature review

1. Introduction

Malaria is a major cause of death especially in developing countries. In 1880, researchers discovered that malaria was transmitted by bites of female Anopheles mosquitoes carrying the *Plasmodium* parasite (Bruce-Chuvatt, 1981). Following that report, Roland Ross in 1897, argued that the parasite could infect the female mosquito. He also observed that, the parasite completes its developmental cycle in the mosquito and when the mosquito takes another blood meal it passes on the parasite (Phillips, 1983). Four species of the Plasmodium genus cause malaria, namely: *Plasmodium falciparum, P. vivax, P. ovale, and P. malariae. P. falciparum* is reported as the most devastating infectious agent of human malaria. At least more than 1 million people die from malaria each year (Snow *et al.,* 2005). Studies conducted suggest that, young children appear to be at the highest risk of death from malaria, especially in Sub-Saharan Africa (Snow *et al.,* 2005). Unfortunately, drugs such as chloroquine face resistance from malaria parasites and this contributes to the high rate of morbidity and mortality due to malaria (Wellems, 2002). The application of Dichlorodiphenyltrichloroethane (DDT) to kill mosquitoes, removal of the potential-breeding grounds of *Anopheline* vectors by the draining of swampland (Sachs and Malaney, 2002), and also the use of bed nets to prevent mosquito bites are amongst the strategies

that have been used to control malaria. However, these efforts face huge challenges in addressing this problem in countries at high risk of malaria (Figure 1.1).

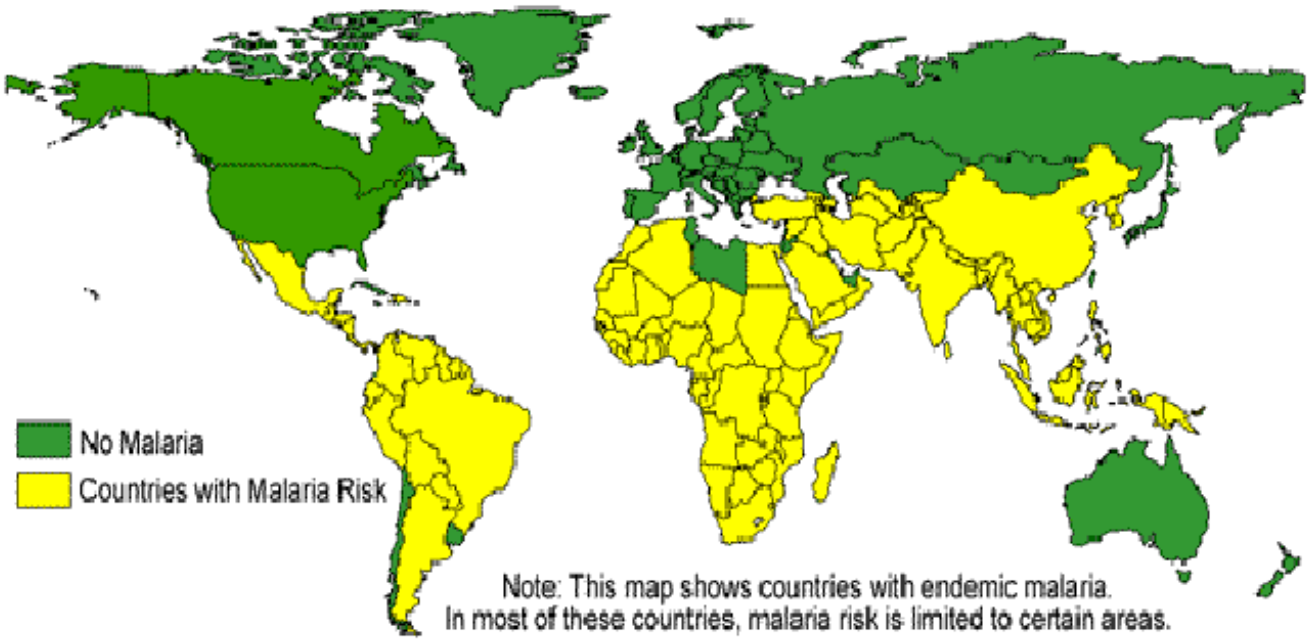


Figure 1.1 The regions where malaria is endemic (Adapted from US Centers for Disease Control and Prevention, 2003).

The search for alternative, effective drugs to combat malaria is ongoing. In coming up with new drugs, an understanding of the structure-function features of the target proteins is important. Therefore, to predict the nature and the function of some proteins, bioinformatics tools are normally used. Ultimately, the structural and functional property of most proteins is confirmed by conducting laboratory experiments during the design of drugs or inhibitors. On the one hand, characterization and identification of drug targets requires the production of the proteins in large quantities. Commonly used heterologous systems for recombinant protein expression include, *Saccharomyces cerevisiae, Pichia pastoris, and Escherichia coli (E. coli)* (Whitford, 2005). *E. coli* is the most famous host for the heterologous expression of recombinant proteins. The

advantages of *E. coli* as a protein expression host include high levels of heterologous expression and scalability of experiments, low cost, fast growth, a lack of post-translational modification and ability to express labelled proteins. However, expression of recombinant proteins in *E. coli* is known to be problematic due to several reasons. Some of the reasons are that some of the recombinant proteins are expressed in truncated forms or become insoluble inclusion bodies (Flick *et al.,* 2004). Mehlin *et al* (2006) analyzed 1000 genes from *P. falciparum* heterologously expressed in *E. coli* and reported that only 337 were successfully expressed. Of these, only 63 were reported as soluble proteins. The high A/T content of *P. falciparum* genes presents the main problem, as it gives rise to poor yields of fully expressed proteins (Flick *et al.,* 2004). Different approaches for improving the production of recombinant proteins have been suggested, but not all of them can be successfully applied for all recombinant proteins (Pandey *et al.,* 2002). There is an urgent need to come up with alternative methods that could be affordable and applicable to different proteins. Some of the strategies used to improve recombinant protein expression in soluble form include use of weak promoters, low temperature (Makrides, 1996), modified growth media (Georgiou and Valax, 1996 ; Makrides, 1996), codon harmonization and fusion with solubility enhancing tags (Davis *et al.*, 1999; Kapast and Waugh, 1999) and co-expression with molecular chaperones (Tresaugues *et al.,* 2004).

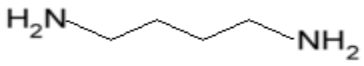
1.1 Molecular chaperones

Molecular chaperones are proteins that assist other proteins to fold properly, in order to form an active three dimensional protein structure (Ellis and Hartl, 1996). They also play an important role by preventing misfolding of other proteins during physiological stress (Lindquist and Craig 1988). *P. falciparum* is known to express a group of highly conserved molecular chaperones

called heat shock proteins (Hsps) under normal and stress conditions during its life-cycle (Gething and Sambrook, 1992). There is mounting evidence suggesting that the same protein acts as molecular chaperones that protect the parasite by being part of its protein folding machinery (Feder and Hofmann, 1999). For example, a cytosolic heat shock protein 70 (Hsp70) from *Plasmodium falciparum* (PfHsp70-1), has been previously shown to exhibit chaperone function when heterologously expressed in *E. coli* cells whose endogenous Hsp70 function was compromized (Shonhai *et al.*, 2005).

1.2 Polyamines as antimalarial drug target

Polyamines are naturally found in almost all organisms. Studies conducted shown that in mammalian cells they are found in millimolar concentration (Pegg and McCann, 1982). They are known to occur as organic polycationic form at physiological pH, because their primary and secondary amino acids are protonated. Therefore, putrescine occurs as divalent, spermidine trivalent and, spermine occurs as tetravalent (Figure 1.2) (Wallace, 1998).



Putrescine

Spermidine

Spermine

Figure 1.2 The structures of putrescine, spermidine and spermine (Adapted from Wallace, 1998)

Polyamines, as positively charged compounds react with negatively charged macromolecules within cells, such as phospholipids and nucleic acids. However, they differ from the normal bivalent cation such as Mg^{2+} or Ca^{2+}. Several studies have shown that polyamines are able to stabilise the DNA helix and other double stranded structures, including stems and loops in ribosomal RNAs, messenger RNAs and transfer RNAs (Tung *et al.*, 1993). These characteristics are thought to be the source for the stimulatory effects of the DNA, RNA and protein synthesis. The positive charge on polyamines is diffuse along the entire length of the flexible backbone chain, which makes electrostatic interactions between polyamines and macromolecules to become more flexible than that of the point charges of the bivalent cations. (Wallace *et al.*, 2003).

1.2.1 Variation between polyamine metabolic pathways in humans and *Plasmodium falciparum*

Polyamine metabolism in mammalian cells is regulated by two separate proteins, S-Adenosylmethionine Decarboxylase and Ornithine Decarboxylase (AdoMetDC and ODC). Mammalian cells are regulated by feedback mechanism and involve multiple routes of synthesis and interconversion (Figure 1.3A). Studies suggest that these complicated regulatory mechanisms enable cells to adapt to considerable changes in extra-and intracellular polyamine concentrations (Muller *et al.*, 2001). The way in which polyamine synthesis is regulated by ODC and AdoMetDC between *Plasmodial* and human systems differ (Figure 1.3). For example, in human cells, the decarboxylases ODC and AdoMetDC are highly regulated, and have short half-lives (<1 h), while in plasmodial systems they have longer half lives, and the pathway also lacks

spermine (Heby *et al.*, 2003; Roberts *et al.*, 2004). This could be the reason why this pathway has been proposed as a drug target.

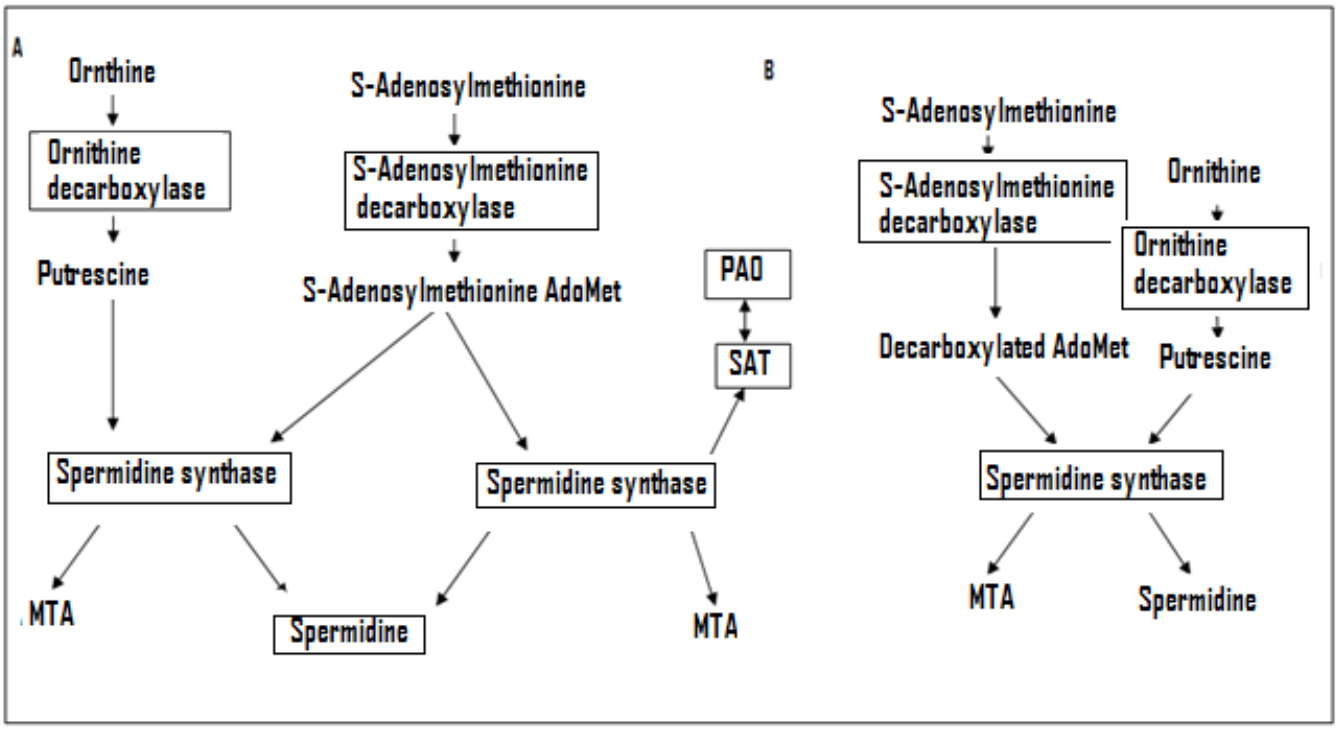


Figure 1.3 Synthesis and regulation of polyamines in Human and *Plasmodial* systems .(A) In human cells, Ornithe decarboxylase (ODC) and S-Adenosylmethionine decarboxylase (AdoMetDC) are highly regulated, and have very short half-lives (<1h), whereas the synthases of SpdS and SpmS are constitutively expressed. Spermidine/Spermine-N^1-acetyltransferase and Polyamine oxidase, provide a pathway for back conversionfrom spermine to spermidine to putrescine. (B) *Plasmodium falciparum* has a bifunctional AdoMetDC-ODC and lacks Spermine synthase. Abbreviations: MTA, 5'methylthioadinosine; SAT, spermine-N^1-acetyltranferase; PAO, polyamine oxidase (Adapted from Heby *et al.*, 2003)

Polyamine synthesis in *P. falciparum* is regulated by a single open reading frame that encodes both rate limiting enzymes in the polyamine pathway, namely Ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC) (Muller *et al.*,2001) (Figure 1.3B). AdoMetDC, is an enzyme that catalyses the formation of polyamines, such as putrescine and spermidine which are needed by parasite for proliferation, differentiation and growth (Heby *et al.*, 2003).

Ornithine decarboxylates to putrescine and subsequent attachment of aminopropyl groups to its terminal amino substituents to form spermidine. The aminopropyl groups are donors of decarboxylated S-Adenosylmethionine (dcAdoMet), in the presence of adenosylmethionine decarboxylase (AdoMetDC) (Figure 1.3B). Both Ornithine Decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC) are rate limiting in this step. Interference with polyamine biosynthesis by inhibition of the rate limiting enzymes (ODC) and (AdoMetDC) has been proposed as potential chemotherapy of cancer and malaria parasite infections (Muller *et al.*, 2001). A previously conducted study suggested that this pathway is unique to *P. falciparum* (Krause *et al.*, 2000).

1.2.2 Structural organisation of the bifunctional AdoMetDC/ODC from *P. falciparum*

A bifunctional enzyme from *Plasmodium falciparum* (PfAdoMetDC/ODC) is in the form of a heterotetramer of approximately 330 kDa, that is formed by 150 kDa heterotetrameric AdoMetDC (α and β subunits) and the 180 kDa homodimeric ODC (Birkholtz *et al.*, 2004) (Figure 1.4). The functional heterotetrameric complex therefore consists of two subunits each of the 160 kDa α-AdoMetDC/ODC and the 9 kDa β-AdoMetDC (Heby *et al.*, 2003; Muller *et al.*, 2000; Wrenger *et al.*, 2001).

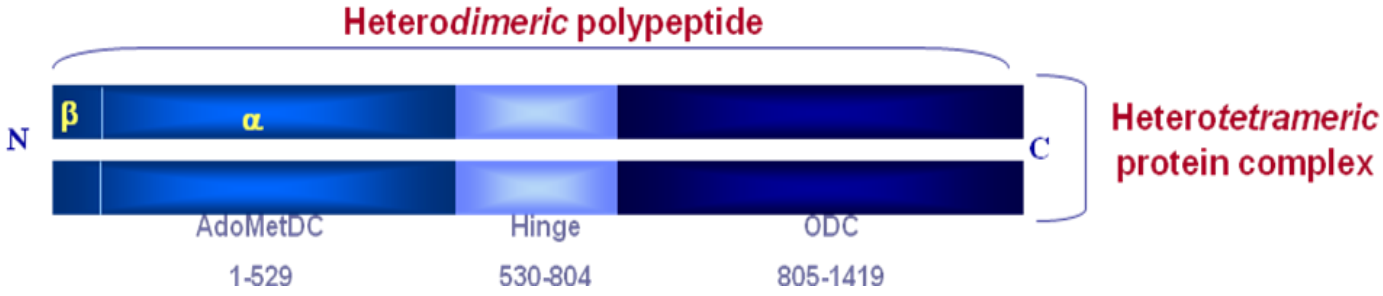


Figure 1. 4: Schematic organisation of the bifunctional AdoMetDC/ODC from *P. falciparum*. Adapted from (Birkholtz, 2002 and Niemand, 2007).

