CHARACTERIZATION OF THE FUNCTIONAL DOMAINS OF FKBP35 FROM *Plasmodium knowlesi*

CARLMOND GOH KAH WUN

BIOTECHNOLOGY RESEARCH INSTITUTE

UNIVERSITI MALAYSIA SABAH

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CARLMOND GOH KAH WUN

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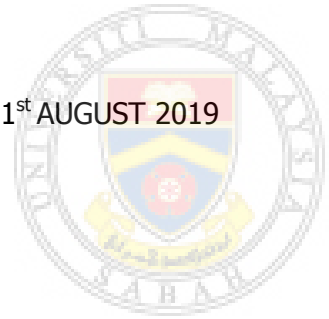
I, Carlmond Goh Kah Wun, student of Biotechnology Research Institute of University Malaysia Sabah hereby declare that my thesis entitled "Characterization of the Functional Domains of FKBP35 from *Plasmodium knowlesi*" is the result of my work and has not been submitted for any degree or professional qualification, except stated, otherwise by reference or acknowledgement. This study was carried out by me for my Master of Science (MSc.) under the guidance and supervision of Dr. Cahyo Budiman and Mdm Sophia Lau Tiek Ying.



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21st AUGUST 2019

CARLMOND GOH KAH WUN
MZ1511001T

CERTIFICATION

NAME	:	**CARLMOND GOH KAH WUN**
MATRIC NO.	:	**MZ1511001T**
TITLE	:	**CHARACTERIZATION OF THE FUNCTIONAL DOMAINS OF FKBP35 FROM *Plasmodium knowlesi***
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VIVA DATE	:	**21st MAY 2019**

CERTIFIED BY,

MAIN SUPERVISOR **SIGNATURE**

Dr. Cahyo Budiman

_____

CO-SUPERVISOR

Mdm. Lau Tiek Ying

_____

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ABSTRACT

High incident of *Plasmodium knowlesi* accompanied by the increase in drug resistance cases in Malaysia Borneo urges us to develop a novel antimalarial drug with no resistance. Despite its advantages as an antimalarial compound without resistance effects, FK506 displayed an immunosuppressive side effect. In attempts to find FK506 replacers, solid fundamental studies on target molecule of FK506 are needed to provide a platform for the development of novel antimalarial drug without the risk of resistance. Previous studies revealed FK506 inhibited function of FKBP35 from *Plasmodium falciparum*. Genome sequence of *P. knowlesi* suggested the presence of its FKBP35 (Pk-FKBP35) in which has high sequence similarity with FKBP35 from *P. falciparum* (Pf-FKBP35) and *P. vivax* (Pv-FKBP35), thus suggesting Pk-FKBP35 is considered a viable target for combating *P. knowlesi*. FKBP35 is a member of peptidyl-prolyl *cis-trans* isomerase (PPIase), consisting FK506-binding domain (FKBD) followed by tetratricopeptide repeated domain (TPRD). There is no study so far on Pk-FKBP35, particularly on the functionality of these domains. In fact, most of the studies on Pf- and Pv-FKBP35 were focused on FKBD region. This is due to the finding that FKBD is the region where FK506 binds and inhibits PPIase catalytic function of this protein. Previous studies on Pf-FKBP35 and Pv-FKBP35 also suggested that this protein might exhibit a dual-function of foldase and chaperone-like activities. However, involvement of the domains of FKBP35 on this dual-function remains poorly understood. High similarity between Pk-FKBP35 and Pf-FKBP35 suggested that Pk-FKBP35 might also exhibit dual-function, yet no experimental evidence was reported. This study aims to understand the regulatory domains for the function of FKBP35 from *Plasmodium knowlesi*. To address, expression system containing gene encoding full-length of Pk-FKBP35 and its derivatives, Pk-FKBD and PK-TPRD were constructed. Each of them was expressed in *Escherichia coli* BL21(DE3). Two steps purifications including Ni-NTA binding affinity followed by gel filtration yielded 109, 162 and 189 mg of proteins from 1 L culture of Pk-FKBP35, Pk-FKBD, and Pk-TPRD respectively. Pk-FKBD showed comparable catalytic PPIase activities with full-length Pk-FKBP35 when tested using synthetic tetrapeptide (Suc-Ala-Leu-Pro-Phe-AMC) with k_{cat}/K_M of 4.7 x 10^5 M^{-1} s^{-1}

