การแสดงออกและการหาคุณลักษณะของเอนไซม์ออโรเทต ฟอสฟอไรโบซิลทรานสเฟอเรส ที่ถูกตัดด้านปลายอะมิโนของเชื้อพลาสโมเดียม ฟัลซิพารัม

นางสาวสวิราศจี พงศะบุตร

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EXPRESSION AND CHARACTERIZATION OF N-TERMINAL TRUNCATION OF *PLASMODIUM FALCIPARUM* OROTATE PHOSPHORIBOSYLTRANSFERASE ENZYME

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Medical Biochemistry

Department of Biochemistry
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Thesis Title	EXPRESSION AND CHARACTERIZATION OF N-TERMINAL TRUNCATION OF PLASMODIUM FALCIPARUM OROTATE PHOSPHORIBOSYLTRANSFERASE ENZYME
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สวิราศจี พงศะบุตร : การแสดงออกและการหาคุณลักษณะของเอนไซม์ออโรเทต ฟอสฟอไรโบซิลทรานสเฟอเรสที่ถูกตัดด้านปลายอะมิโนของเชื้อพลาสโมเดียม ฟัลซิพารัม. (EXPRESSION AND CHARACTERIZATION OF N-TERMINAL TRUNCATION OF PLASMODIUM FALCIPARUM OROTATE PHOSPHORIBOSYLTRANSFERASE ENZYME) อ. ที่ปรึกษา : ศ.ดร. จิระพันธ์ กรึงไกร, 86 หน้า.

พลาสโมเดียม ฟัลซิพารัม เป็นเชื้อก่อโรคมาลาเรียในคนที่ร้ายแรงที่สุด เชื้อชนิดนี้ต้องอาศัยวิถีการสังเคราะห์เบสไพริมิดีนขึ้นมาใหม่เพื่อสร้างเบสไพริมิดีนมาใช้ โดยยีนของออโรเทต ฟอสฟอไรโบซิลทรานเฟอเรส (orotate phosphoribosyltransferase) ซึ่งเป็นเอนไซม์ลำดับที่ 5 ทำหน้าที่เร่งปฏิกิริยาการสร้างออโรทิดีน 5′-โมโนฟอสเฟต (orotidine 5′-monophosphate ; OMP) และไพโรฟอสเฟต (pyrophosphate ; PPi) จาก 5-ฟอสฟอไรโบซิล-1-ไพโรฟอสเฟต (5-phosphoribosyl-1-pyrophosphate ; PRPP) และออโรเทต (orotate) ได้ถูกพบในพลาสโมเดียม ฟัลซิพารัม (*pf*OPRT) และมีลำดับกรดอะมิโนทางด้านปลายอะมิโนเกินมาถึง 66 ลำดับกรดอะมิโน จึงมีลำดับกรดอะมิโนยาวที่สุด (281 ลำดับกรดอะมิโน) เมื่อเทียบกับเอนไซม์ชนิดเดียวกันกับสิ่งมีชีวิตอื่นๆ ซึ่งในพลาสโมเดียม ฟัลซิพารัม ยังไม่ทราบถึงความสำคัญของลำดับกรดอะมิโนที่ยาวออกมา

ในการศึกษาครั้งนี้จึงทำการศึกษายีน *pf*OPRT ที่มีความยาวของลำดับกรดอะมิโนปกติ (full-length *pf*OPRT) และยีน *pf*OPRT ที่ถูกตัดด้านปลายอะมิโนออก (N-terminal truncated *pf*OPRT) โดยทำการโคลนยีนและทำให้ยีนเกิดการแสดงออกเป็นโปรตีน รีคอมบิแนนท์ *pf*OPRT ทั้ง 2 ชนิดในสารสกัดจากเชื้อ *Escherichia coli* และถูกทำให้บริสุทธิ์โดยผ่านนิกเกิล มีทัล แอฟฟินิตี โครมาโทกราฟี (nickel metal-affinity chromatography) จากนั้นทำการวิเคราะห์จาก SDS-PAGE, Western blot รวมทั้งศึกษาการทำงานและความคงตัวของเอนไซม์ต่อไป

ผลการศึกษาพบว่า full-length และ N-terminal truncated *pf*OPRT มีน้ำหนักโมเลกุล 35 kDa และ 30 kDa ตามลำดับ ค่า enzyme specific activity ของ N-terminal truncated *pf*OPRT มีค่าลดลงประมาณ 10 เท่าเมื่อเปรียบเทียบกับ full-length *pf*OPRT ความคงตัวของเอนไซม์ (stability) ของ N-terminal truncated *pf*OPRT ต่ำกว่า full-length *pf*OPRT สรุปได้ว่า N-terminal truncated pfOPRT ซึ่งมีมวลโมเลกุล 30 kDa มี ความไวต่อการเกิดปฏิกิริยา (specific activity) และคงตัว(stability) น้อยกว่า full-length pfOPRT จากข้อมูลเหล่านี้ทำให้ทราบได้ว่าลำดับกรดอะมิโนที่เพิ่มขึ้นมาทางด้านปลายอะมิโนของออโรเทต ฟอสฟอไรโบซิลทรานสเฟอเรสของเชื้อพลาสโมเดียม ฟัลซิพารัมนั้น อาจมีส่วนสำคัญต่ออัตราการเกิดปฏิกิริยาและความคงตัวของเอนไซม์ ซึ่งเป็นที่น่าสนใจและมีโอกาสเป็นเป้าหมายใหม่ในการออกแบบยาต้านมาลาเรียโดยใช้โครงสร้างเป็นพื้นฐาน(structure-based) โดยการควบคุมส่วนที่เกินมานี้น่าจะส่งผลต่ออัตราการอยู่รอดของปรสิตโดยการจำกัดการสังเคราะห์เบสไพริมิดีน

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สาขาวิชา ชีวเคมีทางการแพทย์
ปีการศึกษา 2549

ลายมือชื่อนิสิต สวิราศจี พงศะบุตร
ลายมือชื่ออาจารย์ที่ปรึกษา .....

B9ivjt# # 4874798230 : MAJOR MEDICAL BIOCHEMISTRY

KEY WORD : OROTATE PHOSPHORIBOSYLTRANSFERASE / PLASMODIUM FALCIPARUM / N-TERMINAL TRUNCATED

SAWIRASAGEE PONGSABUT : EXPRESSION AND CHARACTERIZATION OF N-TERMINAL TRUNCATION OF PLASMODIUM FALCIPARUM OROTATE PHOSPHORIBOSYLTRANSFERASE ENZYME. THESIS ADVISOR : PROF. JERAPAN KRUNGKRAI, Ph.D. 86 pp.

Plasmodium falciparum, the causative agent of the most lethal form of human malaria, totally depends on *de novo* pyrimidine biosynthetic pathway. A gene encoding orotate phosphoribosyltransferase (OPRT), the fifth enzyme of the *de novo* pathway catalyzing formation of orotidine 5'-monophosphate (OMP) and pyrophosphate (PP_i) from 5-phosphoribosyl-1-pyrophosphate (PRPP) and orotate, was identified from *P. falciparum* (*pf*OPRT), and was exceptional in that it contained an amino-terminal extension of 66 amino acids, making the longest amino acid sequence (281 amino acids). This unique sequence remains uncharacterized the importance in the parasite.

In this study, the cDNA of the full-length and N-terminal truncated *pf*OPRT gene was cloned and expressed. The both types of the recombinant *pf*OPRT were purified from the *E. coli* lysate by nickel metal-affinity chromatography. After that The both types of the recombinant *pf*OPRT were analyzed by SDS-PAGE and Western blot. Furthermore, the studying in activity and stability were included.

The results revealed that the full-length and N-terminal truncated *pf*OPRT had a molecular mass of 35 kDa and 30 kDa, respectively. The specific activity of the N-terminal truncated *pf*OPRT was decreased about 10-fold when compared with the full-length *pf*OPRT. The stability of the N-terminal truncated *pf*OPRT was lower than the full-length *pf*OPRT. So, it concluded that the N-terminal truncated *pf*OPRT, which had a molecular weight of 30 kDa, had less specific activity and stability than the full-length *pf*OPRT. These evidences provided that the N-terminal extension of *pf*OPRT may have an important role in the activity and stability of this enzyme, therefore it may be the new target for structure-based antimalarial drug design. To block this extension part will take the action on parasite survival by limiting the pyrimidine base biosynthesis.

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ABBREVIATIONS

^{o}C	degree celcius
CO_2	carbon dioxide
EDTA	Ethylendiaminetertraacetic acid
g	centrifugal force
hr	hour
kDa	kilodalton
M	Molar
mg	milligram
min	minute
ml	milliliter
mM	millimolar
µl	microliter
µM	micromolar
M_r	relative molecular weight
N_2	nitrogen
O_2	oxygen
Rpm	revolutions per minute
PMSF	phenylmethanesulfonyl fluoride
Sec	second
SDH	succinate dehydrogenasee
V/V	volume by volume
W/V	weight by volume
DNA	deoxyribonucleic ascid
dNTP	deoxyribonucleotide containing the base adenine,thymine cytosine and guanine, respecpectively
A T C G	Nucleotide sequence containing The base adenine, thymine, cytosine and guanine, respectively

bp	base pair
mg	milligram
O/N	overnight
TE	Tris-ethylene diamine tetraacetic acid
SDS	Sodium dodecyl sulphate
PCR	polymerase chain reaction
BLAST	basic local alignment search tool

CHAPTER I

INTRODUCTION

1. Malaria

Malaria is one the world's most prevalent diseases. Current estimates predict over 200 million cases of malaria annually. The number of clinical cases exceeds 150 million with approximately 2-3 million deaths per year. Most of these victims are infants, and young children[1-3].

Over half the world's population lives in malarious areas[1]. Almost 85% of the world's malaria occurs in sub-Saharan Africa. The vast majority of these cases are *Plasmodium falciparum* malaria, though *Plasmodium ovale* and *Plasmodium malariae* are also present. Malaria is also prevalent in Southeast Asia, India, South and Central America[1-4].

The discovery of the parasite in mosquitoes earned the scientist Ronald Ross the Nobel Prize in Physiology or Medicine, 1902. In 1907 Alphonse Laveran received the prize for his findings that the parasite was present in human blood and that it caused the malaria disease[5, 6].

There are four species of *Plasmodium* which infect humans[1-3, 5, 7].

1. *P. falciparum*
2. *P. vivax*
3. *P. malariae*
4. *P. ovale*

P. falciparum is the most virulent species of malaria since it can cause the death of the host as a result of cerebral malaria, pulmonary or renal failure. In *falciparum* malaria the infected red blood cells become sticky and adhere to the small

vessels in the brain severely restricting the flow of essential nutrients to the brain[1-3, 5, 7, 8].

2. *P. falciparum* orotate phosphoribosyltransferase of pyrimidine pathway

Malaria is one of the most serious and complex health problems facing humanity in the 20th century. Approximately 300 million of the world's people are infected by *P. falciparum* and between 1 and 1.5 million people die every year[1, 3, 5].

Malaria can normally be cured by antimalarial drugs. However, the parasites have developed resistance to certain antimalarial drugs, particularly chloroquine. [2, 3]. The problems of controlling malaria in some countries are aggravated by inadequate health structures and poor socioeconomic conditions. However, the situation has become even more complex over the last few years with the increase in resistance to the drugs normally used to combat the parasite[3]. Therefore, effective antimalarial drugs are still in need of the fight against the disease. This has started a renewed search for new types of drugs with novel targets.