The PfAdoMetDC domain is found at N-terminal domain of the bifunctional peptide (residues 1-529). Like that of the human enzyme, it consists of an $(\alpha\ \beta)_2$ dimer, but unlike other AdoMetDC enzymes, the plasmodial enzyme is not stimulated by putrescine. The ODC sub-domain of the protein is found at the C-terminal of the bifunctional peptide, from residues 805-1419 (Figure 1.4). Polyamines such as putrescine and spermidine play an important role for differentiation, proliferation of cancerous cells and malaria parasite (*P. falciparum*). S-Adenosylmethionine decarboxylase/ornithine decarboxylase (PfAdoMetDC/ODC) has been described as an ideal antimalarial drug target, due to the important role it plays in biosynthesis of the polyamines (Muller *et al.,* 2000). However, the production of this large bifunctional protein to facilitate its biochemical characterization is hampered by the poor yields of recombinant protein. It has previously been proposed that co-expression of target recombinant malaria proteins with molecular chaperones of malarial origin in *E. coli* could improve the yield of the target recombinant proteins (Birkholtz *et al.,* 2008). It was proposed that PfHsp70-1 presumably protected *E. coli* cells against thermal stress by preventing protein aggregation. Therefore, PfAdoMetDC (sub-domain) and PfAdoMetDC/ODC (full-length protein) were co-expressed along with PfHsp70-1 in *E. coli* BL21 star cells to improve the yield and quality of PfAdoMetDC and PfAdoMetDC/ODC proteins.

1.3 Recombinant protein expression, highlight challenges

The over-expression of recombinant protein in host system still remains a problem and in most cases this results in the production of misfolded and aggregated protein. This could be due to the shortage of chaperones in the host system (Kiefhaber *et al.,* 1990). Some properties which makes it difficult to express recombinant proteins in a soluble form of plasmodial are as follows:

proteins with molecular weight greater than 56 kDa, isoelectric point (pI) which is greater than 6 and lack of homology in *E. coli* proteins (Mehlin *et al.,* 2006). In addition the presence of the *Plasmodial* specific inserts and signals peptides could have a negative impact on protein expression (Aguiar *et al.,* 2004; Mehlin *et al.,* 2006; Vedadi *et al.,* 2007). The heterologous expression of *P. falciparum* proteins is also a challenge, due to the codon mismatch. For example study previously conducted on PfAdoMetDC/ODC suggested that the expression of this protein comes out with three contaminating proteins (60 kDa, 70 kDa and 112 kDa) (Neimand, 2007).

Upon purification, the protein yield was poor and the product was contaminated by other protein species. Other studies have proposed that that the production of recombinant protein forms of multi-domain proteins results in their aggregation (Baneyx and Mujacic, 2004; Hartl and Hartl, 2009). This could be due to the incompatibility between the target proteins and host protein folding machinery (molecular chaperones) (Netzer and Hartl, 1997; Chang *et al.,* 2005). On the other hand, it has also been shown that efficient folding should occur in proteins with few domains whose motifs could be easily recognised by chaperones (Agashe *et al.,* 2004). However, numerous techniques have been proposed to improve the recombinant protein production in soluble forms. This includes codon harmonization (matching the codons of the targeted DNA with host system) and co-expression with molecular chaperones.

1.3.1 Molecular chaperones as tools to improve recombinant protein expression

Molecular chaperones can generally be described as proteins that protect non-native proteins to become stable towards their development into an active, three dimensional structure (Hartl and Hartl, 2002). They are known to bind to the hydrophobic patches of non-native proteins in order

to stabilise their elongation upon exiting the ribosomes. The role of molecular chaperones is accomplished by specialized sub-families of these proteins with diverse structures (Hartl and Hartl, 2002). Heat shock proteins form the bulk of the cell's molecular chaperones. Their first recognition takes back when Drosophila salivary gland cells were exposed to heat shock at 37^{o}C and taken back to a normal temperature of 25^{o}C for recovery. It was then noticed that on recovery the cells displayed "chromosomal puffs", due to expression of heat shock proteins. These newly produced proteins were then analysed, and found to have a molecular weight of 70 kDa and 25 kDa. Therefore, these proteins were named 'heat shock proteins' (Schlesinger, 1990). Heat shock proteins can be categorized into different categories namely: Hsp100, Hsp90, Hsp60, Hsp70, Hsp40 and small heat shock proteins (Table 1).

The most interesting characteristic is that molecular chaperone possesses the ability to recognise and bind newly synthesized proteins, in order to prevent misfolding and aggregation. However, the high concentration of misfolded and aggregated proteins could over-whelm the molecular chaperones in the cell. Therefore, cells need a rapid and precise system to eliminate aggregated and misfolded proteins. Some of the molecular chaperones are known to be ATP-independent; therefore, they fall under the so-called, 'holding' group of molecular chaperones. 'Holding' chaperones refer to those whose function is to bind nascent peptides and pass them to ATP-dependent counterparts that are capable of folding the peptides into functional forms. On the other hand, ATP-dependent molecular chaperones are grouped under folding and unfolding forms. Their function is to protect newly synthesized proteins to fold properly and unfold misfolded protein to form a correct structure (Mayer *et al*., 2000; Schubert *et al.,* 2002).

Table 1.1: Major groups of heat shock proteins

Heat shock proteins	Role	Reference
Small heat shock protein	Small heat shock proteins are known to exist in all organisms, their molecular weight ranges between 16-30 kDa.They are up regulated in various diseases such as neurogeneretive disease and damaged motor neurons cell. They are also involved in the maintenance of the native structure of cytosolic proteins	Sun and MacRae, 2005; van den Ijssel., 1999
Heat shock protein 40 (Hsp40)	It acts as a co-chaperone partner of Hsp70. It also recognizes and binds unfolded proteins handing them over to Hsp70 for proper folding. Stimulate the hydrolysis of ATP by ATPase.	Suh *et al.,* 1999; Junbin *et al*., 2008
Heat shock protein 60 (Hsp60)	Heat shock protein 60 is in mitochondrial matrix and is responsible for the transportation and refolding of proteins from cytoplasm to the mitochondrial matrix. It plays an important role as a chaperonin in proper folding of linear amino acid chains to acquire a three-dimensional structure.	Cheng *et al.,* 1990
Heat shock protein 70 (Hs70)	Shielding of newly synthesized proteins, involved in protein translocation, degradation of misfolded proteins and disaggregation of protein units. It also protects cells from thermal stress	Misra *et al*., 2009; Tavara *et al.,* 1996;
Heat shock protein 90 (Hsp90)	Involved in transcriptional regulation, signal transduction, and cell cycle control.	Bagatell and Whitesell , 2004
Heat shock protein 100 (Hsp100)	Is involved in repairing of denatured proteins by resolubilization of heat-denature and misfolded proteins.	Krobitsch *et al*., 1998

1.3.2 Hsp70

Heat shock protein 70 (also known as DnaK in prokaryotic cells) is the most studied type of molecular chaperones. They are produced under both normal and stressful conditions such as high temperature, and toxicity. In eukaryotic cells Hsp70s are involved in various activities such as nascent folding of polypeptides, protein translocation, and disaggregation of protein units, DNA replication and signal transduction (Gambill *et al.,* 1993; Egger *et al.,* 1997; Song *et al.,* 2005). Hsp70 selectively bind to the hydrophobic patches of the substrate protecting it from the crowded environment of the cell for proper folding (Liberek *et al.,* 1991; Qiu *et al.,* 2006). Trigger factor (TF) is the first molecular chaperone to interact with newly synthesized proteins. Previously conducted studies suggest that TF interacts especially with short newly synthesized proteins by recognizing hydrophobic patches exposed in the crowded environment of the *E. coli* system (Patzelt, 2001). However, its role as a folding chaperone is taken over by Hsp70 a homolog of DnaK (a homologue of Hsp70 in prokaryotes) which cooperates with Hsp40 (also called DnaJ in prokaryotes) and nucleotide exchanger GrpE during the synthesis of the proteins (Deuerling *et al.,* 1999; Teter *et al.,* 1999). Table below summarizes some of the co-chaperones partners and co-factors needed and their specific roles in modulating the role of Hsp70

Table 1.2: Hsp70 co-chaperone partners

Names	Roles	References
Hsp40,	Hsp40 recognises a substrate and hands it over to Hsp70 for binding.	Cintron and Toft, 2006 Frydman and Hartl, 1996
Substrate, nature of substrate	Is a newly synthesized protein with hydrophobic stretches exposed in the crowded cellular system.	Peter *et al.*, 2003; Erbse et al., 2004
ATP	Essential for Hsp70 activity	Lopez-Buesa *et al.*, 1998; Rao *et al* .,2010
GrpE/nucleotide exchange factor	Mediates the binding and release of the substrate through binding to Hsp70, thereby affecting nucleotide exchange.	Harrison, 1997
Mg^{2+}	Induces the dissociation of the polypeptide from Hsp70.	Daniel *et al.*, 1991

1.3.3 Structure and functional domains of Hsp70

The heat shock protein 70 (Hsp70), is made up of three distinct domains, with different sizes. The N-terminal domain, also known as ATPase domain is 45-kDa, followed by substrate binding domain, whose size is 15 kDa and a C-terminal domain, with 10 kDa size (Flaherty *et al*, 1990) (Figure 1.3). Substrate binding domain has as groove with affinity to bind neutral and hydrophobic stretches of amino acids (Flaherty *et al*, 1990).

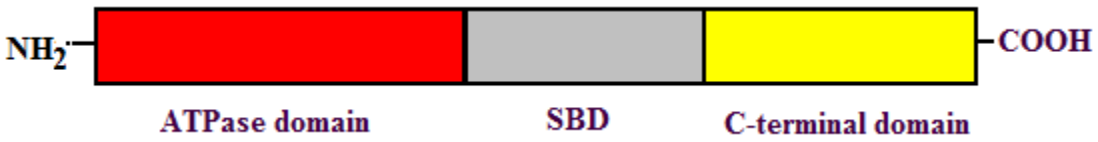


Figure 1.5 The structural domains of Hsp70. The N-terminus consists of an ATPase domain linked to a central substrate-binding domain, and the C-terminus consists of a lid region (adapted from Shonhai, 2007)

The C-terminal domain has alpha helical lid which is involved in binding of the substrate. This means that when Hsp70 is in an ATP bound state the lid is opened but when ATP has been hydrolysed to ADP the lid is closed. Thus Hsp70 has affinity for substrate in its ADP bound state; its affinity for substrate is reduced in the ATP bound state.

1.3.4 Heat shock protein 40 (Hsp40)

Hsp40 is a family of heat shock proteins that occur in different organisms including humans, bacteria and parasites. Their major role is to stimulate the ATPase activity of Hsp70s. Members of the Hsp40/DnaJ family of proteins have three distinct domains, (i) a highly conserved J domain of approximately 70 amino acids, often near the N terminus, that is responsible for the interaction with Hsp70, (ii) a glycine and phenylalanine (G/F)-rich region that putatively acts as a flexible linker; and (iii) a cysteine-rich, zinc finger-containing C-terminal domain (Cheetham and Caplan, 1998) (Figure 1.6).

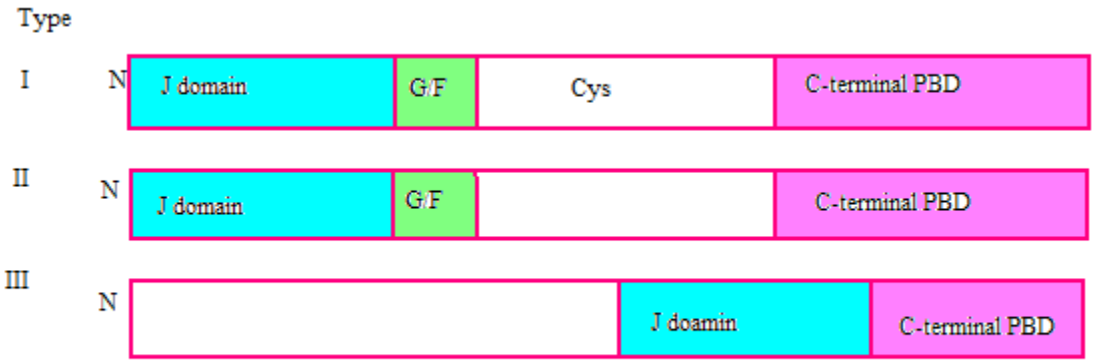


Figure 1.6 The structural domains of Hsp40s.
Linear representation of Hsp40 showing the different sub-domains that characterise each type of Hsp40 protein. The different sub-domains represented here are as follows: the J domain, glycine/phenylalanine-rich region (G/F), cystein-rich region (Cys) and the C-terminal peptide binding domain (C-terminal PBD). The N-terminus is shown as 'N' (adapted from Cheetham and Caplan, 1998).

1.3.5 Function of Hsp70 is regulated by Hsp40

The activity of Hsp70 is regulated by different factors to carry out its function. The hydrolysis of ATP by Hsp70 on its own is very slow; hence this step is potentially rate-limiting. Therefore, co-chaperones play an important role as partners in assisting the Hsp70 to carry out its function with success. Hsp70 carry out its function as follows: (A) Hsp40 binds an incorrectly folded protein (B) Hsp40 targets protein substrate to the ATP-bound Hsp70 and stimulate the hydrolysis of the ATP by ATPase to release inorganic phosphate(Pi) (C) Hsp70 become ADP bound which has high affinity for a substrate to bind (D) Nuclear exchange factor (NEF) regulate the nucleotide exchange during its binding to the Hsp70-sustrate protein complex (E) ATP replaces the ADP, thus resulting in the structural changes of Hsp70 hence the affinity for substrate binding become less, this allows the release of the substrate. Then the correctly folded protein can enter the cycle in the cellular system until it becomes correctly folded (Figure 1.5).

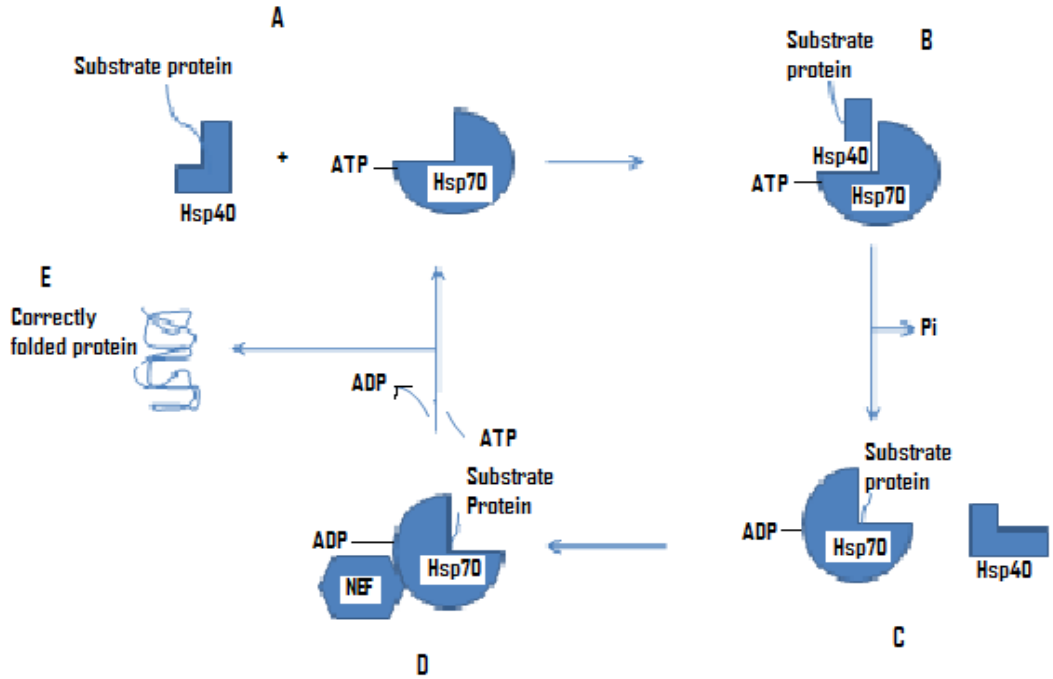


Figure 1.7: **Cycle of Hsp70 chaperone system**. (Adapted from Pesce and Blatch, 2009).