while full-length Pk-FKBP35 yielded k_{cat}/K_M of 5.0 x 10^5 M^{-1} s^{-1}. Meanwhile, no PPIase activity was detected when Pk-TPRD was measured. This suggested that catalytic activity of Pk-FKBP35 resides at its FKBD. Further, this PPIase activity was also confirmed to be inhibited by FK506 with IC_{50} values of 310 and 309 for full-length Pk-FKBP35 and Pk-FKBD, respectively. The binary complex of FK506-FKBP35 was also found to be able to extremely increase the inhibition properties toward calcineurin phosphatase activity. This inhibition was also modulated by FKBD region, while TPRD is apparently important to maximize. Dual-function of Pk-FKBP35 was firstly examined on its foldase activity using RNase T1 as a protein substrate. Pk-FKBP35 demonstrated remarkable ability to catalyze slow-folding of RNase T1 with k_{cat}/K_M of 14.0 x 10^5 M^{-1} s^{-1}. Interestingly, Pk-FKBD was also able to catalyse slow-folding of RNase T1, with k_{cat}/K_M of 3.0 x 10^5 M^{-1} s^{-1}, which was lower than Pk-FKBP35. By contrast, no foldase activity was detected on Pk-TPRD. This suggested that Pk-FKBD is essential for foldase activity. However, the presence of Pk-TPRD is still required to maximize the activity. Meanwhile, Pk-FKBP35 was also confirmed to exhibit chaperone-like activity as indicated by its ability to bind to a folding intermediate of α-lactalbumin, with K_D of 24.39 μM, and prevent DTT-induced insulin aggregation in a concentration dependent manner. Further analysis of chaperone activity on Pk-TPRD and Pk-FKBD revealed that Pk-TPRD is a regulatory domain for binding to a folding intermediate of α-lactalbumin with K_D of 15.75 μM and prevention of DTT-induced insulin aggregation. This study confirmed that both domains of Pk-FKBP35 contributed to different function of this protein. Inhibition of any of this domain is believed to cause disruption of Pk-FKBP35 function and therefore promote the death of parasite cells. Altogether, targeting any of this domain in drug development is considered a good strategy to combat malaria diseases.