Because *P. falciparum* must survive within the human host and proliferate rapidly, so the nucleotide metabolism offers new targets for inhibition of parasite growth.

It has been shown for sometimes that the parasite could only obtain purine nucleotides through the salvage pathway and pyrimidine nucleotides through *de novo* synthesis[9]. The malarial parasite operates pyrimidine biosynthetic pathway for its growth and development in the human host. The first six enzymes, catalyzing the formation of uridine 5'-monophosphate from the starting precursors of HCO_3^-, ATP ,and L-glutamine, were partially characterized. The genes encoding these six enzymes were identified, in order from the first to the sixth step, as CPS II (carbamoyl phosphate synthetase II), ATC (aspartate transcarbamoylase), DHO (dihydroorotase), DHOD (dihydroorotate dehydrogenase),OPRT (orotate phosphoribosytransferase),and OMPDC (orotidine 5'-monophosphate decarboxylase)[10, 11].

From studying about *P. falciparum* orotate phosphoribosyltransferase gene (the gene of the fifth enzyme in the pyrimidine pathway), it was found that amino acid sequence of *P. falciparum* orotate phosphoribosyltransferase had the N-terminal extension sequence more than orotate phosphoribosyltransferase amino acid sequences from other organisms[12, 13]. This exceeding part has not been identified its importance yet. Thus, it is interesting to study about this part of *P. falciparum* orotate phosphoribosyltransferase as the target of antimalarial drug.

3. Aims of thesis

Some reports suggested that enzymes involved in Pyrimidine metabolism, including orotate phosphoribosyltransferase are potential targets for new antimalarial drug. One way to achieve fundamental basis for target-directed development of new antimalarial drugs is to analyze these enzymes in biochemical details. For the N-terminal extension amino acid sequence of *P. falciparum* orotate phosphoribosyltransferase that is interesting, We would like to delete it and compare the properties with the full-length enzyme.

So, the objectives of this thesis are as follow;

1.To clone and express N-terminal truncated *Plasmodium falciparum* orotate phosphoribosyltransferase enzyme.

2.To characterize N-terminal truncated *Plasmodium falciparum* orotate phosphoribosyltransferase enzyme, compared with the full-length enzyme.

CHAPTER II

LITERATURE REVIEW

1. Malaria : a deadly disease.

The World Health Organization collects information on global deaths by International Classification of Disease (ICD) code categories. The following table lists the top infectious disease killers which caused more than 100,000 deaths in 2002 (estimated). 1993 data is included for comparison, and found that the top three single agent/disease killers are HIV/AIDS, Tuberculosis and malaria (Table 2-1)[14].

Malaria is one the world's most prevalent and important tropical parasitic diseases. It is one of the most common infectious diseases and an enormous public-health problem. Malaria is a vector-borne infectious disease that is widespread in tropical and subtropical regions[2, 4].

The discovery of the parasite in mosquitoes earned the scientist Ronald Ross the Nobel Prize in Physiology or Medicine, 1902. In 1907 Alphonse Laveran received the prize for his findings that the parasite was present in human blood and that it caused the malaria disease[5, 6].

Malaria affects huge numbers of people worldwide: up to 300 million clinical cases, mainly children, emerge each year causing 1.5 to 2.7 million deaths[5]. The vast majority of deaths occur among young children in Africa, especially in remote rural areas with poor access to health services and kills one child every 30 seconds. In absolute numbers, malaria kills 3,000 children per day under five years of age. In those children who survive, malaria also drains vital nutrients, impairing their physical and intellectual development[4]. Other high-risk groups are women during pregnancy, non-immune travelers, refugees, displaced persons and laborers entering endemic areas

[2, 3]. Malaria is a public health problem today in more than 90 countries, inhabited by a total of some 2,400 million people - 40% of the world's population[4].

Worldwide mortality due to infectious diseases

Rank	Cause of death	Deaths 2002	Percentage of all deaths	Deaths 1993	1993 Rank
N/A	All infectious diseases	14.7 million	25.9%	16.4 million	32.2%
1	Lower respiratory infections	3.9 million	6.9%	4.1 million	1
2	HIV/AIDS	2.8 million	4.9%	0.7 million	7
3	Diarrhea diseases	1.8 million	3.2%	3.0 million	2
4	Tuberculosis (TB)	1.6 million	2.7%	2.7 million	3
5	Malaria	1.3 million	2.2%	2.0 million	4

Table 2-1 The top three single agent/disease killers are HIV/AIDS, TB and malaria.

Almost all vertebrates, birds, snakes and monkeys, for example, can be infected by *Plasmodium* (malaria) parasites. Different animal species can only be infected by their own specific species of *Plasmodium*[2]. The disease is caused by a group of human parasites which are organisms that need to feed on other organisms in order to survive. The four different parasites that cause human malaria are: *Plasmodium vivax*, *Plasmodium falciparum*, *Plasmodium malariae* and *Plasmodium ovale*[1-3, 5, 7]. It is possible to get infected with more than one type of *Plasmodium* parasite and this occurs in five to seven percent of infections[7]. *Plasmodium* are transmitted by mosquito bites, specifically female mosquitoes, which need a supply of blood to produce and lay eggs[2, 3]. The mosquitoes that transmit human malaria belong to a group called Anopheles. There are some 380 species of anopheline mosquito, but only 60 species are able to transmit the parasite[3]. They contract the disease by taking blood from an already infected person, and later pass on the disease when they bite someone else[5]. Malaria can also be acquired from an infected blood transfusion or even from the shared needles of drug addicts[2].

Symptoms of malaria include fever, shivering, pain in the joints, headache, repeated vomiting, generalized convulsions and coma[1, 2, 7]. If not treated, the most serious kind caused by the *P. falciparum* parasite, can become deadly within two days[5]. *P. falciparum* is the most virulent species of malaria since it can cause the death of the host as a result of: cerebral malaria, pulmonary or renal failure[1-3, 7]. In *falciparum* malaria the infected red blood cells become sticky and adhere to the small vessels in the brain severely restricting the flow of essential nutrients to the brain[1]. The other malaria parasites cause less serious symptoms, but can weaken a person's immune system, making him/her more vulnerable to other infectious, life-threatening diseases[5].

Almost 85% of all malaria cases are in sub-Saharan Africa, it is mainly found in tropical areas such as Africa, Central and South America, South East Asia and the Pacific islands[7]. The vast majority of these cases are *P. falciparum* malaria, though *P.ovale* and *P. malariae* are also present. Malaria is also prevalent in Southeast Asia, India, South and Central America[1].

The geographical area affected by malaria has shrunk considerably over the past 50 years, but control is becoming more difficult and gains are being eroded[4]. Other causes of its spread include global climatic change, disintegration of health services, armed conflicts and mass movements of refugees[4, 5].

2. Biology and biochemistry of malaria

2.1 Life cycle

The human malaria parasite actually consisting of four species of the genus *Plasmodium* (P*. falciparum, P. vivax, P. malariae and P. ovale*) undergoes over a dozen distinguishable stages of development as it move from the arthropod vectors (species of anopheline mosquitoes) to the human host and back again. One way to conceptuallize this complex life cycle is to consider it in three distinct parts; the liver phase, the blood phase and the mosquito phase.

Depending on the developmental stages and species, malaria parasites can be spherical, ring shape, elongated, or crescent shaped and can be ranged in size from 1 to 20 microns in diameter. By comparison, a normal red blood cell has a diameter of about 7 microns.

Although the four species of human malaria parasites are closely related, there are major differences among them. *P. falciparum*, the most pathogenic of the four species, has been found to be more closely related to avian (*P. lophurae, P. gallinaceum)* and rodent species *(P. berghei, P. yoelii. P. chabaudi*) of Plasmodium than to the other primate and human species[15]. The malarial parasites have a life cycle which is split between a vertebrate host and an insect vector.

2.1.1 Liver phase

The liver phase of malaria begins when the female malaria-infected anopheline mosquito injects the sporozoites stage of the parasites and anticoagulant saliva from their salivary gland into the human host during a blood meal to ensure an

even meal. After just a few minutes, the sporozoites arrive at the liver and invade the liver cell (hepatocytes). Over the course of 5 to 15 days, depending on the species, the sporozoites undergo a process of asexual reproduction, mutiplying within the cells and become schizont stage. (This stage is called exo-erythrocytic schizogony) that results in the production of 30,000 " daughter " parasites, namely merozoites. It is the merozoites that, once released from the liver, carry malaria infection into the red blood cell (erythrocytes).

2.1.2 Blood phase

When merozoites are released from the liver into the blood stream, asexual blood-stage reproduction, or erythrocytic schizogony, has begun. Parasites invasion of red blood cells unfolds in four steps: attachment of the merozoites to the erythrocyte, rapid deformation of the red blood cell, invagination of the erythrocyte membrane where the parasites is attached and subsequent envelopment of the merozoites, and the resealing of the erythrocyte membrane around the parasites[16-18].

After invasion, the parasite lies within a membranous parasitophorus vacuole, where it synthesizes nucleic acids, proteins, lipids, mitochondria, and ribosomes and assembles these components into new merozoites[19]. The entire erythrocytic asexual cycle takes between two and three days to run it course, depending on the species. The ring stages mature into trophozoints and then into schizonts. Once merozoite assemble is completed, the erythrocyte ruptures and merozoites are released into the plasma, where they attach to other erythrocytes and begin the process anew. Some merozoites, for reasons not well understood, differentiate into the sexual forms of the parasite, the gametocytes. The factors that determine the sex of the gametocytes are unknown. Gametocytes development takes between 2 days (for *P. vivax*) and 10 days (for *P. .faciparum*). Blood stage parasite are responsible for the clinical manifestation of the disease.

2.1.3 Mosquito phase

When gametocytes are taken up during a mosquito's blood meal a number of factors, including temperature, concentrations of oxygen and carbondioxide, pH, and a mosquito exflagellation factor, are thought to contribute to the maturation of gametocytes. Male microgamete are released during a process called exflagellation. Fusion of the female macrogamete with a single microgamete results in fertilization and the formation of the ookinete. The ookinete migrates to the wall of the mosquito midgut, where it penetrates the peritrophic membrane and epithelium and comes to rest on the external surface of the stomach. Over a period of days, this stage of the parasite matures into an oocyst containing up to 10,000 motile sporozoites. When the oocyst ruptures, the sporozoites enter the mosquito circulation and travel to the salivary glands, where they are injected into the human host when the mosquito feeds. The number of sporozoites that enter the human host during a single blood meal is thought to be highly variable. The life cycle of Plasmodium in the mosquito and man is shown in Figure 2-1.