1.4 Hsp70s from *Plasmodium falciparum*

P. falciparum is known to possess six Hsp70 homologues (known as PfHsp70-1, PfHsp70-2, PfHsp70-3, PfHsp70-x, PfHsp70-y and PfHsp70-z) (Table 3), which localizes to different sub-cellular organelles such as, endoplasmic recticulum, mitochondria, nucleus and cytoplasm (Sargeant *et al.*, 2006; Shonhai *et al.*, 2007). The comparative studies conducted on these proteins suggest that they have been found to have a high percentage of similarities under pair wise sequence identity analysis. PfHsp70-1 (Nucleus and Cytoplasm) and PfHsp70-x (Cytoplasm); have been found to show a high percentage (74%) of identity. PfHsp70-x and putative ER homology PfHsp70, also show (54%) of identity. Also PfHsp70-2 (Endoplasmic recticulum) and mitochondrial homology PfHsp70-3 share (46%) of similarity. It has been suggested that, other proteins or the rest of the proteins share a very low identity between them, suggesting that PfHsp70s share a different range of identity between them might be an indication of the wide range of activities that the chaperones could play across the different organelle locations in which they occur (Shonhai *et al.*,2007).

Table 1.3: Hsp70s from *P.falciparum*

Names	**Cellular Localization**	**Molecular weight (kDa)**	**References**
Hsp70-1	Nucleus and Cytoplasm	74	Sharma, 1992
Hsp70-2	Endoplasmic recticulum	73	Kumar *et al* .,1991
Hsp70-3	Mitochondrion	76	Sargent *et al.*, 2006
Hsp70-x	Cytoplasm	73	Sargent *et al.*, 2006
Hsp70-y	Cytoplasm	100	Sargent *et al.*, 2006
Hsp70-z	Endoplasmic recticulum	108	Sargent *et al.*, 2006

1.4.1 *Plasmodium falciparum* Hsp70-1 protein

Malaria parasite involves two hosts during its life cycle. Cold blooded mosquito parasite vector and warm-blooded human (Figure 1.8). Due to the variations of temperature between the vector and the host, the parasite expresses heat shock proteins to ensure maintenance of the stability of its proteome (Sharma, 1992).

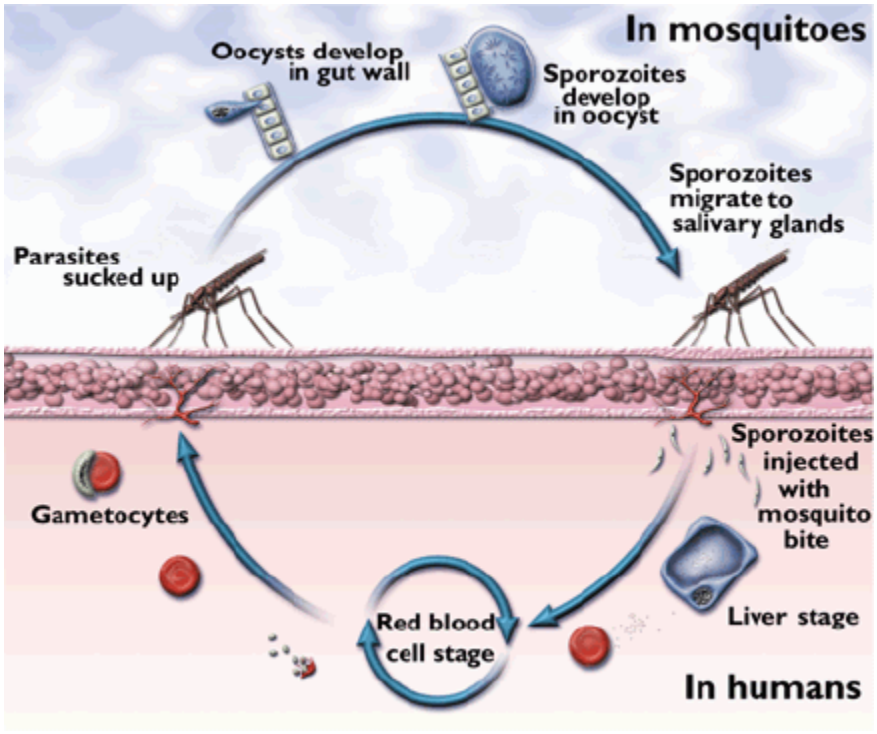


Figure 1.8 Schematic presentation of the *Plasmodium* life cycle

After parasite has been sucked up by mosquito, Oocysts develop in the gut wall of the mosquito, then Sporozoites develop in oocyt, which Sporozoites migrate to salivary glands. When mosquito bites then Sporozoites are injected to human as second host of the parasite. These enter liver cells where they multiply for about 7 to 14 days before producing between 10,000 and 30,000 daughter cells called merozoites, which invade red blood cells. The parasite multiplies in the red cells, which invade red blood cells. The parasite multiplies in the red cells, again by asexual reproduction, to produce 8 and 16 merozoites every 48 or 72 hours, depending on the species of *Plasmodium*. These merozoites are released by the bursting of the red blood cells and the release of toxic substances from within cause the fibrile attacks of the disease. After a number of such cycles, sexual stages, male and female gametocytes, are produced, and these are taken up by a feeding mosquito, in which the *Plasmodium* life cycle is completed by sexual reproduction, resulting in new sporozoites (Adapted from Donald, 2002).

PfHsp70-1 found in cytosol has received most research attention because of the characteristics that it possesses as a chaperone (Kumar *et al.,* 1990). The molecular weight of this protein is approximately 74 kDa and it posses EEVD motif that enables it to interact with co-chaperones that act via C-terminus (Ramya *et al.,* 2006). PfHsp70-1 has been previously shown to exhibit chaperone function when heterologously expressed in *E. coli* cells whose endogenous Hsp70 (DnaK) function was compromized (Shonhai *et al.,* 2005). It was proposed that PfHsp70-1 presumably protected the *E. coli* cells against thermal stress by preventing protein aggregation in *E. coli*.

Molecular chaperones from *P. falciparum* especially cytosolic heat shock proteins 70 (Hsp70-1) could be justified as potential co-expression tools. PfHsp70-1 recognises typical Hsp70 peptide substrates, because of its ability to suppress protein aggregation in vitro (Shonhai *et al.,* 2008). Previously conducted studies suggest that PfHsp70-1 could recognise different substrates from *P. falciparum* including those that are found in apicoplast (LaCount *et al.,* 2005; Botha *et al.,* 2007; Shonhai *et al.,* 2007).

The need for overproduction of target protein in original host system is still a challenge for downstream studies. On the other hand co-expression of molecular chaperones and target proteins from the same species but cloned on different plasmids has been reported to improve the production of some target proteins in *E. coli* (de Marco *et al.,* 2004; Tolia and Joshua-Tor, 2006).

1.5 Research hypothesis

Over-production of recombinant protein is hampered by the formation of aggregates that results in the formation of inclusion bodies. To overcome this problem, previously conducted studies have shown the success in the application of molecular chaperones to improve the production and solubility of the target protein. On the other hand, Shonhai *et al* (2005) argued that heat shock protein 70-1 from *Plasmodium falciparum* demonstrated chaperone characteristics by suppressing the thermosensitivity in *E. coli dnaK756* strain at 43.5^{o}C. Hence, PfHsp70-1 could be used as co-expression partner to improve the expression of *P. falciparum* AdoMetDC and AdoMetDC/ODC in *E. coli*.

Based on this hypothesis the following are the aims of this study:

1) To co-express *P. falciparum* AdoMetDC/ODC and AdoMetDC (wild type or codon harmonized forms) along with PfHsp70-1 in *E. coli* BL21 star cells.
2) To establish the solubility status of the recombinant PfAdoMetDC proteins obtained.
3) To purify PfAdoMetDC and PfAdoMetDC/ODC using strep-tectin column.
4) To determine the yield and quality of PfAdoMetDC and PfAdoMetDC/ODC proteins using sodium dodecyl sulphate polyacrylaminde gel electrophoresis and to conduct Western blot as well as Bradford's assay to determine the concentration of the proteins.

2.0 Materials and Methods

The different plasmid constructs were expressed in *E. coli* BL21 star cells as an expression host system. pASK-IBA3/PfAdoMetDC/ODC (expressing wild type and codon harmonized PfAdoMetDC/ODC) and pASK-IBA3/PfAdoMetDC (expressing wild type and codon harmonized PfAdoMetDC) were kind gifts from Prof .L.M. Birkholtz (University of Pretoria). Figure 3.1 shows the domain organization of the PfAdoMetDC/ODC and its derivatives.

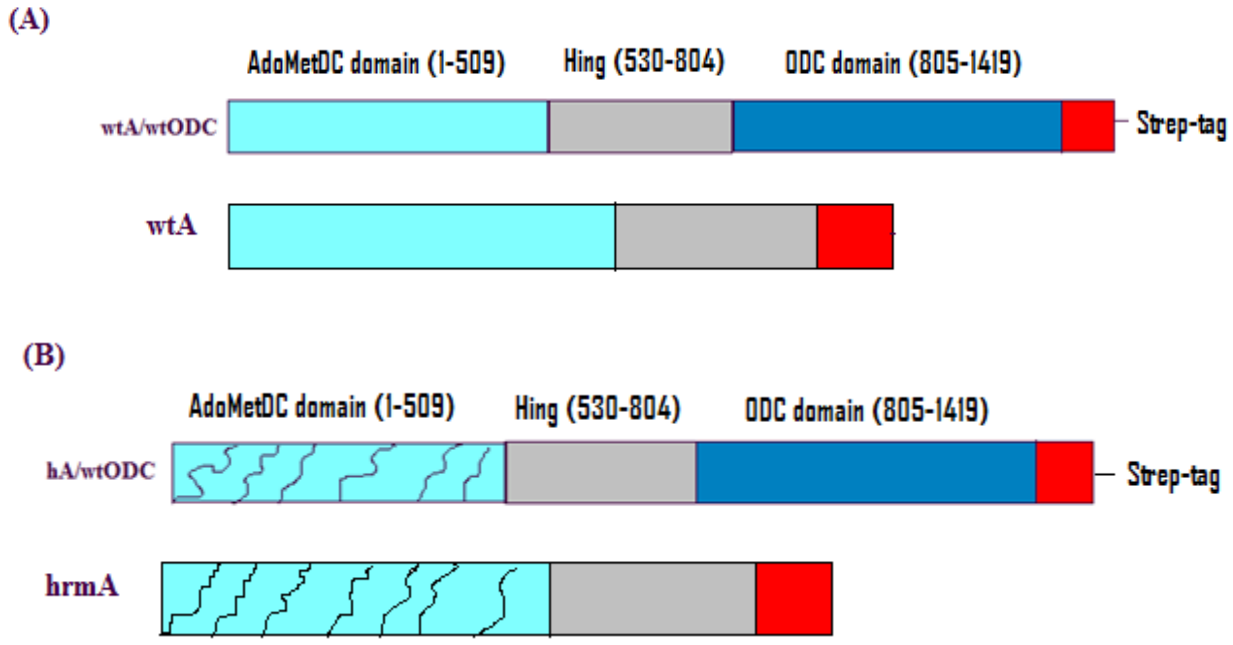


Figure 3.1: Schematic presentation of monofunctional PfAdoMetDC and bifunctional PfAdoMetDC/ODC. (A) bifunctional wild type PfAdoMetDC/ODC and monofunctional wild type PfAdoMetDC (B) bifunctional codon harmonized PfAdoMetDC/ODC and monofunctional codon harmonized PfAdoMetDC with C-terminal Strep-tag II (red colour).

pMRBAD/PfHsp70-1 was prepared and donated by Dr .A. Shonhai (Stephens *et al*., 2011). To facilitate co-expression of the plasmid, it was important to ensure that they had different promoters (araBAD promoter under the control of the araC gene versus tet promoter/operator under the control of the tet repressor gene) and modes of resistance. Thus pMRBAD vector is on

Kanamycin, while pASK-IBA3 vector is on Ampicillin. The pASK-IBA3 vector hosts a C-terminal strep-tag II encoding sequence which ensured that the PfAdoMetDC/ODC and PfAdoMetDC proteins expressed had C-terminal strep-tag II to facilitate their purification.

2.1 Plasmid isolation

A colony of transformed cells was picked from agar plate with appropriate anti-biotic (Ampicillin or Kanamycin) and inoculated in a test tube containing 5 ml 2YT liquid broth (1.6% w/v Tryptone, 0,5% w/v NaCl and 1% w/v yeast) and incubated overnight at 37^0C with shaking at 160 rpm. The culture was then transferred into microcentrifuge tubes and centrifuged at 13000 x g for 1 minute. A pellet cell was resuspended in 100 µl of solution I to which lysozyme was added at final concentration of 2 mg/ml, followed by running microcentrifuge tube along test tube rack to resuspend the pellet. Then micro-centrifuge tubes were incubated for 5 minutes at room temperature and placed on ice for 5 minutes, followed by addition of 200 µl of Solution II, again it was incubated on ice for 5 minutes, immediately 150 µl of Solution III was added. Then microcentrifuge tube was centrifuge for 10 minutes at 13000 rpm (room temperature), then supernatant was transferred into new a micro-centrifuge tube, 300 µl of 100% isopropanol was added, again microcentrifuge tube was let stand for 10 minutes at room temperature, then centrifuged as before (10 minutes,13000 x g at room temperature). Then after that, pellet was washed with 800 µl of cold 70% ethanol, followed by spinning for 2 minutes at 13000 x g (to compact pellet), ethanol was poured off, microcentrifuge tube was dry for 30 minutes in heating block at 37^oC, after that 50 µl of TE buffer was added, DNA was ready for use and it was stored at -80 freezer.

Alternatively the Zyppy ^{TM} Plasmid Miniprep Kit was used to isolate the plasmid DNA following the supplier's instructions.

2.2 DNA quantification

DNA was calculated after the absorbance was measured at absorbance of 260 (A_{260}) from spectrophotometer, using the following equation:

$$\text{DNA concentration } (\mu g/ml) = \frac{OD_{260} \times 100 \text{ (dilution factor)} \times 50\ \mu g/ml}{1000}$$

One absorbance unit at 260 nm is equal to 50 ng/µl double stranded DNA and 33 ng/µl single stranded.

2.3 Restriction analysis of plasmid DNA

To confirm the integrity of the pASK-IBA3/PfAdoMetDC/ODC (Wild type and Harmonised plasmid contains full length gene), and pASK-IBA3/PfAdoMetDC (wild type and harmonized contains sub-domain) and pMRBAD/PfHsp70-1, after the plasmid isolation following above mentioned method (2.2.1). The DNA plasmids were digested using diagnostic restriction enzymes such *Xba* I, *Hind* III, *Bam* HI. Then, the products were analyzed by agarose gel electrophoresis in order to estimate the size of the fragments.

Table 1: DNA digestion set-up

Component	**Volume**
Water nuclease-free	16 µl
10x Fast Digest green buffer/10x Fast Digest buffer	2 µl
DNA	2 µl
Fast Digest enzyme	1 µl
Total Volume	**21 µl**

To prepare 0.8% of agarose, recommended volumes were added 10X TAE buffer (4.84% w/v Tris-HCl, 1.09% v/v glacial acetic acid, 0.3% EDTA) and boiled until the mixture became clear. The mixture was allowed to cool and ethidium bromide was added (10% v/v). A comb was put into the casting tank to create wells and agarose gel was poured. The agarose gel was allowed to solidify and the comb was removed. The gel was put into the electrophoresis tank and filled with 1x TAE buffer. A 5 µl of O'Gene Ruler TM 1 kb DNA Ladder (Fermentas life sciences) was loaded on the first well and 20 µl of DNA samples cut with appropriate restriction enzymes were allowed to run for 1 hour at 100 V on agarose gel electrophoresis.

2.4 Preparation of competent cells

Competant cells were prepared as follows: The *E. coli* glycerol stock cells (15% v/v glycerol) from -80^{o}C were grown onto agar plates (1.6% w/v tryptone, 0.5% w/v NaCl, 1% w/v yeast and 1.5% w/v nutrient agar) without anti-biotic overnight at 37^{o}C. Then a positive colony was picked and inoculated in 5 ml 2YT liquid broth (1.6% w/v tryptone, 0.5% w/v sodium chloride and 1% w/v yeast) which was then incubated overnight in a shaking platform (162 rpm) at 37^{o}C. After 16 hours, 250 µl of culture was diluted into 50 ml fresh 2YT liquid broth (1.6% w/v tryptone, 0.5% w/v NaCl, 1% w/v yeast) and incubated for 1 hour with agitation (162 rpm) at 37^{o}C until an OD_{600} = 0.3-0.6 was reached. Then cells were centrifuged for 10 min at 5000 x g at 4^{o}C in an Epperndoff model centrifuge. The supernatant was removed and the tubes were incubated on ice followed by addition of 10 ml of magnesium chloride (0.1M $MgCl_2$) and allowed to stand on ice for 20 minutes. After 20 minutes tubes were centrifuged again for 10 minutes (4000 x g Eppendorf centrifuge model). The supernatant was removed and 10 ml of calcium chloride (0.1 M $CaCl_2$) was added into tube and followed by incubation on ice for 2-4 hours. After centrifuge

(4000 x g for 10 min) the pellet was dissolved in 3 ml of (0.1 M $CaCl_2$) and 30% glycerol (30% v/v glycerol). After that, cells were aliquoted in 100 µl amounts in eppendorf tubes then stored at -80^{o}C freezer.

