CHAPTER 4

DISCUSSION AND CONCLUSION

Plasmodium falciparum is still a major public health problem in Asia, South/Central America and Africa where it causes around 80 million cases of malaria annually. The strategy to fight against malaria has been limited due to an increasing of malaria parasite resistance to currently used antimalarials. To overcome this problem, urgent need must be focused on finding new and effective antimalarial drugs. This goal can be achieved in two ways: either by focusing on validated targets in order to generate new drug candidates; or by identifying new potential targets for malaria chemotherapy.

Methionine synthase or MS in malarial parasite was described by Krungkrai and his colleagues in 1989 (Krungkrai, Webster, & Yuthavong, 1989). They have shown that methionine synthesis cycle exists in the malarial parasite and that this pathway has less activity compared to the thymidylate synthesis cycle and may serve to regulate the one-carbon transfer reactions of tetrahydrofolate metabolism rather than provide a major source of methionine. They proposed that MS might be a potential target for new antimalarial drugs. Interestingly, the gene coding for MS was not found from Plasmodium Genome Sequencing Project (detailed in PlasmoDB.org), hence it has never been investigated further until now. In this study, the putative sequence of methionine synthase of *P. falciparum* was cloned and expressed in bacterial recombinant system using advantages from malarial genome database and

recombinant technology. The expressed recombinant protein was characterized for the activity of methionine synthase.

P. falciparum MS putative gene, PLF1625w, was identified using cobalamin (vitamin B12) binding domain, which is active domain for MS in other species, as a template for domain search in PlasmoDB. PFL1625w gene contains 1746 basepairs in length and is now described as homocysteine methyltransferase (PlasmoDB). To obtain PLF1625w PCR product, gDNA from *P. falciparum* 3D7 parasite culture was extracted and digested with *Pst*I and *Sal*I, the sites that do not exist inside the gene, to chop down gDNA and help increase the binding of our primers to the specific gene locus. Nested PCR method was used to help amplify the specific PCR product. However, most of the obtained PCR products contained some mutations, when aligned with the gene sequence in the database. This error might be the result of AT-rich nature of the sequence, even though the *Pfu polymerase* was used in the PCR. To overcome this problem, site-directed mutagenesis was performed to correct the points of mutation. The corrected sequence of PFL1625w (MS putative gene) was confirmed by DNA sequencing. It was ligated into pET28α plasmid via *Nco*I and *Sal*I cloning sites. This plasmid was chosen due to their ability to tag expressed protein with histidine residues which are important for protein purification step using nickle column later. Positive colonies was confirmed by colony PCR and restriction digestions. The PFL1625w sequence was re-confirmed by DNA sequencing analysis.

For protein expression, pET28PFL1625w plasmid was transformed into expression bacterial host BL21(DE3). The transformed BL21(DE3) was induced for recombinant protein expression using l 0.4 M IPTG at 37°C for 3 hr. The result showed that there was an extra protein band as compared to the bacterial control, but

only in insoluble form. This might have caused by many reasons such as expressed toxic protein, rare codons, misfolded protein, hydrophobic protein, etc. To help on expression of recombinant protein as soluble form that can be further purifiled and assessed for its activity, different induction conditions were tried.

First, induction of protein at different temperatures was tried since lower temperature would slower the rate of bacterial growth that would result in slower protein expression. Thus, higher soluble protein expression was expected from lower induction temperature. Induction of protein expression was then carried at 16°C for 20 hr, however, our protein band of interest still appeared in insoluble fraction

Second, IPTG concentration was considered to be a variation for this protein expression experiment. Like induction temperature, lower IPTG concentration would slower rate of protein expression which leads to higher expression of soluble protein fraction. Unfortunately, there was no significant shifting from insoluble to soluble protein.

Third, BLRIG(DE3) and BL21(DE3)pLysS were used as host variation for protein expression. BL21(DE3)pLysS can provide a higher level of lysozyme which can substantially increase the lag time and reduce the maximum level of expression of target genes upon induction of T7 RNA polymerase. This damping effect on expression is sufficient that cells containing a target gene whose product is relatively innocuous can continue to grow indefinitely in the presence of IPTG, a property that may be useful in some circumstances. Additionally, BLRIG(DE3) can enable adjustable levels of protein expression throughout all cells in a culture and supply rare codon tRNA which enhance solubility of expressed protein. Still, there is no band

shifting from insoluble lane to soluble lane. However, the changing of expression hosts did not help on expression of soluble fraction

Finally, pET32α plasmid vector, which was designed to have histidine-tag at 5' end of the expressed protein, was used to assess if changing expression plasmid would result in more soluble protein expression. pET32PFL1625w plasmid was transformed into BL21(DE3) and induced with 0.4 M IPTG at 16°C for 20 hr. The result showed that our band of interest still remains on insoluble fraction.

To reassure that our expressed protein in insoluble fraction was PFL1625w, band of interest from polyacrylamide gel was sliced and digested with trypsin before analyzed using LC-MS/MS analysis. The result from LC-MS/MS was matched with the information from NCBI database via MASCOT MS/MS Ions search. The result showed that our expressed protein in insoluble fraction was completely matched with protein ID: gi 124806467 (conserved *Plasmodium* protein (*Plasmodium falciparum* 3D7)) on the NCBI database which is PFL1625w or homocysteine methyltransferase on plasmoDB. This confirmed that we could express PFL1625w protein which is homocysteine methyltransferase or methionine synthase, but not in soluble form that can be used in enzyme activity assay to check its activity.

To test if our expressed protein in insoluble fraction possess a biological activity of homocysteine methyltransferase or methionine synthase, pET28PFL1625w was transformed into RK4536, an *E. coli* K-12 strain that *MetH* and *MetE* genes were knockouted which lead to absent of methionine synthase activity (Banerjee, Johnston, Sobeski, Datta, & Matthews, 1989), and subjected into complementation assay. The result showed that there was no biological activity emerged from pET28PFL1625w-

transformed RK4536 which correlated with our previous results that our protein stayed in insoluble fraction that could not exert any biological activity.

In conclusion, PFL1625w or MS putative gene was successfully cloned and expressed in *E. coli* system. However, the recombinant PFL1625w protein was expressed in insoluble form which result in absent of biological activity and failed to complement the survival of *MetH*- and *MetE*-knockouted bacteria. Further investigation is needed to improve the solubility of PFL1625w recombinant protein in order to be able to study its biological activity which is predicted to be important. The discovery of methionine synthase in *Plasmodium* spp. may lead to identification of novel drug target which is in need in the process of antimalarial drug discovery.