ABSTRAK

PENCIRI-CIRIKAN DAN FUNGSI DOMAIN FKBP35 DARIPADA Plasmodium knowlesi

Insiden kejadian Plasmodium knowlesi yang tinggi serta peningkatan kes rintangan ubatan di Borneo Malaysia, mendorong kami untuk menghasilkan ubat baharu antimalaria tanpa rintangan. FK506 memberikan kesan imunosupresif walaupun kelebihannya sebagai sebatian antimalaria tanpa kesan rintangan. Dalam percubaan mencari penganti bagi FK506, satu kajian terhadap molekul sasaran pada FK506 diperlukan bagi menyediakan platform untuk membangunakan ubat anti-malarial tanpa rintangan. Kajian terdahulu pada Plasmodium falciparum mendedahkan bahawa FK506 menghalang fungsi FKBP35. Urutan genome mencadangkan kehadiran FKBP35 pada P. Knowlesi (Pk-FKBP35) menunjukkan persamaan yang tinggi dengan FKBP35 dari P. falciparum (Pf-FKBP35) dan P. vivax (Pv-FKBP35), justeru mencadangkan Pk-FKBP35 dianggap sebagai sasaran untuk memerangi jangkitan P. knowlesi di Asia Tenggara. FKBP35 adalah ahli isomerase cis-trans peptidyl-prolyl (PPIase), yang merangkumi domain FK506-pengikat (FKBD) diikuti oleh domain berulang tetratricopeptide (TPRD). Setakat ini, belum ada kajian tentang Pk-FKBP35, terutamanya mengenai fungsi domain-domain ini. Kebanyakan kajian hanya dari P. Falciparum FKBP35 dan P. vivax -FKBP35 dan difokuskan kepada bahagian domain FKBD. Ini disebabkan bahawa penemuan mengenai FKBD adalah kawasan di mana FK506 mengikat dan menghalang fungsi katalitik protein PPIase ini. Kajian terdahulu mengenai Pf-FKBP35 dan Pv-FKBP35 mencadangkan bahawa protein tersebut mungkin menunjukan aktiviti dual-fungsi dalam perlipatan dan aktiviti chaperon. Walau bagaimanapun, penglibatan domain FKBP35 dalam dual-fungsi ini masih kurang difahami. Persamaan yang tinggi antara Pk-FKBP35 dan Pf-FKBP35 menunjukkan bahawa Pk-FKBP35 juga berkemungkinan menunjukkan dual-fungsi, namun tiada bukti eksperimen dilaporkan. Kajian ini bertujuan untuk memahami domain kawalan bagi fungsi FKBP35 dari Plasmodium Knowlesi. Untuk menangani masalah ini, sistem expressi yang mengandungi pengekodan gen penuh Pk-FKBP35 dan derivatifnya, Pk-FKBD dan PK-TPRD telah dibina. Setiap daripada mereka dihasilkan dalam bacteria Escherichia coli BL21

(DE3). Dua langkah penapisan termasuklah afiniti pengikatan Ni-NTA diikuti oleh penapisan gel menghasilkan 109, 162 dan 189 mg protein dari 1 L kultur Pk-FKBP35, Pk-FKBD, dan Pk-TPRD masing-masing. Pk-FKBD menunjukkan aktiviti PPIase katalitik yang sama dengan Pk-FKBP35 apabila diuji menggunakan tetrapeptida sintetik (Suc-Ala-Leu-Pro-Phe-AMC) dengan k_{cat} / K_M *4.7 x 105* M^{-1} s^{-1}, *Pk-FKBP35 menghasilkan* k_{cat}/K_M *of 4.7 x* 10^5 M^{-1} s^{-1}. *Sementara itu, tiada aktiviti PPIase pada Pk-TPRD. Ini menunjukkan bahawa aktiviti pemangkin Pk-FKBP35 berada di FKBDnya. Seterusnya, aktiviti PPIase ini juga dihalang oleh FK506 dengan* IC_{50} *nilai 310 dan 309 nm bagi Pk-FKBP35 dan Pk-FKBD. Kompleks gabungan FK506-FKBP35 juga dapat meningkatkan sifat-sifat penghalangan terhadap aktiviti calcineurin phosphatase. Inhibisi ini dimodulasi oleh bahagian FKBD, pada masa yang sama TPRD penting untuk memaksimumkan inhibisi ini. Aktiviti dual-fungsi ini pertama kali dikaji pada aktiviti penglipatannya menggunakan RNase T1 sebagai protein substrat. Pk-FKBP35 menunjukkan keupayaan yang untuk mempercepatkan perlipatan RNase T1 yang lambat dengan* k_{cat}/K_M *of 14.0 x* 10^5 M^{-1} s^{-1}. *Pk-FKBD juga dapat mempercepatkan perlipatan RNase T1, dengan* k_{cat}/K_M *of 3.0 x* 10^5 M^{-1} s^{-1}, *lebih rendah daripada Pk-FKBP35. Sebaliknya, tiada aktiviti perlipatan dikesan pada Pk-TPRD. Ini mencadangkan bahawa Pk-FKBD penting untuk aktiviti perlipatan. Walau bagaimanapun, kehadiran Pk-TPRD masih diperlukan untuk memaksimumkan aktiviti tersebut. Sementara itu, Pk-FKBP35 disahkan untuk menunjukkan aktiviti chaperon seperti yang ditunjukkan oleh keupayaannya untuk mengikat pada perlipatan sementara α-laktalbumin, dengan nilai* K_D *24.39 µM, dan menghalang agregasi insulin yang disebabkan oleh DTT. Analisa lebih lanjut mengenai aktiviti chaperon pada Pk-TPRD dan Pk-FKBD mendedahkan bahawa Pk-TPRD adalah domain kawalan seliaan untuk mengikat kepada pertengahan perlipatan α-laktalbumin dan mencegahan agregasi insulin daripada DTT dengan nilai* K_D *15.75 µM. Kajian ini mengesahkan bahawa kedua-dua domain pada Pk-FKBP35 menyumbang kepada dual-fungsi berlainan bagi protein ini. Penghalangan daripada domain-domain ini dipercayai menyebabkan gangguan fungsi Pk-FKBP35 dan menyumbang kepada kematian sel parasit. Kedua-dua domain ini disarankan sebagai penghasilan ubatan yang dianggap sebagai strategi baik untuk memerangi penyakit malaria.*