2.5 Transformation of competent cells

E. coli strain competent cells BL21 star cells stored at -80^{o}C were thawed on ice. 20 ng of plasmid DNA plasmid were added to 100 µl cells and then followed by incubation on ice for 30 minutes. To induce uptake of the DNA the cells were heat-shocked at 42^{o}C for 45 seconds into heating block and followed by incubation on ice for 2 minutes. 900 µl of 2xYT broth was added to the bacterial cells followed by incubation on shaking platform at 37^{o}C for 1 hour allowing cells to grow in absence of antibiotics. After 1 hour of incubation the culture was plated 50-100 µl onto agar plates supplemented with either 50 ug/ml of kanamycin or 100 ug/ml ampicillin, depending on the expression vector that was used for cloning. The plates were incubated overnight at 37 ^{o}C to confirm transformed cells.

2.6 Recombinant protein expression of bifunctional PfAdoMetDC/ODC

E. coli BL21 star cells were used as a recombinant expression host system for PfAdoMetDC/ODC and PfAdoMetDC proteins that were expressed off the pASK-IBA3 plasmid using the strep-tag purification system, which involves the interaction between biotin and streptavidin. The plasmid encodes for a C-terminal expressed Strep-tag II (WSHPQFEK), which is known to bind to the strep-tactin Sepharose with high affinity, allowing for the isolation of fusion under physiological conditions (IBA GmbH, Germany).

PfAdoMetDC/ODC (full length expressed at 22^{o}C culturing temperature) and PfAdoMetDC (Sub-domain expressed at 37^{o}C culturing temperature) were transformed into *E. coli* BL21 star cells without application of chaperones of PfHsp70-1. pMRBAD/PfHsp70-1 was transformed into the same strain to confirm its expression in this strain before the co-expression. For pMRBBAD/PfHsp70-1 the expression is under the transcriptional control of araBAD in the presence of the sugar L-arabinose (0.2%) (Malgliery *et al*., 2004). Then, the transformed cells were plated onto agar plates supplemented with 100 μg/ml ampicillin, for PfAdoMetDC/ODC and PfAdoMetDC; on one hand transformed cells were plated on agar plates supplemented with 100 μg/ml of kanamycin for pMRBAD/PfHsp70-1. A positive colony was picked and grown overnight in 10 ml liquid broth containing appropriate antibiotic (ampicillin and kanamycin) agitating at 37^{o}C incubator. Then, the saturated culture was diluted 1:100 and grown further until OD_{600} =0.4 was reached at 37^{o}C in shaking incubator.

For both PfAdoMetDC/ODC and PfAdoMetDC the expression is under the transcriptional control of the tet-repressor, of which gives rise to a balanced stoichiometry between the numbers of plasmids and the repressor molecules. The tet operator/promoter is not functionally connected to the genetic background or other inherent bacterial machinery. Therefore the recombinant expression of the protein only occurs after the chemical induction with anhydrotetracycline (AHT), which prevent the 'leaky' expression. Therefore, recombinant protein expression was induced by addition of 200 ng/ml of AHT (IBA GmbH, Germany), and samples were collected hourly at various temperature. The full length PfAdoMetDC/ODC was grown at 22^{o}C for 16 hours whereas the sub-domain PfAdoMetDC was grown at 37^{o}C for 4 hours. Followed by

resuspending the pellet into PBS after spectrophotometric reading and stored at -20^{o}C followed by analysis on SDS-PAGE.

2.7 Co-expression of either PfAdoMetDC/ODC or PfAdoMetDC with pMRBAD/PfHsp70-1

PfAdoMetDC/ODC was transformed with pMRBAD/PfHsp70-1 molecular chaperone in the same competent cells following the above mentioned method (2.2.6). The transformed cells (50-100 µl) were inoculated onto liquid broth supplemented with both anti-biotics (100 µg/ml ampicillin and kanamycin), and incubated overnight at 37^{o}C. Furthermore, a positive colony was inoculated in 2YT liquid broth containing both anti-biotics (50 µg/ml ampicillin and kanamycine). Below is the schematic diagram which summarizes this method that was used for co-expression (Figure 2.1).

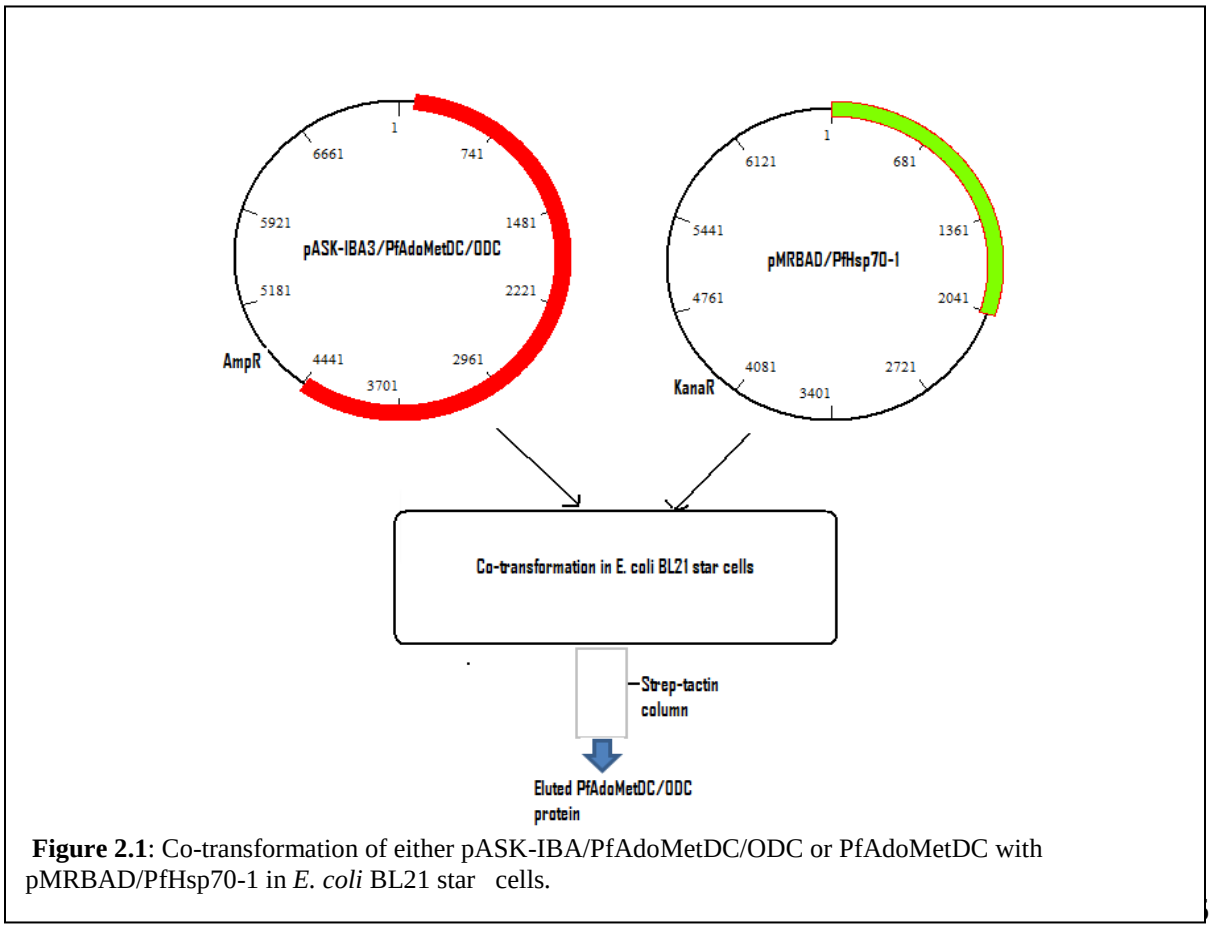


Figure 2.1: Co-transformation of either pASK-IBA/PfAdoMetDC/ODC or PfAdoMetDC with pMRBAD/PfHsp70-1 in *E. coli* BL21 star cells.

Once early–logarithmic growth phase was reached (OD_{600nm} = 0.4), the culture was inoculated with 0.2 % L–arabinose in order to induce the chaperone plasmid inorder to produce a molecular chaperone to be available first in the system before the target protein; a pre–induction sample was taken. The culture was further incubated at 37 ^{o}C with shaking until an absorbance of 0.5 was reached. The culture was then inoculated with 200 ng/ml of AHT in order to induce the PfAdoMetDC/ODC; the cells were grown for 16 hours at 22^{o}C in a shaking platform.

2.8 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of proteins

The short glass plate was put on top of a spacer plate. The plates were put into the green casting frame on a flat surface and the casting frame was clamped and put into the casting stand. The 7.5% of the separating gel (30% bis-acrylamide, 1.5 M Tris pH 8.8, 10% SDS, 10% APS and Temed) was poured between the glasses and distilled water was added on top to level it. The gel was allowed to solidify for 30 minutes. After 30 minutes distilled water was removed and 4% of the stacking gel (30% bis-acrylamide, 0.5 M Tris pH 6.8, 10% SDS, 10% APS and TEMED) was added on top and the comb was put to create wells and allowed to solidify for 30 minutes. The comb was removed after 30 minutes. The gel was put into electrophoresis tank and 1x SDS running buffer was poured into the tank. Diluted protein sample 4:1 was loaded on the second wells after 2 µl of protein ladder was loaded. The gel was allowed to run for 1 hour at 100 V. The gel was put in a container and covered with coomassie brilliant blue solution and left shaking for the 3 hours. Coomassie blue was then followed by distained from the container on the following day and distaining solution was added and allowed to shake until the gel was clear.

Also the Page Silver ^{TM} staining kit (Fermentas Life Sciences) was used following the supplier's instructions, due to the poor visualization of the protein.

2.9 Western blotting

After the SDS-PAGE, PfAdoMetDC/ODC and PfAdoMetDC and molecular chaperones pMRBAD/PfHsp70-1 their expression was confirmed with Western blotting. This was carried out onto nitrocellulose for 1 hour at 100 V. This was followed by visualization of protein with ponceau stain (stain for 2 min and destain with distilled water). Washed twice with TBS (50mM Tris, 150 mM Nacl), then membrane was marked. This was followed by blocking the membrane with 5% of blocking buffer (5 g in 100 ml of TBS) for 1 hour. The membrane was incubated with primary antibody. PfAdoMetDC/ODC and PfAdoMetDC is tagged with Strep-tag II, therefore, polyclonal Strep-tag II antibodies (Merck) were used for its detection. On the other hand, pMRBAD/PfHsp70-1 anti-Hsp70 antibodies were used for its detection. After overnight incubation with primary antibody, the membrane was washed twice with TBS-T for 20 minutes, followed by incubation with secondary antibody in 5% blocking buffer for 1hour on ice with shaking platform. The membrane was washed four times with TBS-T for 15 minutes each. The chemilumiscence reagents were prepared, solution (A) and (B) of which both are light sensitive, therefore, were covered by foil, detection reagent was added to the blot incubated for 60 sec in Chemidock (Bio-Rad).

2.10 Stripping and reprobbing membranes

For co-expressed proteins the membrane was submerged in stripping buffer (100 mM 2-Mercapthoethanol, 2% w/v SDS, 62.5 mM Tris-HCl pH 6.7) and incubated at 50^{o}C for 30 minutes with accessional agitation. The membrane was washed twice for 10 minutes each in PBS or TBS-T at room temperature using large volumes of washing buffer. This was followed by

blocking the membrane in blocking solution for 1 hour at room temperature. The immunodetection was repeated following for the above protocol (Western blot protocol).

2.11 Solubility studies conducted on PfAdoMetDC (wild type and codon harmonized version)

To confirm whether the protein was expressed soluble on its own and in the presence of chaperone PfHsp70-1 in *E. coli* BL21 star cells. The cell suspension were span at 3500 rpm for 20 minutes at 4^{o}C and resuspended in 5 ml lysis buffer (0,01 mM Tris,pH 7.5; 10 mM Imidazole,containing 1 mM (aminoethyl benzenesulforyl floride hydrochloride (AEBSF) and 1 mg/ml of lysozyme). The cells were frovezen overnigth at -08^{o}C. The cells were thawed rapidly and mildly sonicated. The cell lysate was centrifuge at 13000 rpm at 4^{o}C for 20 minutes. The supernantant was collected (soluble fraction) and the pellet was resuspended in 3 ml of phosphate buffered saline solution (pH 7.5) and this constituted the insoluble fraction. This was follwed by analysis of the samples on SDS-PAGE gel.

2.12 Use of Strep-Tactin affinity for protein purification

After packing the column with 1 ml strep-tactin, the column was washed twice with 10 ml of Buffer W (150 mM Nacl, EDTA, 100 mM Tris, pH 8). The supernatant was let to flow three times through the column followed by a wash three times with Buffer W. A protein was then eluted with 3 ml of Buffer E (2.5 mM D-Desthiobiotin in Buffer W, for 100 ml use 0.053575 g). The column was regenerated with 10 ml of buffer R (1 mM 4-hydroxyl azobenzene-2-carboxylic acid (HABA) in Buffer W), which was then washed out with buffer W until the strep-tactin became white or clear, the column was stored at 4^{o}C in buffer W.

2.14 Protein concentration determination

To determine the concentration of the protein after its purification, the Bradford method was used for protein quantification (Bradford, 1976). It is based on the electrostatic binding of the sulfonic groups of the Coomassie Brilliant G-250 dye to basic and aromatic amino acids, which results in the shift in the absorbance of the dye from A_{465} to A_{595}. After the addition of 50ul of protein in 150 ul of the Quick start TM reagent and incubated for 15 minutes the absorbance was read using Multiskan Ascent V1.24. To create a calibration curve, a standard series of protein ranging from 200 ug/ml to 6.25 ug/ml was prepared.

3.0 Results

3.1 Confirmation of plasmids by restriction analysis

Diagnostic endonuclease restriction analysis was conducted on pMRBAD/PfHsp70-1 (Figure 3.2), pASK-IBA3/PfAdoMetDC/ODC and pASK-IBA3/PfAdoMetDC plasmids (Figure 3.2 and Figure 3.3) (Muller *et al*., 2000), after their isolation using Zyppy ^{TM} Plasmid Miniprep Kit following the supplier's instructions. The pMRBAD/PfHsp70-1 construct was digested with *Bam* HI and *Hind* III. The DNA plasmid size is 6803 bp, *Hind* III linearized the plasmid and released a 6803 bp fragment, whereas cutting with *Bam* HI, it released two fragments 4390 bp and 2413 bp long. When the same plasmid was cut with both *Hind* III and *Bam* HI, it released three fragments 4390 bp, 2413 bp and 974 bp long.