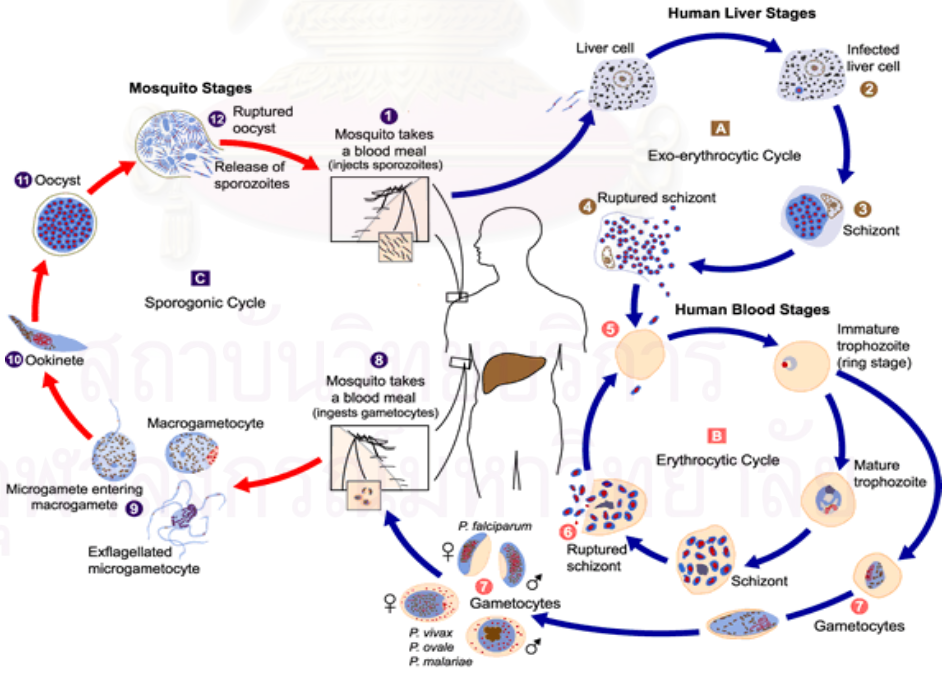

Figure 2-1 Life cycle of the malarial parasite

(From http://www.uni-tuebingen.de/modeling/Mod_Malaria_Cycle_en.html

2.2 Genome organization

Malaria parasites are lower eukaryotes which are haploid for their life cycle with a brief diploid phase prior to meiosis in the mosquito vector. The haploid genome comprises 2-2.5 x 10^7 base pairs of DNA. It is unusual that it has extremely low (G+C) content. It was also found that the repeat element is present in malaria DNA at a very high copy number and appear to be distributed widely throughout the genome [33, 34] as found in all of the eukaryotic genome.

There are three genomes in *Plasmodium.* [35](1)The nuclear genome contains 14 chromosomes. The technique of pulse field gel electrophoresis has allowed researchers to visualize the chromosomes, which range in length from 600 to 3,500 kilobases. Genes for various parasites proteins has been located on individual chromosomes[36, 37]. (2) Mitochondrial genome, known as the 6 kilobase (Kb) tandemly repeated element encodes three genes of subunit I,III of cytochrome oxidase as well as cytochrome b, and fragmented rRNA genes.(3) 35 Kb circular genome contains large and small ribosomal RNA genes, transfer RNA genes and genes encoding rpo B and rpo C subunits of RNA polymerase.

2.3 Biochemistry of malaria

The red cell receptor for *P. falciparum* is probably located on the glycophorin. After invasion infected cell undergoes considerable morphological and metabolic changes. These changes accompanying maturation are related to parasite biochemical activities involving the biosynthesis of protein, DNA, and RNA, carbohydrate metabolism, amino acid, and lipid metabolisms. The relatively recently acquired ability to cultivate *P. falciparum in vitro* has greatly expanded biochemistry to study parasite nutrition and metabolism[20]. The malarial parasite exhibit a rapid growth and multiplication rate during many stages of its life cycle. This necessitates that the parasite, like all other organisms, acquires nutrients and metabolizes these various biological molecules in order to survive and reproduce. A better understanding of the

parasite's metabolism may lead to the development of novel therapeutic strategies which exploit the uniqueness of the parasite.

2.3.1 Carbohydrate metabolism

The erythrocytic stages of malaria do not store glycogen or other reserved polysaccharides, therefore they rely on host supplied glucose. In *P.falciparum*, glucose can be replaced by fructose, but the parasite will not develop *in vitro* when another sugar, such as galactose, mannose, maltose or ribose are substituted[21]. The end products of glucose catabolised vary with the species of *Plasmodium*: simian and rodent malaria infected red blood cells convert 70-85% of glucose to lactate, whereas in red blood cells infected with avain malarial parasites, CO_2, organic, and amino acid as well as lactate are produced[20, 22].

All species of malaria appear to posses the glycolytic enzymes of the Embden-Meyerhoft (glycolysis) pathway. All of the glycolytic enzymes have not been identified in a single species [23] and therefore it is an assumption that the entire pathway exists in all species of *Plasmodium*.

The pentose pathway is not increased on parasitisation of the red blood cells. The existence of glucose-6-phosphate dehydrogenase (G6PD), the first enzyme in the pathway, remains to be further elucidated[24]. Recently, Usanga and Luzzatto [25] reported that *P. falciparum* constains its own enzyme which can be expressed in the G6PD deficient red cells. However, the second enzyme in the pathway, 6-phosphogluconate dehydrogenase, is consistently identified in malarial parasites and is different from that of host red cells[20, 22]. The remaining enzymes in the pathway have not been studied in the parasites.

The parasite has no complete cycle of tricarboxylic acid. The enzyme of the tricarboxylic acid cycle identified with some degree of certainly in avian, human, and rodent malaria is malate dehydrogenase[26]. In contrast, it has been reported that some

strains of *P. berghei* lack of enzyme malate dehydrogenase[22]. Isocitrate dehydrogenase has been identified in *P. lophurae* (avian malaria) *P. falciparum* (human malaria)[27] and *P. knowlesi* (simian malaria)[28], but can not be detected in *P. berghei* (rodent malaria). Succinate dehydrogenase activity has been found in *P. gallinaceum* and *P. lophurae* (avian malaria), but not in *P. berghei*[20].

2.3.2 Amino acid metabolism and protein synthesis

There are four potential sources of amino acids for the intraerythrocytic Plasmodium. (1) *De novo* synthesis from folate mediated reaction e.g. glycine, methionine. (2) CO_2 fixation,which can only supply a limited amount of amino acids e.g. glutamic acid. Only one of the enzymes in the CO_2 fixing pathway, namely phosphoenolpyruvate carboxylase, has been identified in *P. berghei*, however , the presence of CO_2 fixation has been resently reported in *P. falciparum* by Blum and Ginsburg[19]. (3) The free amino acid pools of the blood plasma and erythrocyte. It is found that isoleucine and methionine supplied exogenously are necessary for parasite growth, probably because hemoglobin(Hb) is deficient in these amino acids. The increased uptake of various amino acids by malaria infected red cells has been reviewed by Sherman. However the detailed mechanism of uptake should be further studied. (4) The host cell Hb. Hb is likely to be the major source of amino acids for parasite protein synthesis. It is ingested via the cytostome and then the food vacuoles at the base of the cytostome vacuole containing the protein are pinched off. The degradation of Hb in the food vacuole take place by at least 2 catalytic enzymes : cathepsin D and aminopeptidase. The food vacuoles contain the degradative products of hemoglobin with electron particle, namely malarial pigment (or hemozoin).It has been suggested that the food vacuole is lysosomal particle.

The protein synthesis of malaria appears to be typically eukaryotic : the parasite has its own ribosomes which have a sedimentation constant of 80S and can be dissociated into 60S and 40S, the synthesis is inhibited by cycloheximide and puromycin, but not chloramphenicol or streptomycin[20]. Most proteins of *P. falciparum*

are reported to be synthesized by every stage of growth, and unchanged the cycle through the ring stage following merozoite invasion of the red cells. Some proteins synthesized are found to be dependent on the stages of the parasite[29].

2.3.3 Phospholipid and cholesterol metabolism

Malaria infected red cells show an increase in total lipids, and change in the phospholipid to cholesterol ratio which is associated with parasite membrane[30]. It is found that malaria does not synthesize cholesterol and fatty acids *de novo*[30, 31]. In falciparum malaria cholesterol is probably obtained preformed from the host. The parasite obtains free fatty acids from the host plasma. *P. falciparum* and *P. knowlesi* shows their ability to synthesize phospholipid *de novo* from their precursors such as palmitate, serine, choline, inositol and glycerol–3–phosphate. The metabolism of phospholipids in *P. falciparum* is a unique pathway and may constitute a potentially fruitful chemotherapeutic approach to malaria. Plasmodium also appear to lack the capacity to change fatty acids into their phospholipids, thereby maintaining a lipid fatty acid composition distinct from that of the host cell.

2.3.4 Pyrimidine biosynthesis (Figure 2-2) and purine salvage

There are two possible sources of purine and pyrimidine bases, nucleosides and nucleotides, for nucleic acid synthesis by the intraerythrocytic parasite : synthesis from simple precursors, and using preformed materials from outside the cell. Malaria paraites cannot synthesize purines *de novo*, and must obtain them from the host[20]. Hypoxanthine, obtained from both host plasma and from adenosine metabolism (involving 2 enzymes : adenosine deaminase and purine nucleoside phosphorylase), appears to be the major purine base salvaged by *P. falciparum.* The purine metabolism pathway in the malaria parasite is well characterized, from hypoxanthine to both guanosine and adenosine and adenosine nucleotides. At least 6 enzymes involving in purine metabolism have been identified in *P. falciparum*[9]. Some of the enzymes are

well characterized e.g. adenosine deaminase of *P. falciparum*[38] and of *P. lophurae*[39], purine nucleoside phosphorylase of *P. lophurae*.

Malaria parasites are unable to utilize exogenous pyrimidines, and must synthesize them *de novo*[20]. Thymidylate (TMP) is the precursor of nucleic acid synthesis, and its precursor is in turn deoxyuridylate (dUMP). All of the enzymes necessary for the de novo synthesis dUMP have been identified in *P. berghei* extract[40], *P. falciparum* extract[9, 41, 42]. Only two enzymes, orotate phosphoribosyltransferase and orotidylate decarboxylase, are well characterized in *P. falciparum*. The two pathways of nucleic acid synthesis, purine salvage and pyrimidine *de novo*, render possible targets for the design of novel antimalarial agents.