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LIST OF ABBREVIATIONS

bp	Base pair
FKBD	FKBP binding domain
FKBP35	FK506-binding domain
P. falciparum	*Plasmodium falciparum*
P. knowlesi	*Plasmodium knowlesi*
P. vivax	*Plasmodium vivax*
Pf	*Plasmodium falciparum*
Pk	*Plasmodium knowlesi*
PPIase	Peptidyl prolyl isomerase
Pv	*Plasmodium vivax*
rpm	Revolution per minute
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TEMED	Tetramethylethylenediamine
UV	Ultraviolet ray
EDTA	Ethylenediaminetetraacetic acid

LIST OF SYMBOLS

µg	Micro gram
µm	Micro meter
µM	Micro molar
°C	Degree Celsius
a.u.	Arbitrary unit
g	Gram
kDa	Kilo Dalton
M	Molar
ml	Mili liter
nm	Nano meter

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background Study

Human malaria is an overwhelming disease that caused more than half of the world's population at risk. Malaria disease is caused by a parasite of Plasmodium genus and it can be transmitted from macaque to human, and between human through bites of female *Anopheles* mosquitoes. In some rare cases, this parasite can be transmitted via infected blood transfusion or through placenta delivery from an infected pregnant mother to the infant in the womb (Malhotra *et al.*, 2006). Based on World Health Organization (2018), there were an estimated 212 million new human malaria cases and up to 445,000 deaths in the year 2016 even though anti-malarial drugs and treatments are available. However, until today, there are no valid licensed for vaccines towards malaria, hence raising the global stress in the finding malarial treatment. Studies found that there are 6 Plasmodium species that caused human malaria, which are *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, *P. knowlesi* and *P. Cynomology*. *P. knowlesi* is the latest species causing malaria in human (Daneshvar *et al.*, 2009).

Among the 6 species of Plasmodium parasite, *P. knowlesi* was found caused most of human malaria in Southeast Asia, with high incidences were reported at the Borneo island, Peninsular Malaysia, Thailand, Singapore, Philippines, Vietnam and Myanmar (Lau & Joveen-Neoh, 2011a, 2011b). However, the most widespread of *P. knowlesi* is in Malaysian Borneo region. In the year of 2004, Singh *et al.* (2004) discovered a large number of *P. knowlesi* malaria were misdiagnosed as *P. malariae*

malaria due to their similarity in microscopic examination by PCR which consequently caused delayed in treatment in Sarawak, Malaysia. Since then, the cases of *P. knowlesi* malaria was reported progressively mostly from Southeast Asia region hence confirming the existence of human malaria that is caused by *P. knowlesi*. Apart from that, large numbers of human malaria were reported by travellers whose visited countries in Southeast Asia, especially Sabah and Sarawak states of Malaysia, were caused by *P. knowlesi* (Bronner *et al.*, 2009; Daneshvar *et al.*, 2009; Figtree *et al.*, 2010; Joveen-Neoh, *et al.*, 2011).