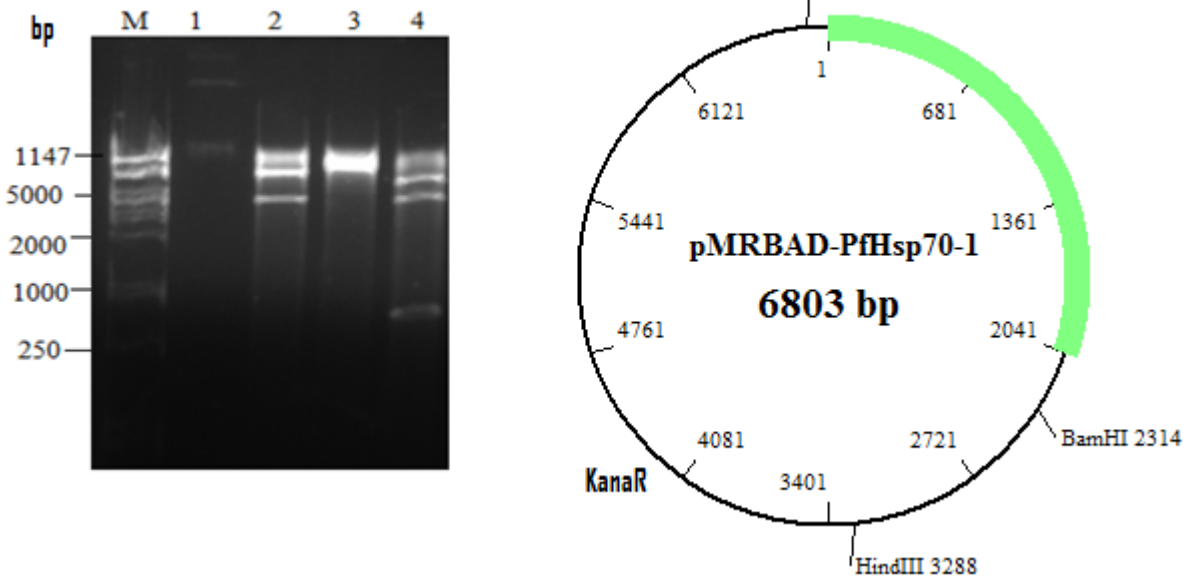


Figure 3.2: Diagnostic restriction analysis of pMRBAD/PfHsp70-1. Ethidium bromide stain of 0.8% agarose gel and restriction map of pMRBAD/PfHsp70-1. The DNA was loaded onto the gel in the following order: lane M, DNA marker; lane 1, represent undigested DNA; lane 2, construct digested with *Bam* HI; lane 3, construct digested with *Hind* III and lane 4, construct digested with both *Bam* HI and *Hind* III.

The pASK-IBA3/PfAdoMetDC/ODC wild type construct was digested with *Hind* III, and *Xba* I. The products were then analyzed on 0.8% agarose gel electrophosis in order to estimate fragment sizes. The size of the full length plasmid DNA is 7405 bp, *Xba* I linearized the plasmid and it released a 7405 bp fragment. When cut with both *Xba* I and *Hind* III it released fragments, of sizes 3093 bp, 786 bp and 452 bp (Figure 3.3).

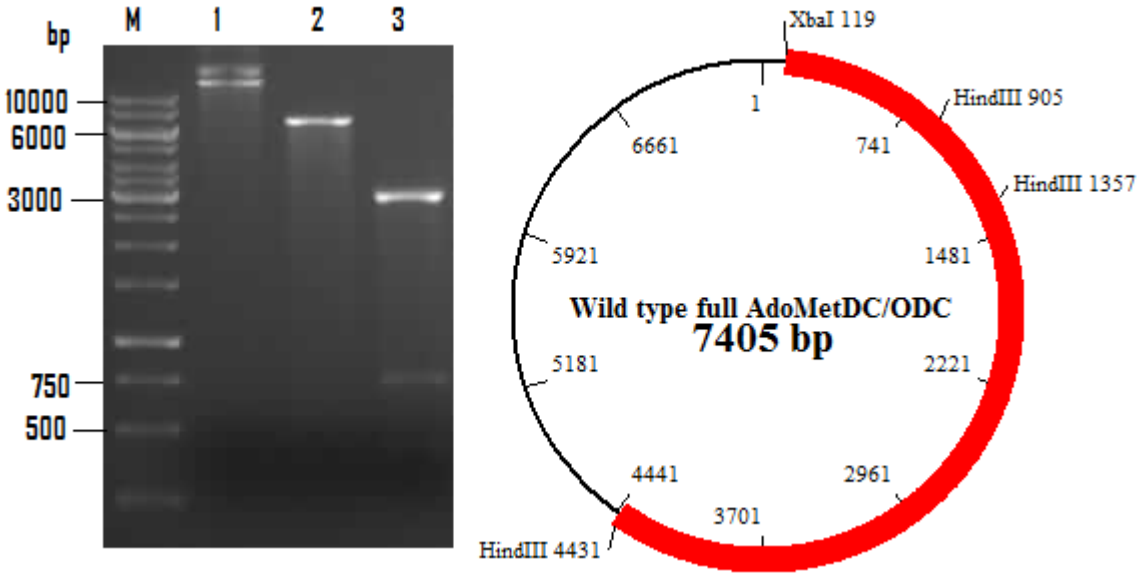


Figure 3.3: Diagnostic restriction analysis of pASK-IBA3/AdoMetDC/ODC (wild type). Ethidium bromide stain of 0.8% agarose gel and restriction map of pASK-IBA3/AdoMetDC/ODC (wild type). The DNA was loaded onto the gel in the following order: lane M, DNA marker; lane 1, is an undigested DNA; lane 2, represent DNA digested with *Xba* I; lane 3, DNA digested with both *Xba* I and *Hind* III.

A pASK-IBA3/PfAdoMetDC/ODC codon harmonized construct was isolated and digested with *Xba* I and *Hind* III. The products were analyzed on 0.8% agarose gel to estimate the fragment

sizes. The full length plasmid DNA is 7405 bp. *Xba* I linearized the plasmid construct and released 7405 bp fragment, when cut with both *Hind* III and *Xba* I it released the fragments around 4312 bp and 3093 bp (Figure 3.4).

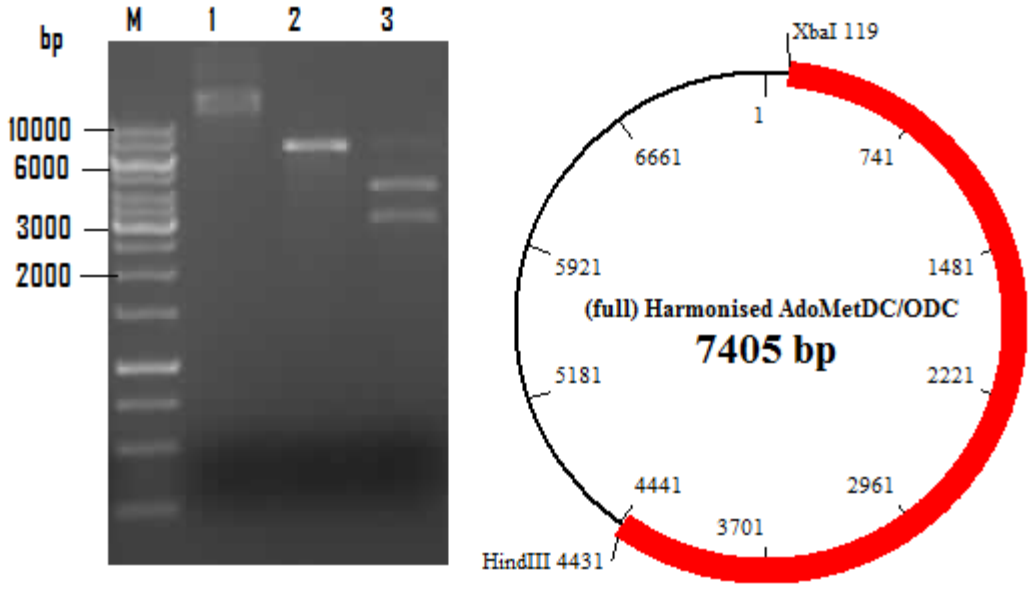


Figure 3.4: Diagnostic restriction analysis of pASK-IBA3/AdoMetDC/ODC (codon harmonized). Ethidium bromide of 0.8 % stain agarose gel and restriction map of pASK-IBA3/AdoMetDC/ODC (hrm). The DNA was loaded onto the gel in the following order: lane M, is a DNA marker; lane 1, is an undigested DNA; lane 2, represent DNA digested with *Xba* I; lane 3, DNA digested with both *Hind* III and *Xba* I.

A pASK-IBA3/PfAdoMetDC construct was isolated and digested with *Hind* III and *Xba* I. The size of this construct is 4699 bp; hence the expected fragment when cut with *Xba* I is 4699 bp

because it only cuts once. When cut with *Xba* I and *Hind* III the expected fragments are around 3092 bp, 787 bp, 452 bp and 368 bp (Figure 3.5).

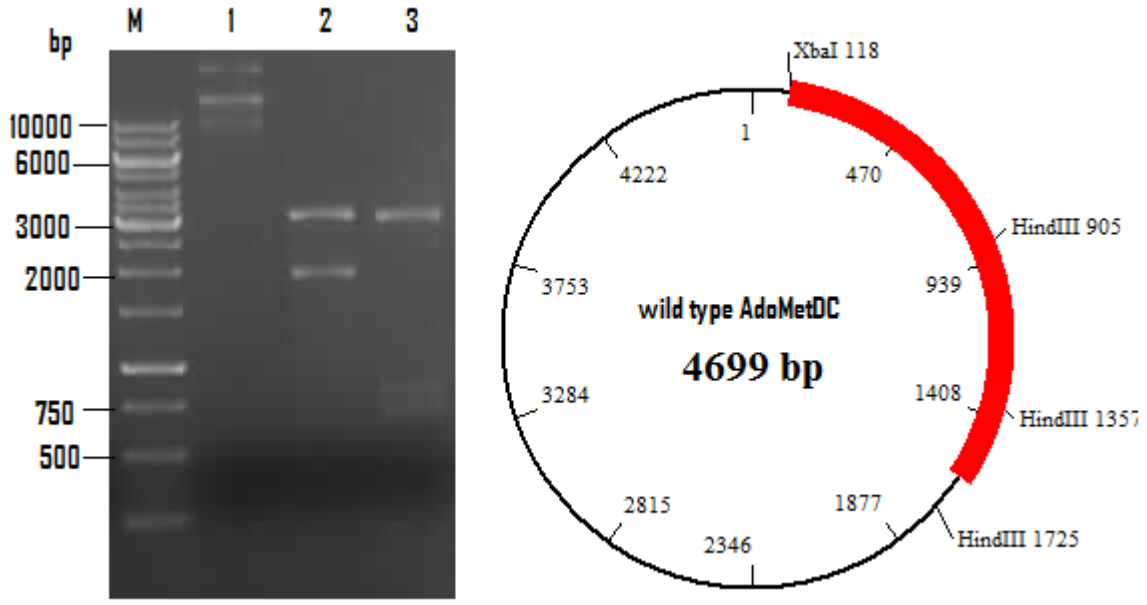


Figure 3.5: Diagnostic restriction analysis of pASK-IBA3/AdoMetDC (wild type). Ethidium bromide of 0.8 % stain agarose gel and restriction map of pASK-IBA3/AdoMetDC (wt). The DNA was loaded onto the gel in the following order: lane M, DNA marker; lane 1, is an undigested DNA lane 2, represent DNA digested with *Xba*I; lane 3, construct digested with both *Hind* III and *Xba* I.

Also the pASK-IBA3/PfAdoMetDC codon harmonized was digested with *Hind* III and *Xba* I after its isolation to confirm its integrity. The size of pASK-IBA3/PfAdoMetDC is 4864 bp, as *Xba* I only cuts once. When cut with both *Hind* III and *Xba* I it released the two fragments 3093 bp and 1771 bp (Figure 3.6).

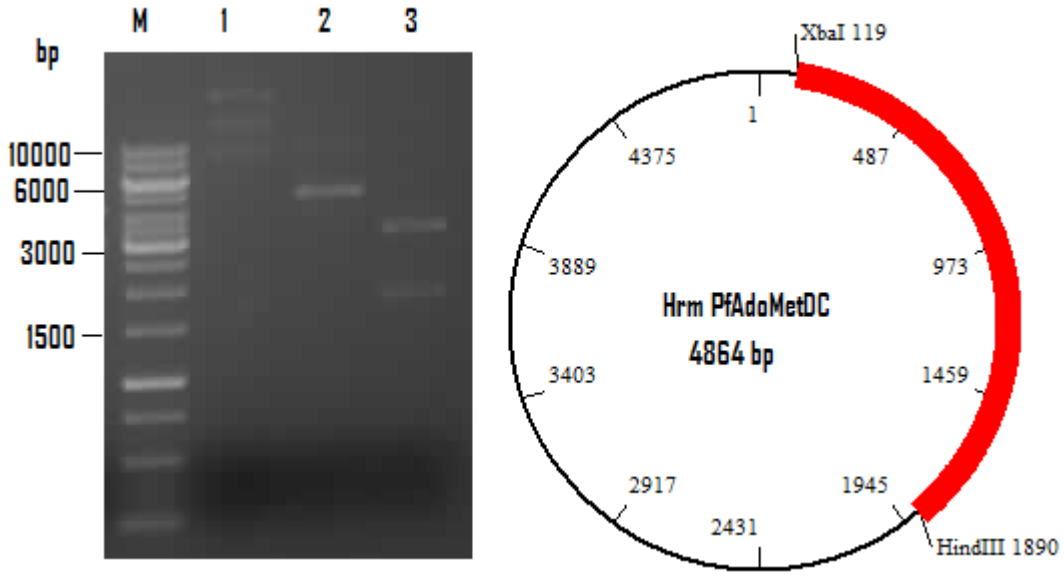


Figure 3.6: Diagnostic restriction analysis of pASK-IBA3/AdoMetDC (harmonized). Ethidium bromide stain agarose gel and restriction map of pASK-IBA3/AdoMetDC (hrm). The DNA was loaded onto the gel in the following order: lane M, DNA marker; lane 1, is an undigested DNA lane 2, represent DNA digested with *Xba* I; lane 3, construct digested with both *Hind* III and *Xba* I.

3.4 Recombinant expression of PfHsp70-1 in *E. coli* BL21 star cells

pMRBAD/PfHsp70-1 as a molecular chaperone was recombinantly expressed on its own in *E. coli* BL21 star cells in order to confirm its expression and possible toxicity to the cells (Figure 3.7).

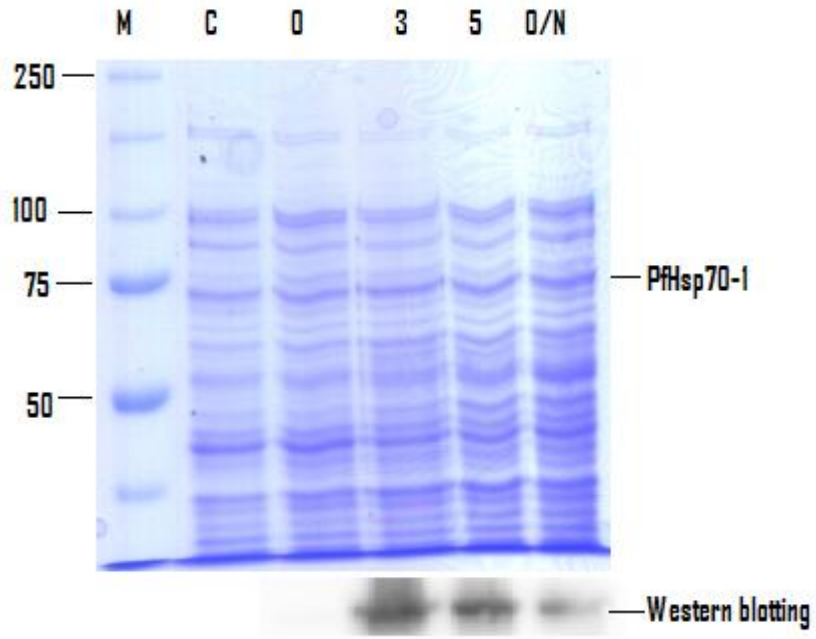


Figure 3.7: Recombinant expression of pMRBAD/PfHs70-1 in BL21 star (DE3) *E. coli* cells. Lane M, molecular weight markers; lane C, control; lane 0, sample taken before the induction; lane 3 and 5, samples taken after the induction with L-arabinose at the final concentration of 0.2%. Lane O/N; sample taken overnight. This was confirmed by carrying out Western blotting, using anti-PfHsp70 antibodies (lower panel).

Fig 3.7 confirms that PfHsp70-1 was successfully expressed based on the Western blot data. The expression of the protein was thus relatively low. The expression of PfHsp70-1 did not lead to cell toxicity as the cells were able to multiply during its expression.

3.5 Recombinant expression of wild type PfAdoMetDC sub-domain

PfAdoMetDC protein was recombinantly expressed on its own to confirm its expression in *E. coli* BL21 star cells and the expected size of the protein is around 60 kDa (Figure 3.8).