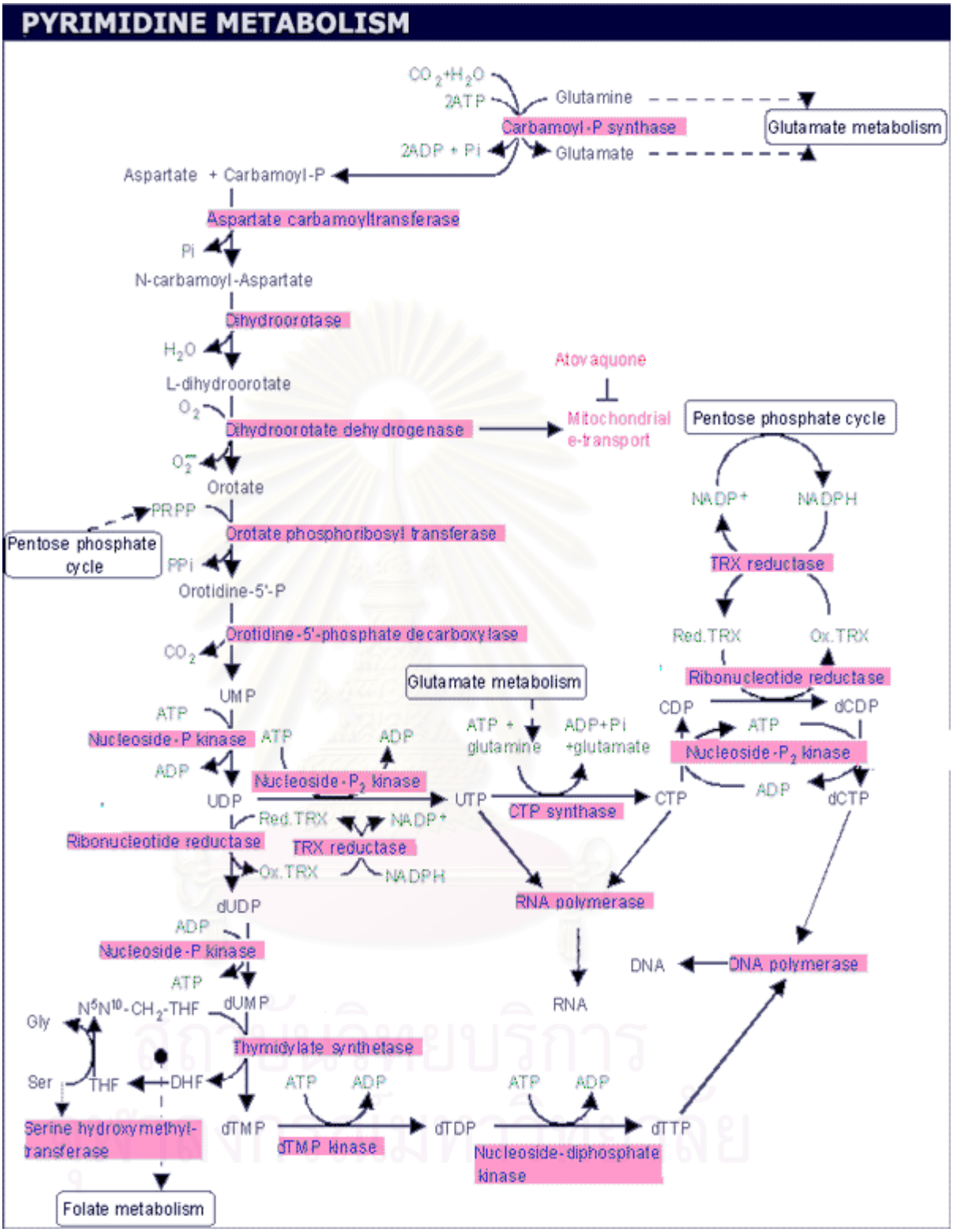

Figure 2-2 Pyrimidine pathway (from http://sites.huji.ac.il/malaria)

2.3.5 Energy transformation and mitochondria

There has been considerable debate about whether the erythrocytic stages of mammalian malaria parasite posses mitochondria. The energy producing organelles essential for all life forms. The falciparum parasite uses glucose as its primary energy source. In fact, glucose utilization is significantly greater in the infected erythrocyte than in the uninfected cell. Progress has been made in characterization of all enzymes involved in glycolysis in *P. falciparum*. However, there is no evidence supporting the presence of a tricarboxylic acid cycle, a key energy producing process of the mitochondria.

The presence of mitochondria in the erythrocytic asexual stages of *P.falciparum* has recently been shown, but the actual function is not well understood[43]. Recent advances in the molecular biology of the mitochondria DNA of malaria parasites may help to untravel the role of the mitochondria[44]. Antibiotics used to treat falciparum infection, such as tetracyclines, clindamycin, and erythromycin appear to work by blocking the development of parasite mitochondria[45]. Of great interest in this regard is the recent finding that mitochondria DNA of *P.falciparum* encodes an RNA polymerase and is sensitive to rifampicin, potentially explaining the antimalarial activity of this drug[46].

The erythrocytic stages of many mammalian malaria parasites appear not to derive their metabolic enegy through classical electron transport. The mitochondria may participate in ion transport, but the role of the organelle in metabolism is unclear. It is not known whether components analogous to those present in the mammalian terminal electron transport system function in the malaria parasite, and for what purpose. Since the organism, like many other parasitic protozoans and all parasitic worms so far studied has rather limited terminal respiration. Mammalian malaria parasites are aerobic fermenters, capable of partially decomposing metabolic substrates to fermentation products, but are unable to oxidize them completely, it would appear that terminal

respiration is either absent or rate limiting in the parasite. Available evidence supports the view that malaria parasites are microaerophillic, homolactate fermenters[47].

3. Antimalarial drugs[1, 2, 7, 48]

(1) Chloroquine was a very effective drug both for treatment and prophylaxis. It was first used in the 1940s shortly after the Second World War and was effective in curing all forms of malaria, with few side effects when taken in the dose prescribed and it was low in cost. Unfortunately, most strains of falciparum malaria are now resistant to chloroquine.

(2) Alebrin (Mepacrine) was developed in the early 1930s. It was used as a prophylactic on a large scale. It is now considered to have too many undesirable side effects and is no longer used.

(3) Mefloquine (Lariam) was effective against malaria, resistant to other forms of treatment. Widespread resistance has now developed and this, together with undesirable side effects, have resulted in a decline in its use.

(4) Halofantrin (Halfan) is an effective antimalarial drug introduced in the 1980s, Unfortunately, resistant forms are increasingly being reported and there is some concern about its side effects.

(5) Malarone was released in the late 1990s. At present it is a very expensive drug.

(6) Artemisinin was a high rate of treatment failures. It has been reported and it is now being combined with mefloquine for the treatment of *falciparum* malaria.

The emergence of multi-drug resistant strains of parasites is also exacerbating the situation. Via the explosion of easy international travel, imported cases of malaria are now more frequently registered in developed countries. Malaria is re-emerging in areas where it was previously under control or eradicated e.g. in the Central Asian Republics of Tajikistan and Azerbaijan, and in Korea[4]. Drug resistant malaria has become one of the most important problems in malaria control in recent years, and it necessitates the use of drugs which are more expensive and may have dangerous side effects. In areas

such as Thailand and Vietnam, mosquitoes of the *Anopheles dirus* and *Anopheles minimus* species spread the drug resistant parasites. Drug resistant *P. falciparum* was first reported in Thailand in 1961. Various *P. falciparum* "strains" have now attained resistance to all commonly used and generally available antimalarial drugs[48].

4. *Plasmodium falciparum* orotate phosphoribosytransferase enzyme

Orotate phosphoribosyltransferase (EC 2.4.2.10), the fifth enzyme of *de novo* pyrimidine biosynthesis has several synonym such as orotate phosphoribosyl pyrophosphate transferase, orotic acid phosphoribosyltransferase, orotidine 5'-monophosphate pyrophosphorylase, orotidylate pyrophosphorylase, and orotidylic acid phosphorylase, etc. The systematic name is orotate-5'-phosphate phospho-alpha-D-ribosyltransferase. This enzyme play important role in pyrimidine pathway[49]. It can be found in many organisms such as *Salmonella typhimurium*, *Saccharomyces cerevisiae*, *Thermus thermophilus*, *Toxoplasma gondii*,and *Escherichia coli*[50].

Orotate phosphoribosyltransferase was found in *de novo* pathway catalyzing formation of orotidine 5'-monophosphate (OMP) and pyrophosphate (PPi) from 5-phosphoribosyl-1-pyrophosphate (PRPP) and orotate, according to the following reaction [50] ;

orotidine 5'-phosphate + diphosphate = orotate +5-phospho-alpha-D-ribose 1-diphosphate

This *P. falciparum* reaction is the random sequential kinetic mechanism[12]. In contrast to enzyme from yeast, which is bi bi ping pong mechanism[51]. The orotate phosphoribosyltransferase enzyme of *E. coli* [52]and *S. typhimurium*[53] are also found in *de novo* pyrimidine synthesis, too.

P. falciparum orotate phosphoribosyltransferase has 67000 Da in molecular weight[12]. Like the *E. coli*[54] and *S. cerevisiae*, [51], *P. falciparum* orotate

phosphoribosyltransferase enzyme is dimer. Each unit of *P. falciparum* orotate phosphoribosyltransferase is about 33400 Da[12].

For *P. falciparum* orotate phosphoribosyltransferase inhibition study, there is no inhibition by SH-group reagents[55]. The inhibitors which are the substances in orotate phosphoribosyltransferase reaction such as orotidine 5'-phosphate, diphosphate, orotate, and 5-phospho-alpha-D-ribose 1-diphosphate have different properties. Orotidine 5'-phosphate is product inhibitor, competitive to 5-phospho-alpha-D-ribose 1-diphosphate, competitive to orotate. Diphosphate is product inhibitor, noncompetitive to orotate, competitive to 5-phospho-alpha-D-ribose 1-diphosphate. Orotate is product inhibitor, noncompetitive to diphosphate and 5-phospho-alpha-D-ribose 1-diphosphate is product inhibitor, competitive to diphosphate and orotidine 5'-phosphate[12].

The stability of *P. falciparum* orotate phosphoribosyltransferase has been studied. The recombinant enzyme is stable for at least 3 months at 4^{O}C or -20^{O}C in presence of 1 mM dithiothreitol and 10% glycerol[12], but in absence of dithiothreitol and glycerol, 50% of activity is lost during overnight storage at 4^{O}C. The native enzyme complex is stable for at least 6 months at -20^{O}C or -80^{O}C in 50 mM Hepes, pH 7.4, 300 mM NaCl, 5 mM dithiothreitol, 20% glycerol. At 4^{O}C, 50% of the activity was loss about 50% within 4 weeks[56].

5. Background of the experimental approach

This study aim to clone and heterologously express full-length and truncated *P. falciparum* orotate phosphoribosyltransferase gene in a bacterial system and to compare the characterization of the expressed enzymes. The DNA fragment of truncated *P. falciparum* orotate phosphoribosyltransferase gene from PCR amplifications would be clone to pDrive cloning vector and allowed to multiply in DH5α competent cells, then the positive clone would be subjected to DNA sequence and analyze by BLAST family of program. After sequencing, the DNA fragment carrying truncated uridine phosphorylase gene would be subcloned into pQE30Xa expression

vector and heterologously expressed in a M15 competent cells. The full-length gene would be cloned to pQE30Xa directly and allowed to multiply in M15 competent cells. Finally, the expecting enzyme would be purified and analyzed by SDS-PAGE and Western blot then enzyme specific activity and stability would be performed.

5.1 The pDrive cloning vector (Figure 2-3)

The pDrive cloning vector use in this study is a linear form with a U-base overhang at each end, ready-to-use for direct ligation of PCR products. This vector allows ampicillin and kanamycin selection, as well as blue/white colony screening. The vector contains several unique restriction endonuclease recognition sites around the cloning site, allowing easy restriction analysis of recombinant plasmids. The vector also contains a T7 and SP6 promoter on either side of the cloning site, allowing in vitro transcription of cloned PCR products as well as sequence analysis using standard sequencing primers. In addition, the pDrive cloning vector has a phage f1 origin to allow preparation of single-strand DNA(The data according to QAIGEN handbook).

5.2 The pQE30Xa expression vector (Figure 2-4)

The pQE30 expression vector is an expression vector. It is the low-copy plasmid that have 6xHis-tag coding sequence either 5'or3' to the cloning region and have the synthetic Ribosomal binding site, RBSII, for high translation rate and allows ampicillin section. This vector obtains several unique restriction endonuclease recognition sites around the cloning site allowing easy restriction analysis of recombinant plasmids. The pQE-30 Xa vector is similar to pQE-30, but also encodes a Factor Xa Protease recognition site which is bracketed by the 6xHis-tag coding region on the 5' side and the multiple cloning site on the 3' side. 5'-end cloning using the blunt-end *Stu*I restriction site allows insertion of the gene of interest directly behind the Factor Xa Protease recognition site, without any intervening amino acid codons. Factor Xa Protease cleaves off the 6xHis-tag peptide behind the arginine residue of the protease recognition site (The data according to QAIGEN handbook).