Due to the rapid development of malaria parasite, available antimalarial drugs such as chloroquine and artemisinin begins to exhibit resistant effect (Alag *et al.*, 2010) hence increases the rate of malarial disease infection. William *et al.* (2011) reported that 39% of the malarial patients in Queen Elizabeth Hospital (QEH), Kota Kinabalu suffered from severe *P. knowlesi* malaria, while six of them have died. While Cox-Singh *et al.* (2008) stated that four fatal cases were reported in Sarawak caused by *P. knowlesi* between the years 2004 – 2005. This brought the urge in discovering a new anti-malarial drug for *P. knowlesi* in addition to the most used anti-malarial drug caused resistant to the parasite (Kotaka *et al.*, 2008). One of the ways in drug design is through re-visiting and re-designing the existing anti-malarial drug.

Based on previous studies, malarial activities of *P. falciparum* and *P. vivax* parasite were found to be inhibited by FK506 drugs, this lead to the binding target site of FK506 on Pf- and Pv-FKBP35, a member in FKBP family in both *P. falciparum* and *P. vivax*, which also conserved in *P. knowlesi*, named as Pk-FKBP35 (Alag *et al.*, 2010). Tacrolimus or commonly known as FK506 is an immunosuppressive drug that usually used for prevention of rejection during organ transplant, surprisingly it shows antimalarial activity. FK506 is a 23-membered macrolide lactone that found to binds at peptidyl-prolyl *cis-trans* isomerase (PPIase) domains of various FK506-binding proteins (FKBPs), including FKBPs of Plasmodium species, hence promising malarial drug target site (Alag *et al.*, 2010; Kotaka *et al.*, 2008). However, due to

the immunosuppressive effect of this FK506 drug, novel antimalarial drug replacing FK506 must be developed. To address, comprehensive studies on the function of pk-FKBP35 should contributed to accelerate the development of FK506 replacers.

FKBP35 is a 35 kDa FKBP member protein that can be identified in most *Plasmodium* species, which known to involve in malaria infection (Alag *et al.*, 2009). Pf- and Pv-FKBP35 exist as a multi-domain protein containing N-terminal FK506-binding domain (FKBD) followed by tetratricopeptide domain (TPRD) with a putative calmodulin binding site (Alag *et al.*, 2013; Kumar *et al.*, 2005). FKBD was known to have *cis-trans* peptidyl-prolyl isomerase (PPIase) activities, which function in the conversion of *cis-trans* rotamers of peptidyl-prolyl amide bones (Alag *et al.*, 2010); while TPRD believed to exhibit chaperone function, which both of these functions are related in protein folding (Bianchin *et al.*, 2015). Pf-FKBP35 also found to interact with calcineurin in a dependent and independent manner in the presence of FK506, this highly represents the potential drug target site (Kumar *et al.*, 2005; Monaghan & Bell, 2005). The high sequence similarity of FKBP35 protein in *P. knowlesi* with both *P. falciparum* and *P. vivax* assuming Pk-FKBP35 holding both FKBD and TPRD hence increase the possibility of Pk-FKBP35 exhibit both PPIase and chaperone activities (Kotaka *et al.*, 2008; Yoon, *et al.*, 2007). Due to the multi-domains of FKBP35, it is believed FKBP35 exhibit dual-function which are PPIase activity and chaperone function, that held in each of the domain.

PPIase is a highly conserved protein family that can be found in almost all living organisms. It functions as an enzyme that accelerates the rate-limiting *cis-trans* isomerisation of Xaa-Pro peptide bones. This process occurs during protein folding reactions, especially during refolding of denatured proteins, de novo protein synthesis as well as during the formation of biologically active conformations of polypeptides (Budiman *et al.*, 2012; Schiene-Fischer & Yu, 2001). Based on their inhibitor specificity, PPIase was divided into three families, which are cyclophilins (CyPs), FK506-binding proteins (FKBPs), and parvulins, however all of them having different sequences and structures. The evolving of these proteins was believed to