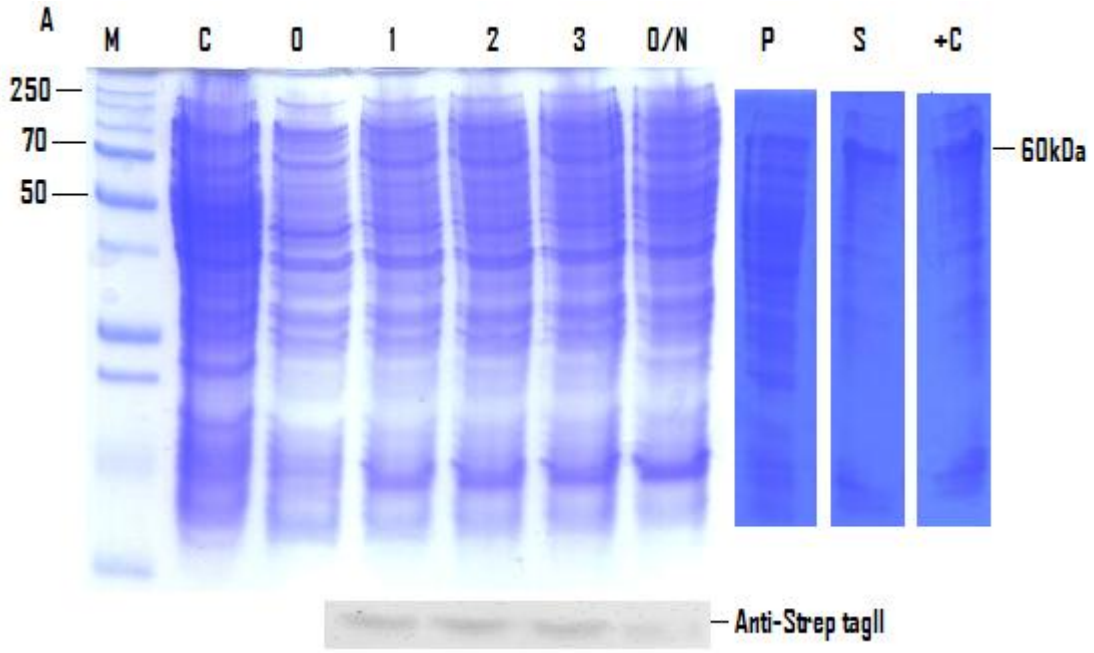


Figure 3.8: SDS-PAGE analysis of PfAdoMetDC (wild type) recombinant expression. (A) Represent PfAdoMetDC wild type recombinant expression in BL21 (DE3) *E. coli* cells (upper panel). Lane M, molecular weight markers, lane C, control, lane 0, sample taken before induction, lane 1-3, samples taken after induction with Anhydrotetracycline (200ng/ml), lane O/N, sample taken overnight. Western blotting using: anti-strep-tag II antibody (lower panel). Solubility studies, lane P, pellet; lane S, soluble fraction, lane + C, positive control.

Protein expression was analyzed on 7.5% SDS-PAGE gel electrophoresis and the protein production was confirmed by Western blot using anti-strep tag II antibodies (mouse raised). Solubility studies were conducted to check the state of the protein in the absence of PfHsp70-1. The protein was found in the pellet and supernatant, implying that the protein is partially soluble (Figure 3.8, lanes P and S). Solubility of proteins during their expression is important as their production in aggregated form leads to protein misfolding and complicates their purification.

3.6 Recombinant expression of codon harmonized PfAdoMetDC sub-domain

Monofunctional PfAdoMetDC codon harmonized version was initially expressed in the absence of a molecular chaperone (Figure 3.8B) and the expected size of the protein is 60 kDa.

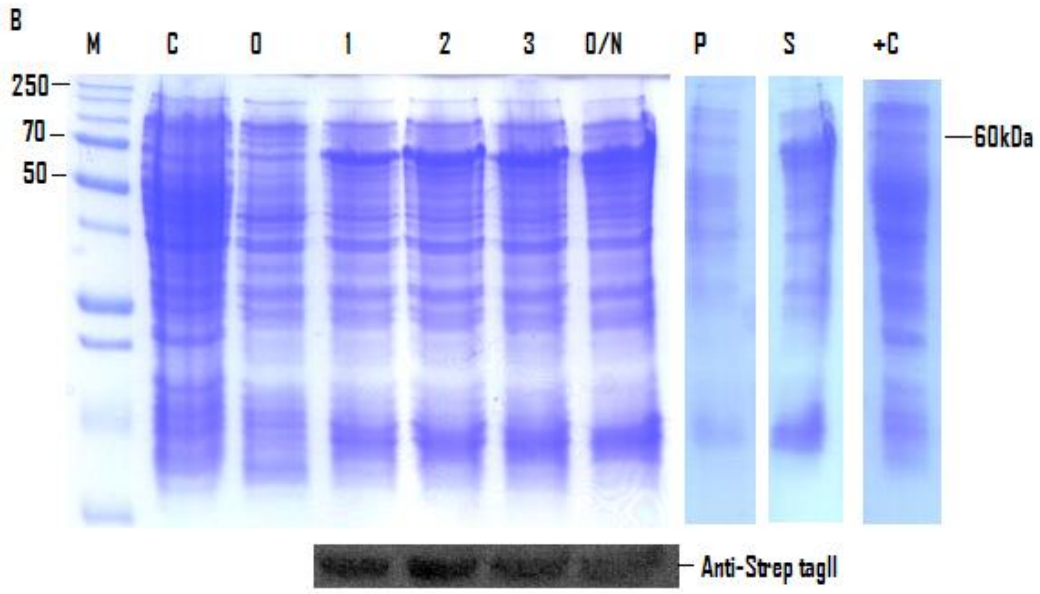


Figure 3.8: SDS-PAGE analysis of PfAdoMetDC (codon haromized) recombinant expression (B) recombinant expression of PfAdoMetDC harmonized type in BL21 star (DE3) *E. coli* cells (upper panel). Lane M, molecular weight markers, lane C, control, lane 0, sample taken before induction, lane 1-3, samples taken after induction with Anhydrotetracycline (200 ng/ml), lane O/N, sample taken overnight. Western blotting using: anti-strep-tag II antibody (lower panel). Solubility studies, lane P, pellet; lane S, soluble fraction, lane + C, positive control.

Protein expression was confirmed by SDS-PAGE gel electrophoresis and Western blot using anti-strep tag II anti-bodies (mouse raised). A protein species of approximately 60 kDa was produced based on SDS-PAGE analysis. The protein was produced in a soluble form (Figure 3.8; lanes P and S). The expression of the codon harmonized form of the protein in soluble form as opposed to the expression of the wild type form which was partially insoluble. Perhaps codon harmonization led to the smooth translation of the PfAdoMetDC.

3.7 Recombinant co-expression of PfAdoMetDC sub-domain

Both PfAdoMetDC wild type and codon harmonized were co-expressed along with pMBRBAD/PfHsp70-1 in *E. coli* BL21 star cells (Figure 3.9A). This was done to establish whether PfHsp70-1 would improve the expression yield and solubility of PfAdoMetDC protein.

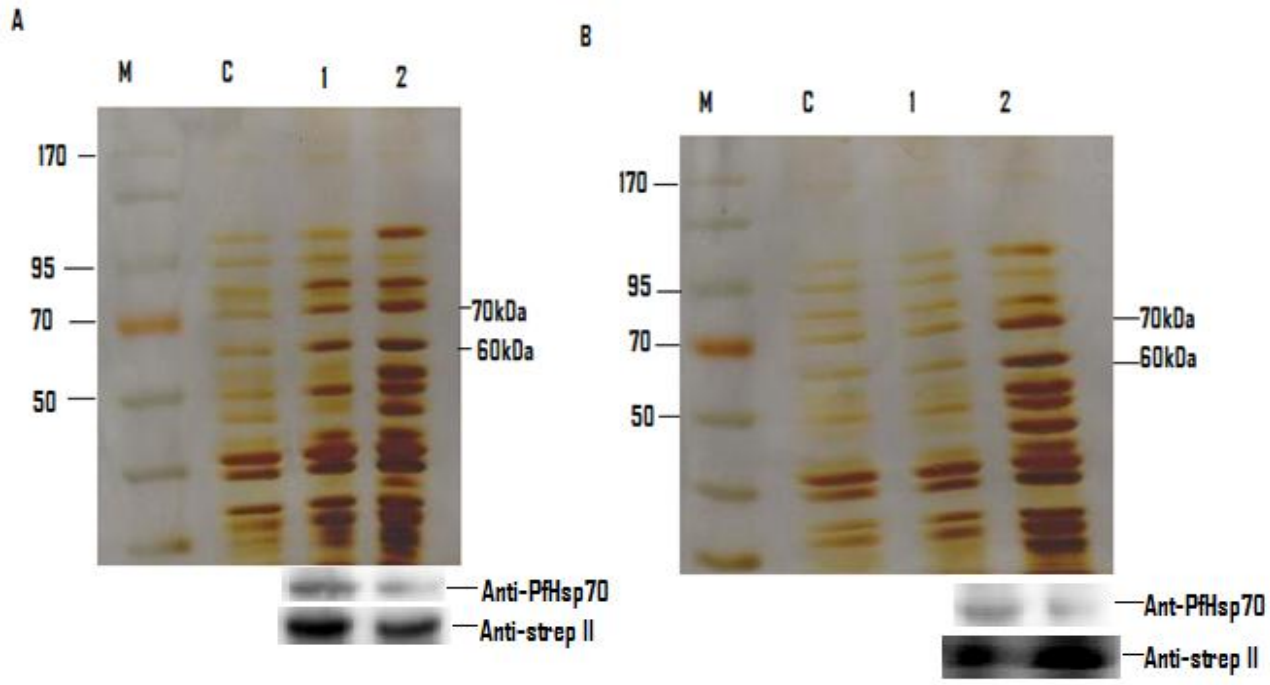


Figure 3.9: SDS-PAGE analysis of co-expressed PfAdoMetDC with PfHsp70-1. (A) Represent PfAdoMetDC wild type recombinant co-expression in *E. coli* BL21 cells. Lane; M, protein markers; lane C, control; lane 1, sample taken before induction; lane 2, sample taken after induction. (B) Recombinant co-expression of PfAdoMetDC codon harmonized type in *E. coli* BL21 star cells. Lane; M, protein markers; lane C, control; lane 1, sample taken before induction; lane 2, sample taken after induction. Western blotting using: anti-strep-tag II antibody (lower panel) for PfAdoMetDC and Anti-PfHsp70 antibodies.

Both wild type and codon harmonized forms of PfAdoMetDC were successfully expressed in *E. coli* BL21 star cells along with PfHsp70-1. The expressions of the PfHsp70-1 and target proteins were apparent based on SDS-PAGE gel and Western analysis (Figure 3.9).

3.8 Solubility study conducted in co-expressed wild type PfAdoMetDC

After recombinant co-expression, the status of the protein was confirmed by conducting the solubility studies. Indeed the co-expression of PfHsp70-1 improved the solubility of wild type PfAdoMetDC compared to the solubility of the same protein in the absence of PfHsp70-1 (Figure 3.8). Thus PfHsp70-1 may have facilitated the expression of PfAdoMetDC as soluble produced protein by facilitating folding process during its translation.

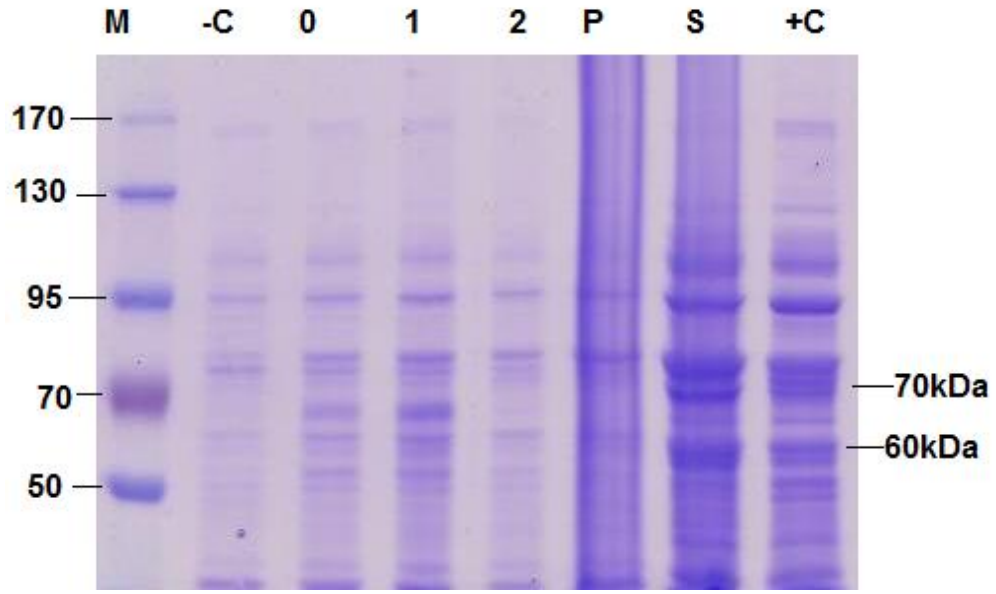


Figure 3.10: Solubility studies of co-expressed PfAdoMetDC wild type. Lane M, is prestained marker; lane – C, is negative control; lane 0, is a sample taken before induction; lane1; is a sample taken after induction; lane 2 is a sample taken after 4 hours of growth; lane P, is pellet that was washed with PBS; lane s, is supernatant and + C is positive control.

3.9 Solubility study conducted on codon harmonized PfAdoMetDC expressed in the presence of PfHsp70-1

PfAdoMetDC protein was co-expressed with PfHsp70-1, and its solubility was confirmed. Based on the SDS-PAGE result (Figure 3.11) the product was largely soluble. Thus PfAdoMetDC (harmonized) was produced as soluble product both in absence (Figure 3.10) and in the presence of PfHsp70-1 (Figure 3.11). Therefore, it is not clear whether PfHsp70-1 may have improved the folding process of PfAdoMetDC (harmonized). Solubility of recombinant proteins does not always imply that the product is properly folded. Thus it is possible that PfHsp70-1 may have improved the folded status of PfAdoMetDC (harmonized). Whether this is the case, only activity assay on the proteins produced in the absence and presence of PfHsp70-1 can confirm this.

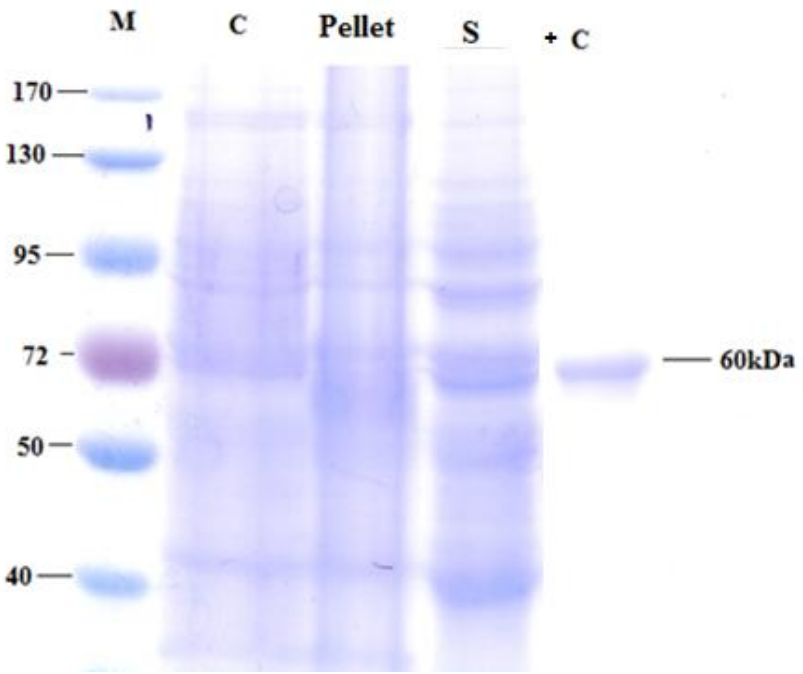


Figure 3.11: Solubility studies of co-expressed PfAdoMetDC codon harmonized sub-domain. Lane M, is prestained marker; lane C, is a negative control; lane P, is a pellet that was washed with PBS; lane s, is supernatant and lane + C, is a positive control.

3.10 Recombinant expression of bifunctional PfAdoMetDC/ODC (wild type) in the absence and presence of chaperone

The expected size of the full length bifunctional PfAdoMetDC/ODC was 160 kDa. Based on the SDS-PAGE analysis, production of this protein in *E. coli* BL21 star cells could not be confirmed (Figure 3.12A). Due to technical constrains, no Western blot analysis was conducted to verify if the protein was expressed at low concentrations.