5.3 The M15 competent cells

The *E. coli* strains M15 contain the low-copy plasmid pREP4 which confers kanamycin resistance and constitutively express the lac repressor protein encoded by the lacI gene . The pREP4 plasmid is derived from pACYC (cloning vector) and contains the p15A replicon. Multiple copies of pREP4 are presence in the host cells that ensure the production of high levels of the lac repressor protein which binds to the operator sequences and tightly regulates recombinant protein expression. The pREP4 plasmid is compatible with all plasmids carrying the ColE1 origin of replication, and is maintained in *E. coli* in the presence of kanamycin at a concentration of 25 μg/ml. *E. coli* strain M15 (pREP4) is permits high-level expression may be useful for the production of protein that are poorly expressed in M15 (pREP4). The M15 strain derived from *E. coli* K12 and do not harbor any chromosomal copy of the lacI mutation, so pREP4 must be maintained by selection for kanamycin resistance.

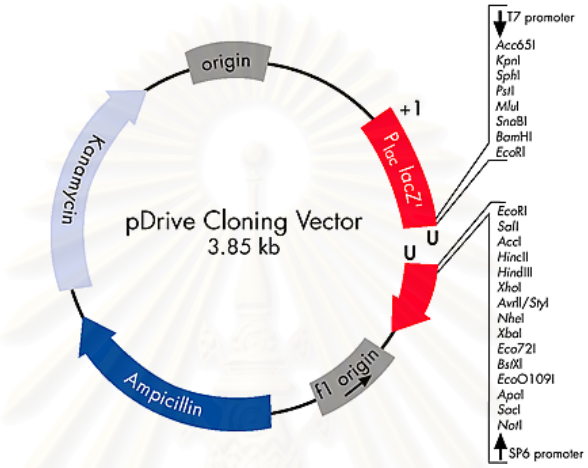

Figure 2-3 Map of the pDrive cloning vector from QIAGEN PCR cloning handbook.

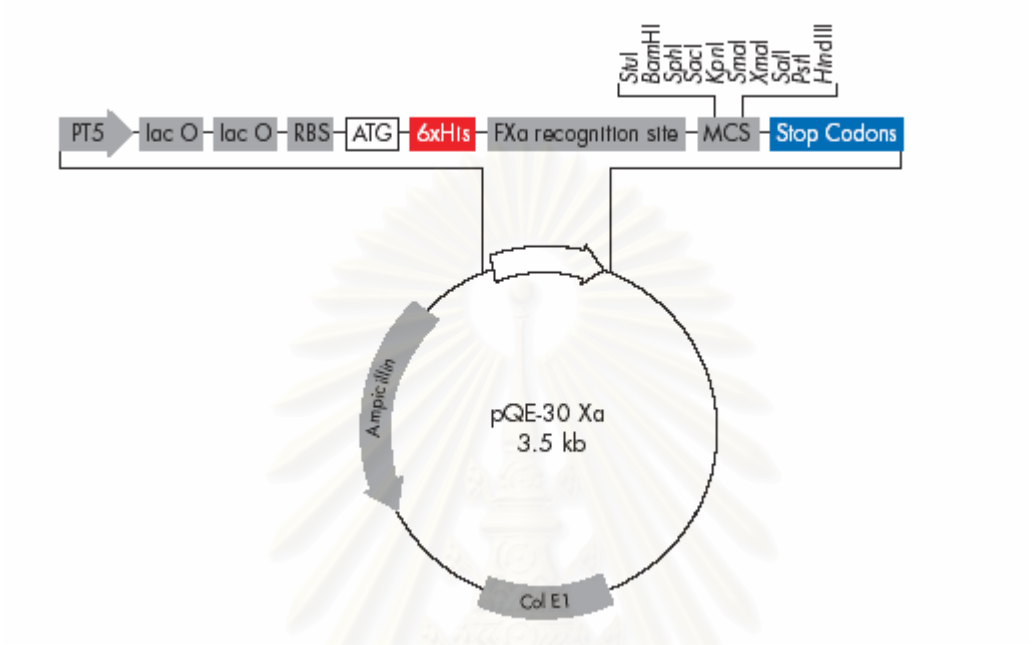

Figure 2-4 Map of the pQE30Xa expression vector from QIAGEN QIA expressionist handbook.

CHAPTER III

MATERIALS AND EQUIPMENTS

1. Materials

(1) Alcohol lamp

(2) Beaker

(3) Cuvette

(4) Cylinder

(5) Flask

(6) Glass pipette

(7) Microcentrifuge tube

(8) Microcentrifuge tube rack

(9) Nitrocellulose membrane

(10) Parafilm

(11) Petridish

(12) Pipette rack

(13) Pipette tip

(14) Plastic wrap

(15) Platinum loop

(16) Polaroid film

(17) Polypropylene conicol centrifuge tube

(18) Scapel

(19) Screw cap tube

(20) Spreader

(21) Stirring-magnetic bar

(22) Test tube

(23) Thermometer

2. Equipments

(1) Balance
(2) Deep freeze -20^{O}C, -80^{O}C
(3) DNA thermal cycler
(4) Heat block
(5) Incubator
(6) Microcentrifuge
(7) pH meter
(8) Polaroid camera
(9) Power supply
(10) Refrigerator 4 ^{o} C
(11) Sonicator
(12) Spectrophotometer
(13) Stirring hot plate
(14) Thermostat shaking-water bath
(15) UV transilluminator
(16) Vortex
(17) Water bath

3. Reagents

3.1 General reagents

(1) Acetic acid glacial
(2) Acrylamide
(3) Agar
(4) Agarose
(5) Ammonium persulfate (APS)
(6) Ampicillin sodium salt
(7) Blocking buffer
(8) Blotting buffer
(9) Bovine serum albumin

(10) Butanol

(11) Coomassie brilliant blue

(12) Dimethyl formamide

(13) Dithiothreitol

(14) Ethanol, absolute

(15) Ethidium bromide

(16) Ethylene diamine tetraacetic acid (disodium salt) (EDTA)

(17) Glucose

(18) Glycerol

(19) Glycine

(20) Horsradish peroxidase staining solution

(21) Isopropanol

(22) Isopropyl-β-D-thiogalactoside (IPTG)

(23) Kanamycin

(24) Magnesium chloride

(25) Magnesium sulfate

(26) Methanol, absolute

(27) N, N Methylene-bis-acrylamide (BIS)

(28) Mercaptoethanol

(29) Orotic acid

(30) Phenol-chloroform

(31) 5-Phospho-alpha-D-ribose 1-diphosphate

(32) Sodium acetate

(33) Sodium chloride

(34) Sodium dodecyl sulfate

(35) Sodium hydroxide

(36) Sodium phosphate

(37) N, N, N', N'-Tetramethylethylenediamine (TEMED)

(38) Triton-X-100

(39) Tris base

(40) Tris HCl

(42) Tryptone

(43) TBS buffer

(44) TBS-Tween/Triton buffer

(45) Yeast extract

3.2 Reagent Kits

(1) QIAGEN PCR Cloning Kit (QIAGEN)

(2) QIAGEN Plasmid Midi and Maxi Kit (QIAGEN)

(3) QIAquick PCR Purification Kit (QIAGEN)

(4) QIAquick GEL extraction Kit (QIAGEN)

(5) QIAexpress Kit (QIAGEN)

(6) Ni-NTA Spin column Kit (QIAGEN)

3.3 Enzymes

(1) RNase (Promega)

(2) *Taq* polymerase (Promega)

(3) *BamH*I (Promega)

(4) *Hind*III (Promega)

(5) DNA Ligase (Promega)

(6) Anti-His Antibodies conjugates (Biorad)

CHAPTER IV

METHODS

1. Identification of orotate phosphoribosyltransferase gene

The bioinformatics underlying NCBI resources was used to study on enzyme orotate phosphoribosyltransferase and model organisms. The selected model organisms were identified for their orotate phosphoribosyltransferase gene using database of the National Center for Biotechnology Information (NCBI) available at http://www.ncbi.nlm.nih.gov/Genbank/ (Figure 4-1)

All genes were then analyzed for sequence homologies using the alignment program (ClustalW) available at http://www.ebi.ac.uk/clustalw/ (Figure 4-2). The result homology sequences were then used to search for nucleotide sequence of a candidate gene of *P. falciparum* orotate phosphoribosyltransferase.

The primer for amplifying the candidate DNA by PCR were designed from searching results.

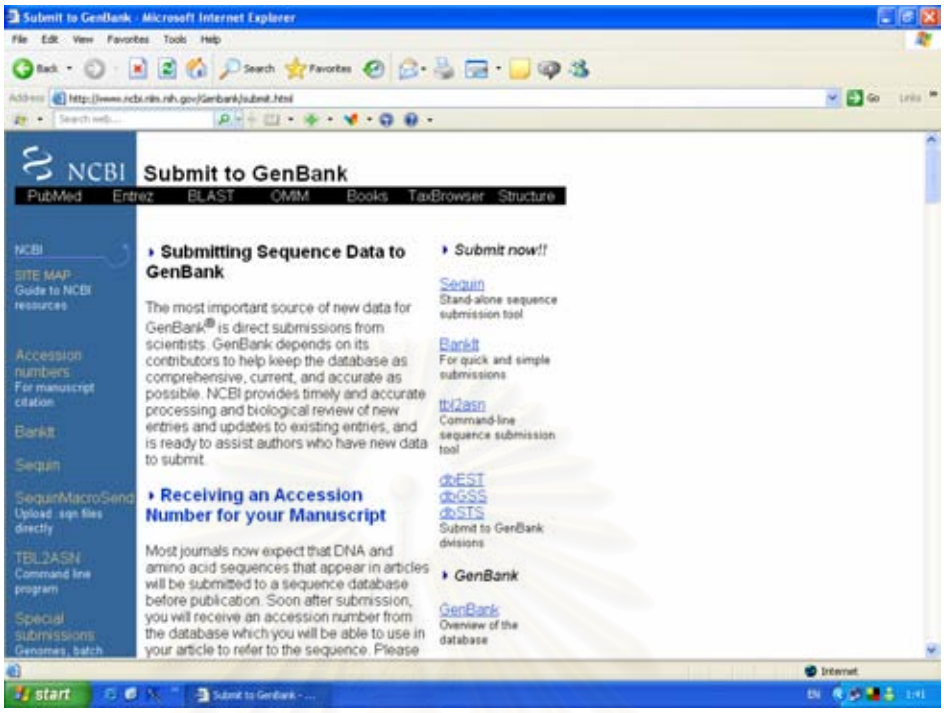

Figure 4-1 Web page of http://www.ncbi.nlm.nih.gov/Genbank/

Figure 4-2 Web page of alignment program.

2. Specimens

Plasmodium falciparum (3D7) orotate phosphoribosyltransferase gene that was cloned in pQE30Xa (figure 4-3).The gene contains 846 bp and express the protein having 281 amino acids (33 kDa).

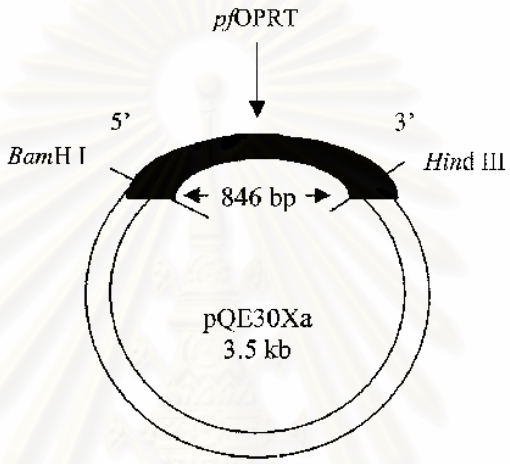

Figure 4-3 *Plasmodium falciparum* (3D7) orotate phosphoribosyltransferase gene that was cloned in pQE30Xa.