recognize the specific signature of proteins sequences and supervise *in vivo* protein folding (Schiene-Fischer & Yu, 2001). Studies found that PPIase activity of CyPs can be inhibited by cyclosporine while FKBPs can be suppressed by FK506 and rapamycin. Hence, they usually used as target inhibitor in immunosuppressive drugs development. FKBPs can be found in most Plasmodium species, this indicates that they show PPIase activities. Most of FKBPs family members, including Pf- and Pv-FKBP35 showing PPIase activities (Kumar *et al.*, 2005; Monaghan & Bell, 2005), hence there is a high probability of Pk-FKBP35 having PPIase activity. However, the natural function of FKBPs still poorly understood.

On the other hand, chaperone is a type of protein that functions to assist the proper folding of a protein. Misfolding or aggregation of protein thereby by disrupting the cell function and lead to diseases such as Alzheimer and Parkinson diseases (Caughey & Lansbury Jr, 2003). Hence chaperone plays an important role in preventing as well as refolding of the misfolded protein (Buchner, 1996). Chaperone can be reusable as it will release the properly folded protein. Besides, chaperone tend to control the folding state not only under normal conditions, but as a protection for the protein during extreme/stress conditions such as in different temperature or unsuitable pH that might lead to protein self-destruction hence protein unfolding/aggregation (Hoffmann, *et al.*, 2004). Because of this, most of the chaperones are heat shock protein (HSP) since it can endure in different temperature states. Chaperone usually assists in post-translational mechanisms that can be easily found on endoplasmic reticulum where it provided space for the newly synthesized polypeptide to fold properly. Based on Hoffmann *et al.* (2004), there are two systems of chaperones, foldase and holdase. Foldase support protein folding in ATP-dependent manner while holdase prevent aggregation by binds to protein folding intermediate state, but not support in protein folding.

Some PPIase family member exhibited chaperone function in addition to their PPIase catalytic activity (Alag *et al.*, 2013; Kumar *et al.*, 2005; Yoon *et al.*, 2007). Interestingly, the chaperone function is mostly found in multi-domain PPIase.

In this group of PPIase, catalytic activity and chaperone function are often found to be separately regulated by different domains. FKBP35 from *P. falciparum* was reported to have a dual-function (Kotaka *et al.*, 2008). Given the high similarity between Pf- and Pk-FKBP35, the later protein assumes to exhibit dual-function. Nevertheless, experimental evidence remains to be confirmed. Even more, whether the dual-function of Plasmodium FKBP35 is specifically regulated by different domain is unknown.

In this study, we demonstrated full-length Pk-FKBP35 exhibited both PPIase and chaperone activities comparable to those Pf- and Pv-FKBP35, in addition to identify regulatory domains for the catalytic and chaperone activities of this protein.

1.2 Problem Statement

As a worldwide threat, Malaria needs more attention and efforts to reduce the infection cases. In Southeast Asia, especially at Borneo region, most of the malaria was caused by *P. knowlesi*. There are antimalarial drugs available at the market however most of them were found resistance toward malarial disease and this became a threat to global efforts in controlling and eliminating malaria. Better inhibitor toward FKBP35, a malarial target gene, in combating malarial disease must be developed. However, there are only studies of FKBP35 from other species such as *P. falciparum* and *P. vivax* but no *P. knowlesi*. Moreover, most of the studies only focus on the catalytic part, but less study on dual-function on both PPIase and chaperone activities. Hence comprehensive understanding towards dual-functions of Pk-FKBP35 should be studied.

1.3 Hypothesis

It is hypothesised that Pk-FKBP35 exhibit remarkable PPIase activity which is regulated by PK-FKBD, by accelerating the isomerisation of tetrapeptide; it also hypothesized that Pk-FKBP35 and Pk-FKBD also regulate the function of foldase by accelerating the isomerisation of RNase T1. Lastly, it is hypothesized that Pk-FKBP and Pk-TPRD exhibit chaperone by binding to protein intermediate and prevent protein aggregation.