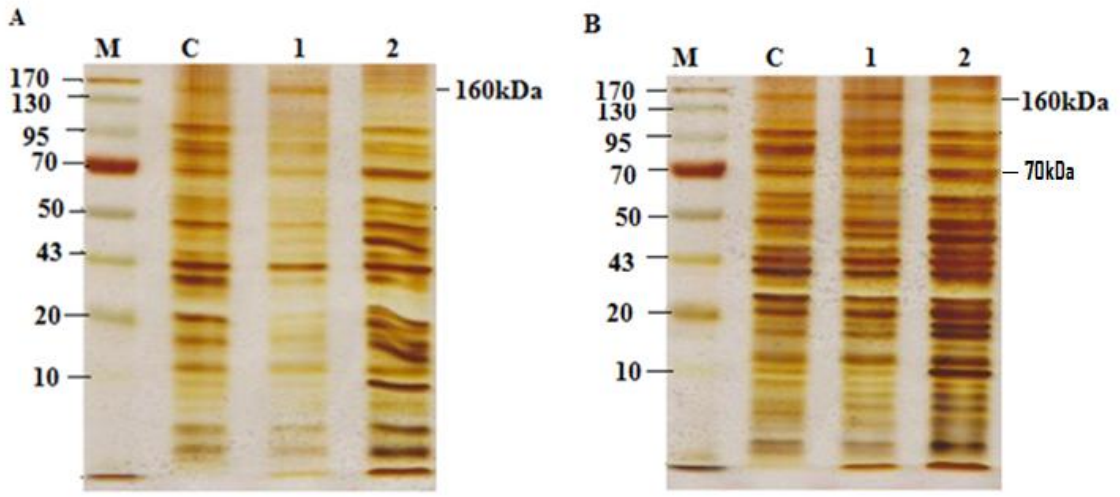


Figure 3.12: Recombinant expression of bifunctional PfAdoMetDC/ODC. (A) Represent PfAdoMetDC/ODC wild type recombinant expression in *E. coli* BL21 cells. Lane; M, protein markers; lane C, control; lane 1, sample taken before induction; lane 2, sample taken after induction. (B) Recombinant co-expression of PfAdoMetDC/ODC wild type in *E. coli* BL21 star cells. Lane; M, protein markers; lane C, control; lane 1, sample taken before induction; lane 2, sample taken after induction.

PfAdoMetDC/ODC was recombinantly co-expressed in *E. coli* BL21 star cells along with PfHsp70-1 (Figure 3.12B). In this case PfHsp70-1 expression was initiated first in order to be available on system before the induction of PfAdoMetDC/ODC as a target protein. The

production of PfHsp70-1 was apparent by SDS-PAGE analysis, as there was a band corresponding to about 70 kDa, expressed after induction. However, the expression of the full length PfAdoMetDC/ODC (wild type) protein could be ascertained by SDS-PAGE. There was no evidence of the production of a 160 kDa species upon induction of the culture (Figure 3.12B, lanes 1, 2). Again, due to technical constrains, Western blotting could not be conducted to ascertain whether the protein was not produced at low levels. However, it is not surprising that the full length PfAdoMetDC/ODC protein may not have been expressed since multi-domain proteins are generally known to be problematic to express (Baneyx and Mujacic, 2004; Hartl and Hartl, 2009).

3.11 Purification of recombinant PfAdoMetDC/ODC and PfAdoMetDC proteins expressed in the absence of PfHsp70-1

All constructs were initially expressed and purified in the absence of PfHsp70-1. Figure 3.13 show recombinant bifunctional wild type PfAdoMetDC/ODC and codon harmonized PfAdoMetDC/ODC and monofunctional PfAdoMetDC (wild type) and PfAdoMetDC (codon harmonized) recombinantly affinity purified proteins. Both forms of constructs, either full length or sub-domains were recombinantly expressed with a C-terminal Strep-tag II to allow the affinity purification (using strep-tactin column). The full length protein PfAdoMetDC/ODC (wild type and harmonized) size should be around 160 kDa while the PfAdoMetDC sub-domain is around 60 kDa. It was hoped that purification, would promote concentration of the proteins if expressed. However, there was no evidence that PfAdoMetDC/ODC (wild type or codon harmonized) was expressed (Figure 3.13; lanes wA/wO and hA/wO). No species of proteins were observed in those lanes migrating as species of 160 kDa size. However, PfAdoMetDC (60 kDa) was purified

for both wild type and codon harmonized versions, infact as expected, codon harmonized version of PfAdoMetDC was purified in higher yield than wild type form. However, in either case, the proteins were purified along with a lot of background contaminants. The failure to purify PfAdoMetDC/ODC successfully has been reported before (Niemand, 2007). It was suggested that the origins of these proteins were smaller versions of the bifunctional protein not expression vector itself.

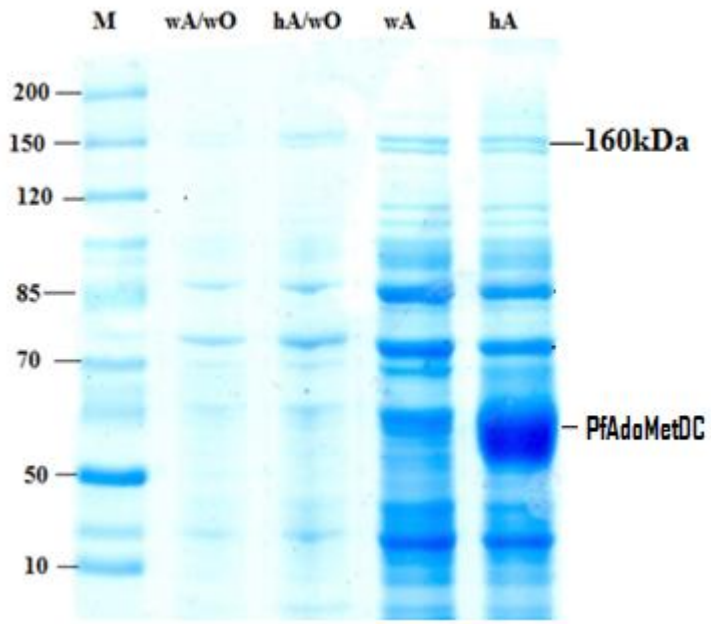


Figure 3.13: Recombinatly affinity purified PfAdoMetDC/ODC, PfAdoMetDC using Strep-tactin column. The protein eluates were analyzed using 7.5% of separating gel and visualized with colloidal coomassie. Lane M, unstained protein markers, lane; wA/wO (wild type PfAdoMetDC/ODC) protein; lane hA/wO (codon harmonized PfAdoMetDC/ODC); lane wA, wild type PfAdoMetDC; lane hA, codon harmonized PfAdoMetDC sub-domains.

3.12 Purification of recombinant PfAdoMetDC protein co-expressed with PfHsp70-1

As previously reported, the wild type PfAdoMetDC was produced in the absence of PfHsp70-1 (Figure 3.14, lane wA). In the presence of PfHsp70-1, the protein was less expressed upon co-expression (Figure 3.14, lane hA+70). In either case, *E. coli* background protein was retained on the Strep-tactin column. However, the co-expression of PfAdoMetDC (harmonized) in the presence of PfHsp70-1 was followed by neat purification of PfAdoMetDC (Figure 3. 14, lane hA+70). The protein that was expressed in the absence of PfHsp70-1 purified along with a lot of background *E. coli* proteins (Figure 3.14, lane hA). Thus it could be argued that PfHsp70-1 may have facilitated folding of PfAdoMetDC (harmonized) exposing its Strep-tag which bound to the column, outcompeting non-specific bounders. Thus PfHsp70-1 may have not only facilitated folding of PfAdoMetDC (codon harmonized), but led to the column stripping off non-specific binders. Further studies are required to understand how PfHsp70-1 manifested the dramatic results that were observed.

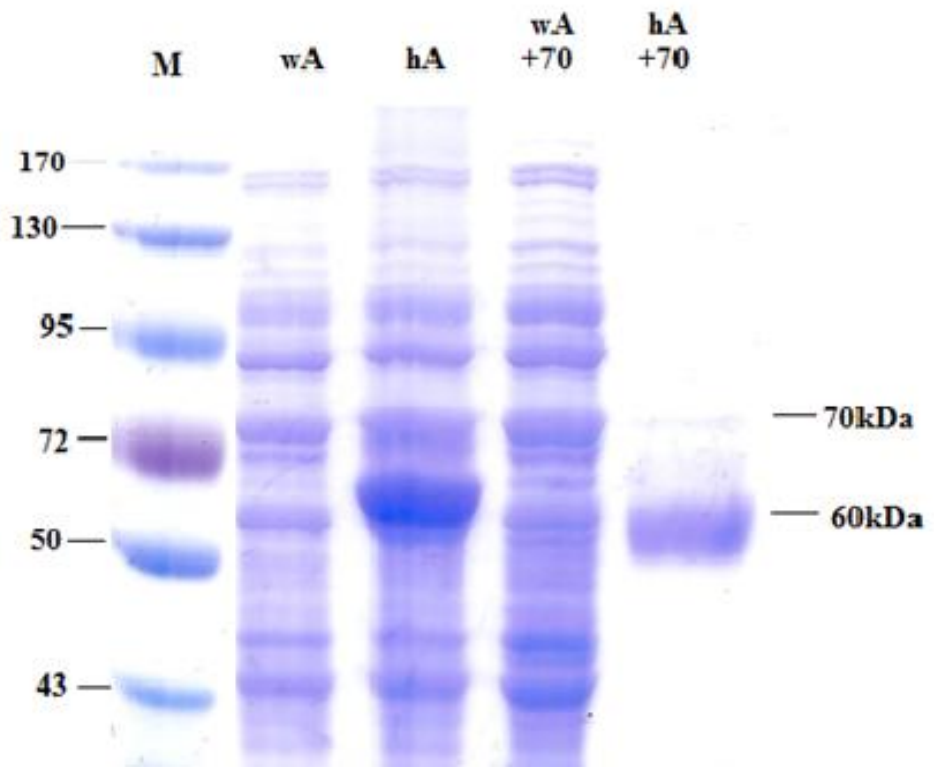


Figure 3.14: **Shows the recombinant affinity purification of PfAdoMetDC protein.** Lane M, is a prestained protein marker, lane wA, wild type PfAdoMetDC; lane hA, codon harmonized PfAdoMetDC and co-expressed PfAdoMetDC (wt and hrm) with pMRBAD/PfHsp70-1. The monofunctional PfAdoMetDC domain is around 60 kDa. The protein eluates were analyzed using 7.5% of separating gel and visualized with colloidal coomassie.

3.13 Purification of PfAdoMetDC/ODC protein expressed with PfHsp70-1

PfAdoMetDC/ODC (either wild type or codon harmonized forms) were expressed in *E. coli* BL21 star cells in the absence or presence of PfHsp70-1. The proteins were then purified on Strep-tectin column. However, the expression of the wild type protein was not confirmed both in the absence and in the presence of PfHsp70-1(Figure 3. 15 lanes wA/wO and wA/wO+70), however, a species of around 70 kDa was recovered on the column. This could have been *E. coli* DnaK. In the presence of PfHsp70-1, it seemed PfAdoMetDC/ODC (wild type) was expressed and recovered on purification as a band of 160 kDa could be seen in the lane representing

PfAdoMetDC/ODC plus PfHsp70-1 (Figure 3. 15, lane wA/wO+ 70). Again the contaminating species of approximately 70 kDa was more enhanced (possibly representing the mixture of *E. coli* DnaK and PfHsp70-1). The purification of harmonized PfAdoMetDC/ODC was attained better. For, a protein expressed in the absence of PfHsp70-1 as compared to the lysate containing the PfAdoMetDC/ODC plus PfHsp70-1 it seems PfHsp70-1 may have confounded the folding of this multi-domain protein, thus further complicating its purification. Thus background *E. coli* protein observed in the presence of PfHsp70-1 (lane hA/wO+70) could reflect that PfHsp70-1 promoted aggregation of full-length PfAdoMetDC/ODC (harmonized). It is known that Hsp70 proteins tend to hold on to their misfolded species for a long time and thus compromises folding of multi-domian species (Baneyx and Mujacic, 2004; Hartl and Hartl, 2009). The fact that PfHsp70-1 aggregated the purification process of full-length PfAdoMetDC/ODC, whilst it facilitated purification of the monofunctional PfAdoMetDC is intriguing. It is thus possible that PfHsp70-1 facilitated folding of smaller protein species whilst it promoted aggregation of the full-length, multi-domain equivalent.

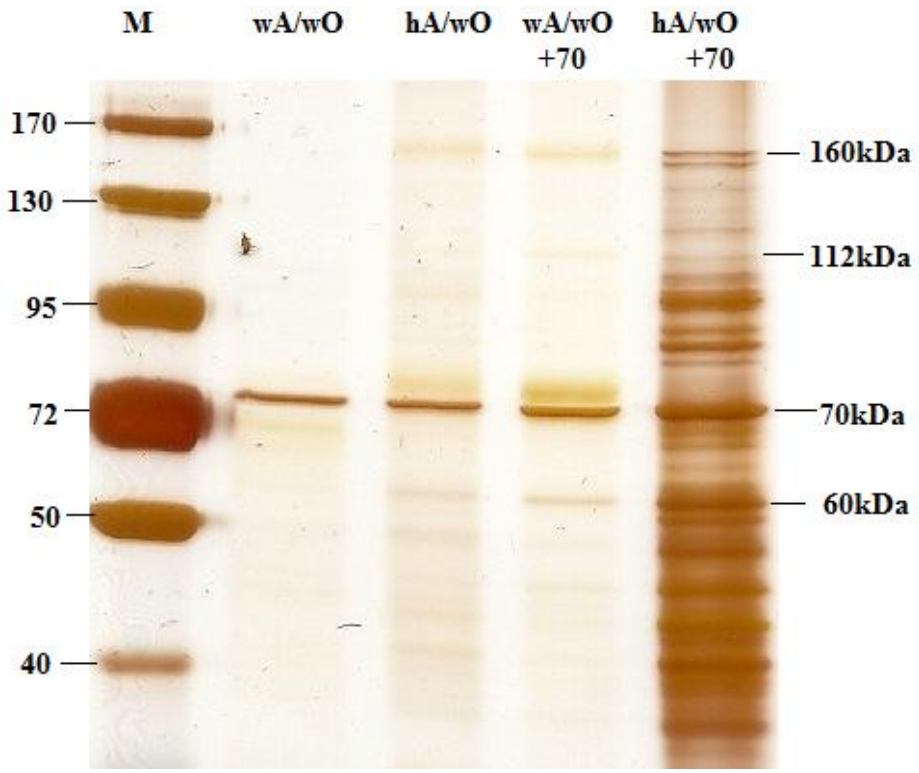


Figure 3.15 Co-expression of bifunctional PfAdoMetDC/ODC as strep-tag proteins. SDS-PAGE of bifunctional PfAdoMetDC/ODC proteins. Lane 1: Prestained protein marker, lane wA/wO (wild type PfAdoMetDC/ODC) protein; lane hA/wO (codon harmonized PfAdoMetDC/ODC); lane wA/wO+70 (wild type PfAdoMetDC/ODC co-expressed with pMRBAD/PfHsp70-1); lane hA/wO (codon harmonized PfAdoMetDC/ODC co-expressed with pMRBAD/PfHsp70-1). The protein eluates were analyzed using 7.5% of separating gel and visualized with silver staining.

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4. Discussion

Although, *E .coli* is a reliable host for the expression of recombinant proteins, not all recombinant proteins are produced successfully in this system. It has been advocated that endogenous *E. coli* chaperones tend to fail in coping with the responsibility of folding recombinant proteins expressed in the system (Martinez-Alonso *et al*., 2010). The current study showed that both codon harmonization and the role of molecular chaperones are important in determining the yield and folding fate of target protein expressed in *E. coli*. PfAdoMetDC (wild type) could not be produced in *E. coli* BL21 star cells. In addition, both full length PfAdoMetDC/ODC (wild type) codon harmonized versions were not expressed both in the absence and presence of chaperone PfHsp70-1. On the other hand, codon harmonized PfAdoMetDC sub-domain was expressed both in the absence and presence of PfHsp70-1. Furthermore, upon purification of codon harmonized PfAdoMetDC co-expressed with PfHsp70-1, the protein was recovered at much higher levels of purity. Therefore, this study confirms that codon harmonization and indeed the presence of appropriate chaperone machinery are important factors in the production of recombinant protein in *E. coli*.