3. Calculation of DNA concentration

The DNA concentration was quantitated by measuring the absorbance (OD) at 260 nm. An OD_{260} of 1 corresponds to approximately 50 μg/ml for double-strand DNA. So that DNA concentration was calculated from the following equation ;

DNA concentration (μg/ml) = OD_{260} x 50 x dilution factor

4. Candidate gene amplification by polymerase chain reaction (PCR)

PCR was used to amplify DNA encoding full-length and truncated *P. falciparum* orotate phosphoribosyltransferase. Primer 5.2 and primer 5.3 are used for increasing

full-length *P. falciparum* orotate phosphoribosyltransferase, but primer 5.1 and primer 5.2 are used for increasing truncated *P. falciparum* orotate phosphoribosyltransferase. The 5' ends of Primer 5.2 and primer 5.3 contain *Hind III* and *Bam*HI restriction sites, respectively. Like primer 5.3, primer 5.1 contains *Bam*HI restriction sites, too. The volume of 50 μl of PCR reaction, is prepared in a 0.5 microcentifuge tube, consisted of 34.5 μl of sterile distilled water, 2 μl of *P. falciparum* DNA, 5 μl of 10XPCR buffer, 4 μl of 2.5 mM dNTP, 2 μl of each 10 μM primer and 0.5 μl of *Taq* polymerase. The automated thermal cycler was used according to the time and temperature program as indicated in Table 4-1. Subsequently, 2 μl of PCR product was analysed using agarose gel electrophoresis.

Temp. / Stage	95°C	48°C	55°C	68°C	Cycle
1	3 min	1 min	-	2 min	1
2	1 min	-	1 min	3 min	40
3	1 min	-	1 min	10 min	1

Table 4-1 PCR program.

5. Agarose gel electrophoresis

The DNA fragments can be analysed by submarine agarose gel electrophoresis. The concentration of agarose gel depends on the size of DNA. In this thesis, 1% (w/v) agarose gel in 1 X TAE was used. The agarose gel in 1 X TAE was melted, poured into an electrophoretic tray and allowed to set at RT. The 1 X TAE was used as an electrophoretic buffer.

The DNA sample was mixed with1μl of loading dye and loaded onto the prepared gel in 1 X TAE running buffer at 0.80 v/cm and stoped when dye front reached the end of gel. The gel was stained with ethidium bromide (EtBr) solution for 5 min and

then destained with water for 15-30 min. The pattern of DNA bands were observed under the UV light box.

6. Purification of PCR product using QIA quick PCR Purification Kit

A 5 volume of PB buffer was added to 1 volume of the PCR product and the tube was vortexed. The solution mixture was transfered to the microspin cup that seated in a receptacle tube, centrifuged at 13,000 rpm for 1 min and the PB buffer was discarded. Then, the microspin cup was added with 750 μl of PE washing buffer and centrifuged at 13,000 rpm for 1 min, the PE buffer was discarded and the cup was centrifuged again. The microspin cup was transferred to a fresh microcentrifuge tube and added with 30 μl of EB buffer directly onto the top of the fiber matrix at the bottom of the microspin cup. The tube was incubated at RT for 1 min and centrifuged at 13,000 rpm for 1 min, then the microspin cup was discarded. The purified PCR product was analyzed by submarine agarose gel electrophoresis.

7. Purification of DNA using QIA quick Gel Extraction Kit

The DNA fragment was excised from the agarose gel with clean, sharp scapel. Weight the gel slice in a tube and added with 3 volume of buffer QG to 1 volume of gel and the mixture was then incubated at 50^{O}C for 10 min or until gel melting, the mixture was added with 1 volume of isopropanol to 1 volume of gel and loaded into a Qai Quick spin column with the bottom outlet capped and centrifuged at 13,000 rpm for 1 min, then discarded the flow-through. The column was added with 0.5 ml of buffer QG through the bottom outlet capped of column and centrifuged at 13,000 rpm for 1 min, then the flow-through was discarded and the column was washed once with 0.75 ml of buffer QG. The DNA was eluted with 30 μl of buffer EB and analyzed by submarine agarose gel electrophoresis.

8. Cloning of the PCR product into the pDrive cloning vector

8.1 DNA ligation

The PCR product was ligated into the pDrive cloning vector by the ligation reaction that was performed in a total volume of 10 μl. The ligation reaction contained 1 μl of pDrive cloning vector, 2 μl of PCR product, 2 μl of sterile distilled water and 5 μl of 2x ligation master mix. The ligation mixture was spin briefly to mixed and incubated at 16^{O}C for 2 hrs, then proceed with transformation or stored at -20^{O}C until use.

8.2 *E. coli* transformation

The aliquot 50 μl of *E. coli* competent cells, strain DH5α, and SOC medium were thaw on ice, then the ligation mixture was transfered to the cells, gently mixed by hand and incubated on ice for 5 min. the tube was heated at 42^{O}C waterbath without shaking for 1 min and incubated on ice for 5 min. the mixture was then added with 250 μl of SOC medium, gently mixed and incubated at 37 ^{O}C for 45 min. The mixture was centrifuged at 6,000 rpm for 1 min and removed the upper phase of mixture, then plate the transformation mixture onto the LB-ampicillin agar (LB agar with 100 μg/ml ampicillin which had spread with IPTG) and incubated at RT until the transformation mixture had absorbed into agar. Invert the plate and incubated at 37^{O}C O/N.

9. Plasmid extraction from *E.coli*

9.1 Small scale plasmid preparation using Qiagen Plasmid Kit Mini Kit

A single white colony of the recombinant bacteria was grown at 37^{O}C O/N with shaking motion in 3 ml of LB broth containing 100 μg/ml ampicillin. The cells were harvested at 6,000 rpm for 5 min. the cells pellet was resuspended in 100 μl of ice-cold solution I, then the cell suspension was added with 200 μl of freshy prepared solution II and incubated on ice 5 min the mixture was added with 150 μl of ice-cold solution III,

gently mixed by invert and incubated on ice for 5 min, then bacterial chromosome and cells debris were removed by centrifugation at 10,000 rpm for 5 min. The supernatant was removed to a fresh tube and was then added with 0.7 volume of ice-cold isopropanol to 1 volume of the supernatant, gently mix and incubated at RT for 10 min. The tube was centrifuged at 10,000 rpm for 5 min, discard supernatant. The pellet was washed once with ice-cold 70 % ethanol, air dry and dissolved in 200 μl of sterile distilled water which added with 0.5 μl of RNase, gently mix and incubate at 37^{O}C for 1 hr. The mixture was then added with an equal volume of phenol/chloroform (1:1,v/v), mix by vortex and incubated at RT for 5 min, then centrifuged at 10,000 rpm for 10 min. The aqueous phase was removed which carefully to a fresh tube and precipitated with 3M sodium acetate and ice-cold absolute ethanol at -20^{O} C O/N. The DNA pellet was collected at 12,000 rpm for 10 min and washed once with ice-cold 70% ethanol. After drying, the pellet was resuspended in EB solution and stored at -20^{O} C.

9.2 Medium scale plasmid preparation using Qiagen Plasmid Midi Kit

A single white colony of the recombinant bacteria was grown at 37^{O}C O/N with shaking motion in 3 ml of LB broth containing 100 μg/ml ampicillin. The overnight culture was transferred to flask with 50 ml LB-ampicillin medium and incubated at 37^{O}C for 3 hrs with vigorous shaking. The cells were harvested at 6,000 rpm for 15 min and resuspend in 4 ml of P1 lysis buffer. The cells suspension was added with 4 ml of P2 buffer, gently mixed by invert and incubated at RT for 5 min. The mixture was added with 4 ml of ice-cold P3 buffer, gently mixed and incubated on ice for 15 min and was then centrifuged at 12,000 rpm for 45 min, at the same time, the tip was equilibrated with 4 ml of QBT buffer. The supernatant was transferred directly onto the top of the fiber matrix of the tip and was then washed twice with 10 ml of QC buffer. The tip was added with 5 ml of QF buffer to elute DNA. The plasmid DNA was precipitated with 3.5 ml of isopropanol at 15,000 rpm for 30 min and washed once with 70% ethanol, air dry and resuspended in sterile distilled water.

10. Restriction endonuclease digestion

The recombinant plasmid DNA was analyzed by digestion with restriction enzymes. The digestion reaction was performed following the conditions of restriction enzymes, in this study, were consisted of *Bam*HI and *Hind*III. The digestion-reaction was performed in a total volume of 20 µl using 15.3 µl of sterile distilled water, 2 µl of reaction buffer, 0.2 µl of BSA, 2 µl of plasmid DNA, 0.25 µl of each restriction enzymes (*Bam*HI and *Hind*III) and incubated at 37^{O}C for 3 hrs. When the reaction was completed, the digestion product was analyzed by submarine 0.8% agarose gel elctrophoresis.

11. Automated DNA sequencing and analysis of DNA sequence

The candidate sequence (*P. falciparum* orotate phosphoribosytransferase sequence) was determined by the dideoxy chain termination method using an automated Applied Biosystems Procise sequencer(Bioservice unit of NSTDA). The sequence homologies were analyzed using the alignment program of the EBI available at http://www.ebi.ac.uk/clustalw/

12. Recombinant protein expression and purification of *P. falciparum* orotate phosphoribosyltransferase from *E.coli*

The construct plasmid, pDrive carrying PCR fragment of *P. falciparum* orotate phosphoribosyltransferase gene, was subcloned into pQE30Xa expression vector. First, the construct plasmid was double digested of restriction enzymes, *Bam*HI and *Hind*III. Second, *P. falciparum* orotate phosphoribosyltransferase gene was purified from the digestion product using QIAquick Gel Extraction Kit and was then ligated into pQE30 expression vector, using 1 µl of pQE30Xa expression vector, 1 µl of orotate phosphoribosyltransferase DNA, 3 µl of sterile distilled water and 5 µl of 2x Ligation Master Mix. The ligation mixture was spin briefly to mix and incubated at 16 ^{O}C O/N and was then transformed into the M15 competent cells using LB-ampicillin-kanamycin media. The recombinant plasmid were analyzed by restriction analysis of

small-scale preparation method. The expression of *P. falciparum* orotate phosphoribosyltransferase gene in *E.coli* was induced by IPTG. First, the positive clones from M15 transfomants were inoculated into 3 ml of LB- ampicillin-kanamycin medium and incubated at 37^{O}C O/N with vigorous shaking, the O/N culture was then diluted to ratio of 1:100 with LB- ampicillin-kanamycin medium and incubated at 37^{O}C O/N with vigorous shaking until OD_{600} was about 0.4-0.5. Second, the culture was added with IPTG to a final concentration of 1 mM and was then incubated at 18^{O}C O/N with vigorous shaking. Finally, the cells were harvested at 6,000 rpm for 10 min and washed twice with ice-cold PBS and freeze until use.

All protein purification steps were performed on ice. Frozen cell pellets were resuspended in 1 ml of lysis buffer added with lysozyme to 1 mg/ml, gently mixed by hand and incubated on ice. The mixture was sonicated on ice for 6 times (30 sec burst / 15 sec cooling), taking care to avoid foaming. Crude homogenate was centrifuged at 12,000 rpm for 20 min at 4^{O}C. The supernatant (~8–10 ml) was loaded onto a 2 ml bed volume of Ni^{2+}-NTA-agarose affinity gel equilibrated with buffer A (50mM NaH_2PO_4, pH 8.0; 300mM NaCl; 10mM imidazole). The column was washed with 10 ml of buffer B (50mM NaH_2PO_4, pH 8.0; 300mM NaCl; 20mM imidazole), and then eluted with 2ml of buffer C (50mM NaH_2PO_4, pH 8.0; 300mM NaCl; 250mM imidazole). All samples, including lysate, flow-through, washing fraction and eluate were analyzed on 12% SDS-PAGE (Appendix A) and protein were visualized by staining with Coomassie blue.

Protein concentrations of 1st eluted fraction was determined by the method of Bio-Rad Protein Assay (Bradford) and using bovine serum albumin as a standard. The standard protein was measured absorbance at 595 nm versus reagent blank and a standard curve was plotted. The unknowns were read from standard curve.

13. Procedure for semi-dry Western blot (according to instruction manual protocol from Biorad)

13.1 Procedure for semi-dry-transfer

Gel from SDS-PAGE was washed with water and blotting buffer. Then, place filter paper, Nitrocellulose membrane, gel and filter paper with avoiding air bubbles, and started the current of 80 mA (or 12V) for 1 hour.

13.2 Procedure for immunodetection with Anti-His Antibodies conjugates (chromogenic method)

Membrane from semi-dry-transfer was washed with TBS buffer at RT, and incubated for 1 hour in blocking buffer. Next, the membrane was washed in TBS-Tween / Triton buffer and TBS buffer. Afterwards, the membrane was incubated in Anti-His HRP Conjugate solution (10 µl/ 10 ml dilution of antibody or conjugate stock solution in blocking buffer) at RT for 1 hour. After that, washed membrane in TBS-Tween / Triton buffer and TBS buffer again, and stain with AP or HRP staining solution until the signal is clearly visible. Finally, stop the reaction by rinsing the membrane with water and air dry.

14. Procedure for enzyme assay of orotate phosphoribosyltransferase

The activity of orotate phosphoribosyl transferase was assayed by following the decrease of substrate, PRPP, according to the modified method of Krungkrai et al. The reaction was monitored by the decreasing absorbance at 295 nm on a Shimadzu spectrophotometer equiped with 37^{O}C thermostat waterbath. The assayed mixture contained 50 mM Tris HCl pH 8.0, 100mM $MgCl_2$, 2.5 mM DTT, 5mM OA, and 50µl of the enzymatic preparation. The reaction was initiated by addition of 5 mM PRPP in a final volume of 1 ml of the assayed mixture.

The specific activity of orotate phosphoribosyl transferase was calculated by the following equation ;

$$\text{specific activity (nmol/min/mg)} = \frac{\text{enzyme activity per fraction (nmol/min)}}{\text{protein per fraction (mg)}}$$

15. Enzyme molecular mass determination

The molecular mass of *P. falciparum* orotate phosphoribosyltransferase was estimated by running SDS-PAGE compared with the standard protein marker.

CHAPTER V

RESULTS

1. Identification of orotate phosphoribosyltransferase gene

The alignments of the *P. falciparum* orotate phosphoribosyltransferase amino acid sequence with other organisms have been shown in Figure 5-1 from Krungkrai et al, 2004. They found that *P. falciparum* orotate phosphoribosyltransferase amino acid sequence had N-terminal extension of 66 amino acids, making the longest amino acid sequence (281 amino acids). *P. falciparum* orotate phosphoribosyltransferase amino acid sequence was similar to *Escherichia coli*(60%),*Salmonella typhimurium*(56%), *Homo sapiens*(30%), and *Trypanosoma cruzi*(28%), respectively from the most to the least. From the bioinformatics underlying NCBI resources, we had selected other *Plasmodium spp.* to study on orotate phosphoribosytransferase amino acid sequences. The enzymes, including *P. chabaudi* orotate phosphoribosyltransferase, *P. berghei* orotate phosphoribosyltransferase, and *P. yoelii* orotate phosphoribosyltransferase , were identified for their amino acid sequences (Figure 5-2).

All amino acid sequences was analyzed for sequence homology by the alignment program, ClustralW. *P. berghei* orotate phosphoribosyltranferase amino acid sequences was found to have the most similarity to *P. falciparum* sequence with about 65% homology. (Figure 5-3)

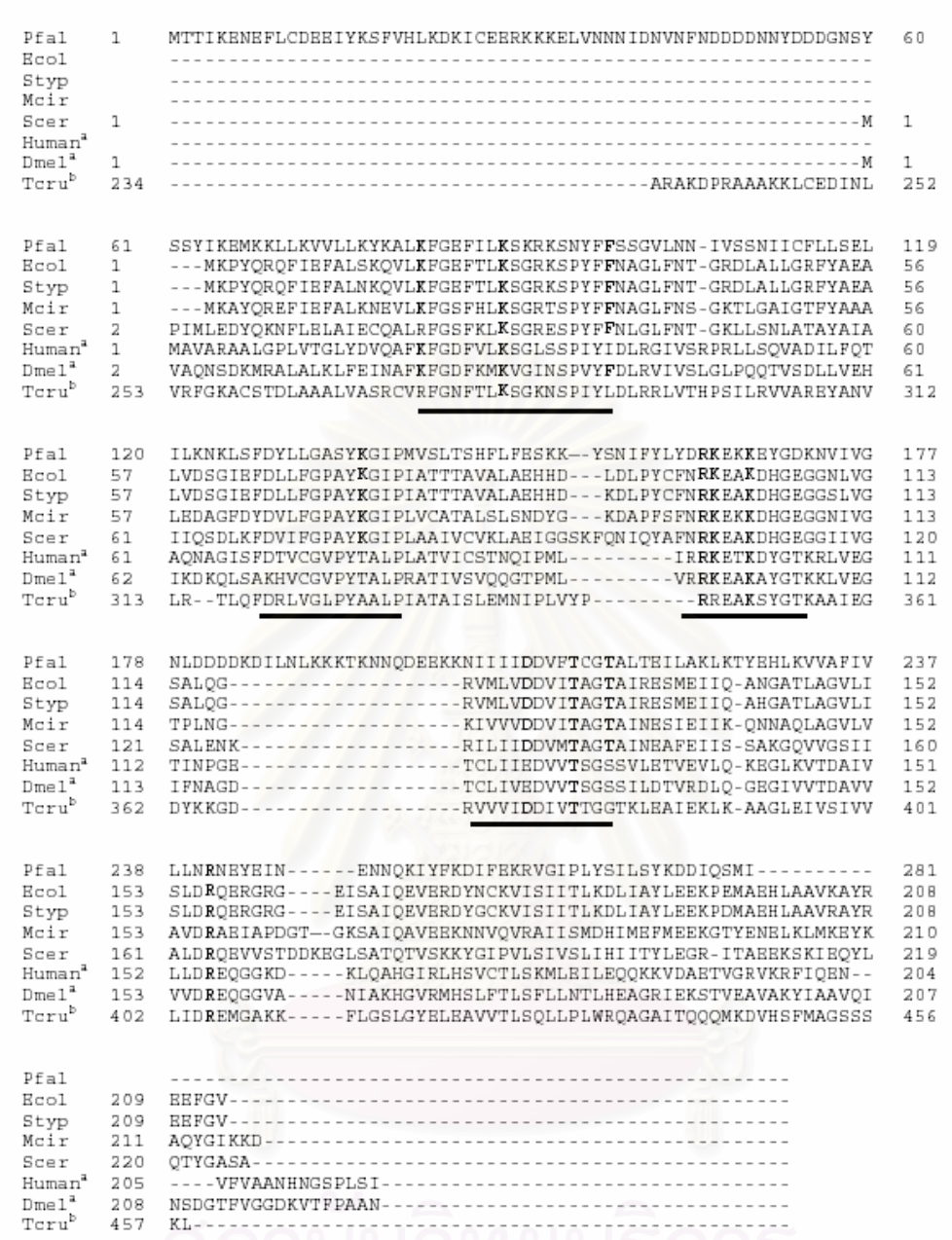

Figure 5-1 Alignments of the *P. falciparum* OPRT amino acid sequence with other organisms. Active site residues are shown in bold and the consensus sequences showing residues that are most commonly found among the OPRT sequences are shown in underline. Species names and accession numbers are as follows: *Plasmodium falciparum*, Pfal (AB074430); *Escherichia coli*, Ecol (X00781); *Salmonella typhimurium*, Styp (Z19547); *Mucor circinelloides*, Mcir (AJ002318); *Saccharomyces cerevisiae*, Scer (X14795); *Homo sapiens*, Human (J03626); *Drosophila melanogaster*, Dmel (Q01637); *Trypanosoma cruzi*, Tcru (AB017765). The alphabet “a” indicates the N-terminally OPRT domain (Human and Dmel) and “b” indicates the C-terminally OPRT domain (Tcru) of the bifunctional UMP synthase protein.

```
CLUSTAL W (1.83) multiple sequence alignment


chabaudi        MEEHNKEAHHISEEELHK-------KYNELCKKIELGK-----------AHENSDE---- 38
yoelii          MDENNKEIKNID-EELHN-------RYNELCKRIELGN-----------DNKNCDD---- 37
berghei         ------------------------------------------------------------
falciparum      -MTTIKENEFLCDEEIYKSFVHLKDKICEERKKKELVNNNIDNVNFNDDDDNNYDDDGNS 59


chabaudi        ----IKEMKKLLVDTLIKYKAILFGNFVLKSKKTSHYYVSTGFLNNAISSNIVSFLISNL 94
yoelii          ----IKEMKNLLIDALIKYEAIKFGDFILKSKRKSKYFVSTGFLNNAISANIISFLISNL 93
berghei         ----------------------------------------------ISANIVSFLISNL 13
falciparum      YSSYIKEMKKLLKVVLLKYKALKFGEFILKSKRKSNYFFSSGVLNNIVSSNIICFLLSEL 119
                                                             :*:**:.**:*:*

chabaudi        ILSKNLSFDYLFGASYKGIPIVTLTSHFLLNTNKFHNVFYLYDRKEKKDYGDASVIIGNL 154
yoelii          ILSKNIHFDYLFGASYKGIPIVSLTSHFLLNTNKFHNIFYLYDRKEKKEYGDKTIIVGNI 153
berghei         ILSKNIAFDYLFGASYKGIPIVSLTSHFLLNTNKFHNIFYLYDRKEKKEYGDKTIIVGNI 73
falciparum      ILKNKLSFDYLLGASYKGIPMVSLTSHFLFESKKYSNIFYLYDRKEKKEYGDKNVIVGNL 179
          **.::: ****:********:*:******::::*: *:**********:*** .:*:**:

chabaudi        EEN---------HIGSAQVEKKTDKKKVIVIDDVFSYGTALTDIFNKIKAFDYLEIVACI 205
yoelii          KENDQDCV-INNNCCNPQFEKKN-KKKVIIIDDVFTCGTALTEIFNKMKYYPDFTSVACI 211
berghei         KESSQDCV-I--NSCNPQFEKKK---KVIIIDDVFTCGTALTEIFNKMKAYEYLQVVACI 127
falciparum      DDDDKDILNLKKKTKNNQDEEKK---NIIIIDDVFTCGTALTEILAKLKTYEHLKVVAFI 236
          .:.         :  . * *:*.   ::*:*****: *****:*: *:* :  :  ** *

chabaudi        VILNRNEHEINEKNEKIYFKDKFEQKHNIPVYSVISYNDDISHLIK 251
yoelii          SSS---------------------------------------- 214
berghei         VLLNRNEHEINENNEKVYFKDLFEQKYNIPIYT------------ 160
falciparum      VLLNRNEYEINENNQKIYFKDIFEKRVGIPLYSILSYKDDIQSMI- 281
```