The expression of PfHsp70-1 was not apparent when analyzed on SDS-PAGE (Figure 3.7). This could be due to the fact that pMRBAD vector is a low copy number. Shonhai *et al* (2005) expressed the same protein expressed off a pQE30 plasmid vector and the protein was produced at much higher levels. Furthermore, Shonhai *et al* (2005) expressed this protein at 37^{o}C culture temperature. Consequently, it could be argued that the low copy number of pMRBAD plasmid vector hosting PfHsp70-1 encoding sequence and lower culture temperature used (22^{o}C) in this study may explain the lower expression of PfHsp70-1 observed. Nonetheless, the lower

expression of PfHsp70-1 may not have been counter-productive as production of excess Hsp70 protein is known to be toxic to cells (Kyratsous *et al*., 2009).

The unsuccessful production of PfAdoMetDC/ODC (wild type and codon harmonized forms) both in the presence and absence of PfHsp70-1 could have been mainly due to several reasons. The wild type form of the protein may not have been expressed, purify due to codon mismatch. *P. falciparum* genome is known to be A/T rich and thus expression of malarial protein in *E. coli* is a huge constrain on the translational machinery of *E. coli*. Supposing, codon mismatch was the only constrain, it would have been expected that the codon harmonized version would have been produced in the presence of PfHp70-1. However, the fact that PfAdoMetDC/ODC (codon harmonized) was not produced is evidence that codon mismatch was not the only constrain at play. PfAdoMetDC/ODC is a huge protein (160 kDa) and consists of two main domains. Huge size of this protein and its multi-domain structure may have placed a challenge to the protein folding pathway. Multi-domain proteins are known to be problematic to express in *E. coli* (Baneyx and Mujacic, 2004; Hartl *et al*., 2009). In addition, the addition of Hsp70 expression of recombinant multi-domain proteins has not been successful in the past (Baneyx and Mujacic, 2004; Hartl and Hartl, 2009). This was largely attributed to the Hsp70 binding to multi-domain proteins for too long at the ribosomal exit, thus confounding protein translocation.

Monofunctional PfAdoMetDC (wild type) as a target protein was recombinantly expressed in the absence of PfHsp70-1 (Figure 3.8A). The expression of the protein was not apparent when analyzed by SDS-PAGE gel and solubility studies conducted shows that the protein was partially soluble. It is possible that the high level of codon mismatch between *P. falciparum* and *E. coli* as an expression host system may have a negative impact on protein production (Birkholtz *et al*., 2008). However, the expression of this protein (albeit lower concentration) is in stuck contrast to

the complete lack of expression of the full length protein. Thus the expression of PfAdoMetDC (wild type) as opposed to the complete lack of expression of full length PfAdoMetDC/ODC (wild type) may be due to the fact that the smaller sub-domain was easier to fold, and hence its production was improved. Hence, the expression of the protein was confirmed by Western blot using anti-strep tag II antibodies and the protein was detected as being expressed.

The successful expression of PfAdoMetDC (codon harmonized) was confirmed both in the absence and presence of PfHsp70-1. The expression of this protein was vastly improved compared to its wild type equivalence, both in the absence and presence of PfHsp70-1. Codon matching may have facilitated the smooth translation of this protein. The presence of PfHsp70-1, not only improved the expression of the PfAdoMetDC (codon harmonized) but also facilitated its purification. The conceived improved expression (due to codon matching) and the folding facility (due to PfHsp70-1) may have both contributed to the outcome observed. In agreement with this, it has recently been reported that PfHsp70-1 successfully improved the production of *Plasmodium falciparum* cyclohydrolase (GTP) as a drug target. It was proposed that molecular chaperone prevented the formation of aggregates and non-productive self-association of the protein (Stephens *et al*., 2011).

Data from purification attempts of full length protein and sub-domains of the target proteins expressed sought to further confirm expression of the proteins. PfAdoMetDC/ODC (wild type and codon harmonized) purification results could not unequivocally confirm expression of these protein, although faint bands of 160 kDa were observed (Figure 3.13; lanes wA/wO and hA/wO). However, the authenticity of these species could not be confirmed by Western blotting due to the technical constrains.

PfAdoMetDC (both wild type and codon harmonized) expressed in the absence of PfHsp70-1 were largely purified with accompanying background proteins (Figure 3.13; lanes wA and hA). However, the co-expression of PfAdoMetDC (codon harmonized) with PfHsp70-1 seemed to have greatly improved the purity of the target product (Figure 3. 14; lane hA+70). It is not clear how PfHsp70-1 improved the purification of PfAdoMetDC (codon harmonized), whereas the PfAdoMetDC (wild type form) was recovered with lot of contaminants protein.

4.1 Conclusion and Future work

Altogether, this study has highlighted the important role of molecular chaperones that could play in both the expression and purification of recombinant proteins in *E. coli*. Based on these findings, PfHsp70-1 seems to promote the folding of PfAdoMetDC (codon harmonized version). In addition, the use or the presence of PfHsp70-1 led to better purification of the PfAdoMetDC (harmonized version). In future, we hope to optimize the co-expression studies to improve the production and purification of the full-length PfAdoMetDC/ODC protein and to confirm whether PfHsp70-1 improves the quality of the PfAdoMetDC/ODC protein obtained by conducting activity assays.

Appendix A

Special chemical reagents

Name of reagents	Vendor/Supplier
Agarose	Merck
Ammonium persulphate	Sigma-Aldrich
Ampicillin	Calbiochem
Anti-strep antibodies	IBA Germany
Chemiluminescence Western blotting kit	Amarsham
2-mercaptoethanol	Merck
Bovine serum albumin	Melford
Bromophenol blue	Merck
Calcium chloride	Merck
Coomasie brilliant blue R250	Merck
Ethidium bromide	Merck
Glacial acetic acid	Merck
Glycerol	Merck
Glycine	Merck
Imidazole	Merck
Lambda DNA	Fermantas Life sciences
Lysozyme	Merck
Methanol	Merck
Magnesium chloride	Merck
Monoclonal mouse anti-His antibody	Merck
Phenylmethylsufonyl fluoride	Merck
Polyacrylamide	Merck
Ponceau S	Sigma-Aldrich
Restriction enzymes	Merck
Sodium chloride	Merck
Sodium dodecyl sulphate	Merck
Sodium hydroxide	Merck
TEMED	Sigma-Aldrich
Tris	Merck
Tryptone	Oxoid, England
Tween 20	Radchem
Yeast	Merck
Broth	Merck
Nutrient agar	Merck
Strep-tagged Antibody	Merck
Glucose	Merck
L+Arabinose	Merck
Anhydrotetracycline	Merck
Kanamycine	Merck

Strep-tactin	IBA Germany
Buffer W	IBA Germany
Buffer E	IBA Germany
Buffer R	IBA Germany
Silver staining kit	Fermantas life sciences
Protein ladder	Fermantas life sciences
Anti-strep tag Antibodies	Merck

Appendix B (Preparation of solutions)

PBS buffer (pH 7.4)

137 mM sodium chloride, 27 mM potassium chloride , 4.3 mM sodium hydrophosphate 1. and 1.4 mM potassium hydrophosphate were dissolved in ddH_20 and autoclaved.

TE buffer (pH 8.0)

0.1211 g Tris and 0.0370 g EDTA 100 ml were mixed together with distilled water and autoclaved.

Sodium hydroxide

115 g of sodium hydroxide was dissolved in 250 ml of distilled water.

SDS (10%)

1 g of SDS was dissolved in 10 ml of distilled water

DNA extraction solutions

Solution 1 (pH 8.0)

0.9008 g glucose, 0.628 g Tris chloride and 0.372 g EDTA were mixed together and the volume was made up to 100 ml

Solution 2

2ml of 10M sodium hydroxide and 10 ml of SDS were mixed together and the volume was made up to 100 ml

Solution 3 (pH 5.0)

29.442 g of potassium chloride was dissolved in distilled water and the volume was made up to 100 ml.

30% glycerol

30 ml of glycerol was mixed together with 70 ml of distilled water to make 100 ml of 30% glycerol.

IPTG

1 g of IPTG was dissolved in 4.2 ml of distilled water and filtered through filter into 1 ml eppendorf tube then stored at -20 ^{0}C

10X TAE buffer (pH 7.8)

48.4 g of Tris, 20 ml of 0.5M EDTA and 11.42 ml of glacial acetic acid were mixed together with distilled water and made up to 1000 ml.

Ampicillin

1 g of ampicillin was dissolved in 10 ml of distilled water and filtered through a filter unit into eppendorf tube100 μg/ml

Ampicillin agar plates

1.6 g of pancreatic tryptone, 1.5 g nutrient agar, 0.5 g of sodium chloride and 1 g of yeast were mixed together with distilled water and the volume was made up to 100 ml in a flask and autoclaved. The mixture was allowed to cool and 10 μl of ampicillin was added and poured into Petri dishes and allowed to solidify.

Liquid broth

1.6 g of pancreatic tryptone, 0.5 g of sodium chloride and 1 g of yeast were mixed together with distilled water and the volume was made up to 100 ml in a flask and autoclaved.

Ammonium persulphate (10%)

1 g of Ammonium persulphate was dissolved in 10 ml of distilled water.

Acrylamide stock solution

300 ml of distilled water was placed in a beaker and 150 g of acrylamide was added to distilled water while stirring and 4.0 g of bisacrylamide was added while stirring and allowed to dissolve. The volume of the solution was made up to 500 ml with distilled water. The acrylamide/Bisacrylamide solution was filtered with a cloth and placed in a dark container and stored in the fridge.

Ethanol 70%

70 ml of alcohol was mixed with 30 ml of distilled water and the volume was made up to 100 ml

0.1M Calcium chloride

1.47 g of calcium chloride was dissolved in 100 ml of distilled water

0.1M Magnesium chloride

2.0 g of magnesium chloride was dissolved in 100 ml of distilled water

Ethidium bromide (10 mg/ml)

1 g of ethidium bromide was dissolved in 100 ml of distilled water and stored in a bottle covered with aluminium foil

Tris

1.5M Tris (pH 8.8)

36.3 g of Tris was dissolved in 200 ml of distilled water

0.5M Tris (pH 6.8)

12.11 g of Tris was dissolved in 200 ml of distilled water

Destaining solution

800 ml of distilled water, 7.5 ml glacial acetic acid and 100 ml of ethanol were mixed together and the volume was made up to 1000 ml

Short protocol of the Strep-Tactin chromatography cycle

1. After the protein extract has entered the *Strep*-Tactin matrix, wash column 5 times with 1 CV (column volume) Buffer W.
2. Elute recombinant protein by the addition of 6 times 0.5 CV Buffer E.
3. Regenerate the column by the addition of 3 times 5 CV Buffer R.
4. Equilibrate the column by the addition of 2 times 4 CV Buffer W prior to the next purification run.
5. Store the column at 4° C overlaid with 2 ml Buffer W or R.

Buffer composition

Buffer W (washing buffer)	Buffer E (elution buffer)	Buffer R (regeneration buffer)
100 mM Tris-Cl pH 8.0 150 mM NaCl 1 mM EDTA	100 mM Tris-Cl pH 8.0 150 mM NaCl 1 mM EDTA 2.5 mM desthiobiotin	100 mM Tris-Cl pH 8.0 150 mM NaCl 1 mM EDTA 1 mM HABA (hydroxy-azophenylbenzoic acid)

Appendix C

Protein concentration determination

Materials:

Protein serial dilution (200, 100, 50, 25, 12.5, 6.25 µg/ml)

Bradford

Elisa plate

Needle

Method:

(1) Load 150 µl Bradford
(2) Load 50 µl serial dilution and blank in duplicate
(3) Load 50 µl protein samples in duplicate
(4) Burst bubbles with needle
(5) Incubate for 15'
(6)Read absorbance at 595 nm

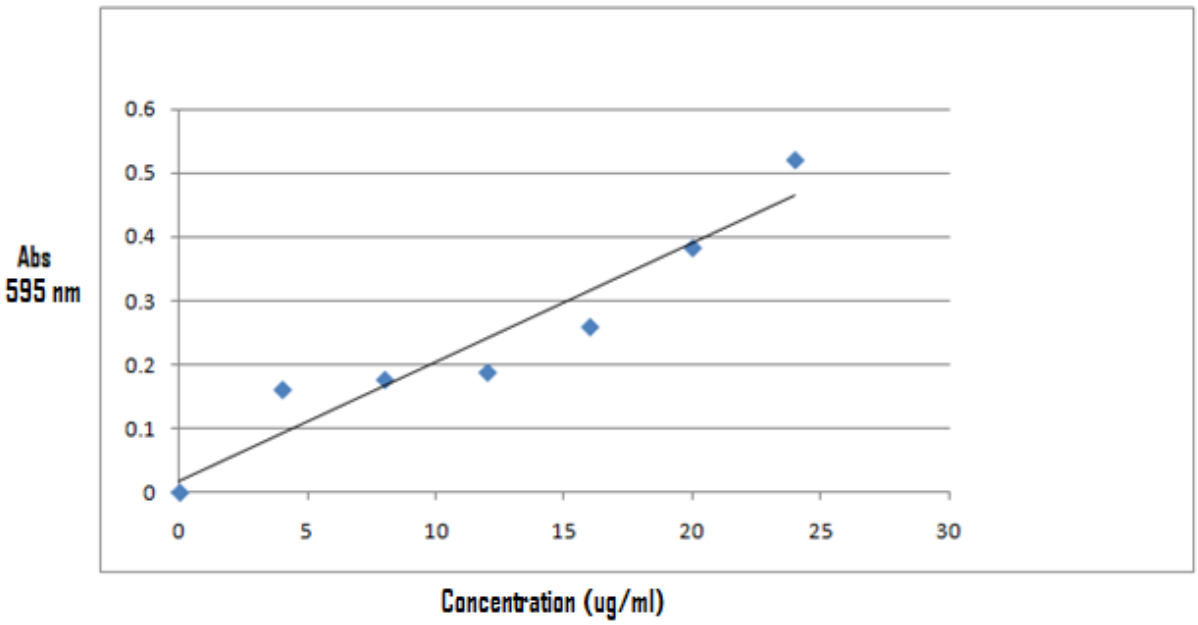

Figure 1: Protein standard curve. Bradford's reagent was used to determination concentration of PfAdoMetDC protein expressed without pMRBAD/PfHsp70-1. The standard curve had an R^2 value of **0.9108** and the equation used was y=0.007x.

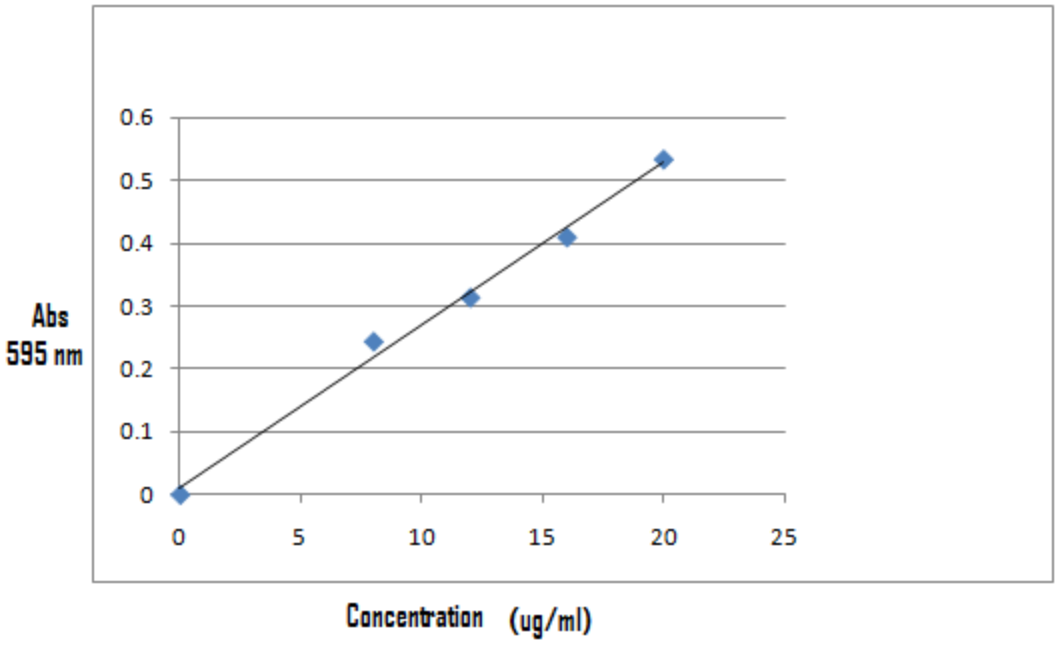

Figure 2: Protein standard curve. Bradford's reagent was used to determination concentration of PfAdoMetDC protein co-expressed with pMRBAD/PfHsp70-1. The standard curve had an R^2 value of **0.9885** and the equation used was y=0.004x.

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