Figure 5-2 Alignments of the *P. falciparum* orotate phosphoribosyltransferase amino acid sequence with other *Plasmodium* parasite. The other amino acid sequence, including *P. chabaudi* orotate phosphoribosyltransferase, *P. berghei* orotate phosphoribosyltransferase, and *P. yoelii* orotate phosphoribosyltransferase, were identified for their amino acid sequences.

```
CLUSTAL W (1.83) Multiple Sequence Alignments


Sequence format is Pearson
Sequence 1: falciparum      281 aa
Sequence 2: chabaudi        251 aa
Sequence 3: berghei         160 aa
Sequence 4: yoelii          214 aa
Start of Pairwise alignments
Aligning...
Sequences (1:2) Aligned. Score:  55
Sequences (1:3) Aligned. Score:  65
Sequences (1:4) Aligned. Score:  53
Sequences (2:3) Aligned. Score:  72
Sequences (2:4) Aligned. Score:  64
Sequences (3:4) Aligned. Score:  72
Guide  tree                file  created:      [/ebi/extserv/clustalw-
work/interactive/2007032805/clustalw-20070328-05550525.dnd]
Start of Multiple Alignment
There are 3 groups
Aligning...
Group 1: Sequences:   2      Score:3839
Group 2: Sequences:   3      Score:2773
Group 3: Sequences:   4      Score:3652
Alignment Score 4309
CLUSTAL-Alignment     file     created          [/ebi/extserv/clustalw-
work/interactive/2007032805/clustalw-20070328-05550525.aln]
```

Figure 5-3 Alignment scores of the orotate phosphoribosyltransferase amino acid sequences *in P. falciparum, P. chabaudi, P. berghei*, and *P.yoelii* .

2. Synthesis of candidate DNA by PCR

PCR was used to amplify DNA encoding *P. falciparum* orotate phosphoribosyltransferase. Primers for DNA amplification by PCR were designed by using the data from the open reading frame and addition of restriction sites for enzymes, *Bam*HI and *Hind* III, into the forward and reverse primers, respectively.
The 5' ends of primer 5.1 and 5.3 containing *Bam*HI restriction site and the 5' ends of primer 5.2 containing *Hind*III restriction site. The forward primer 5.3 and reverse primer 5.2 were used to amplify the full-length *P. falciparum* orotate phosphoribosyltransferase gene, and the forward primer 5.1 and reverse primer 5.2 were used to amplify the truncated *P. falciparum* orotate phosphoribosyltransferase gene(Figure 5-4).

The DNA fragments of *P. falciparum* orotate phosphoribosyltransferase gene were generated by PCR using primers to start the reaction. The PCR products of full-length *P. falciparum* orotate phosphoribosyltransferase gene were approximately 846 bp and the PCR products of truncated *P. falciparum* orotate phosphoribosyltransferase gene were approximately 648 bp (Figure 5-5).

3. Cloning of the PCR product into the plasmid pDrive

The PCR product of full-length *P. falciparum* orotate phosphoribosyltransferase gene was cloned into pQE30Xa directly, but the PCR fragment of truncated *P. falciparum* orotate phosphoribosyltransferase gene was cloned into the plasmid pDrive cloning vector (Figure 5-6). The recombinant plasmid carrying the DNA of the truncated *P. falciparum* orotate phosphoribosyltransferase homolog was transform into DH-5α competent cells (Figure 5-7). Twelve colonies were selected for restriction analysis. The plasmids prepared by rapid alkaline minipreparation were digested with *Bam*HI and *Hind* III. Nine clones which carrying the insert of about 648 bp were identified, as clone 11,13,15-16,18-22 (Figure 5-8). The nucleotide sequence of one positive clone was determined.

Figure 5-4 Primer design for DNA amplification by PCR

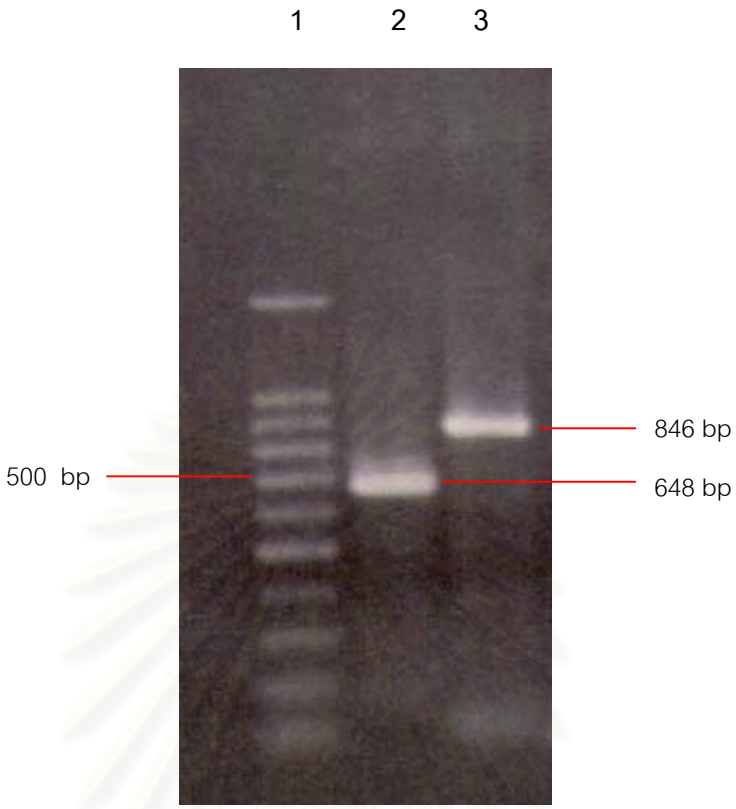

Figure 5-5 PCR amplification of fragment of the full-length and truncated *P. falciparum* orotate phosphoribosyl transferase. The numbers refer to molecular sizes. Lane M is molecular size marker. Lane 1 is PCR product of the truncated *P. falciparum* orotate phosphoribosyltransferase and lane 2 is PCR product of the full-length *P. falciparum* orotate phosphoribosyl transferase.

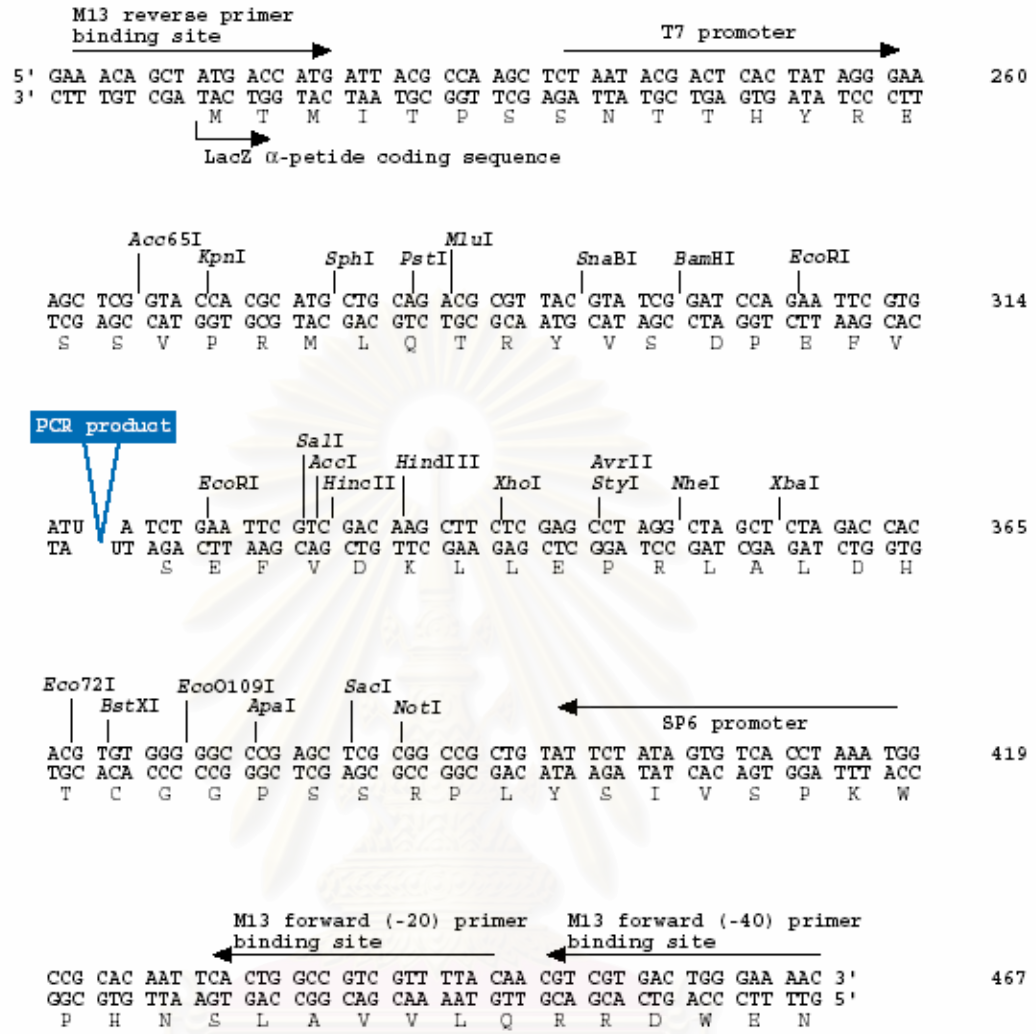

Figure 5-6 Construction of pDrive cloning vector inserted with the PCR fragment of *P. falciparum* orotate phosphoribosyltransferase gene.

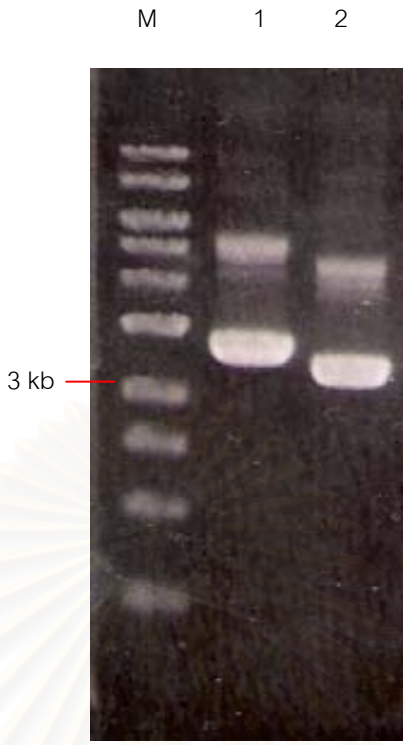

Figure 5-7 Identification of recombinant plasmids, pDrive carring PCR fragment of *P. falciparum* truncated orotate phosphoribosyltransferase gene. The numbers refer to molecular size. Lane M is molecular size marker. Lane 1 and 2 are uncut construct plasmid. Interestingly, the plasmid in lane 1 is higher than lane 2.

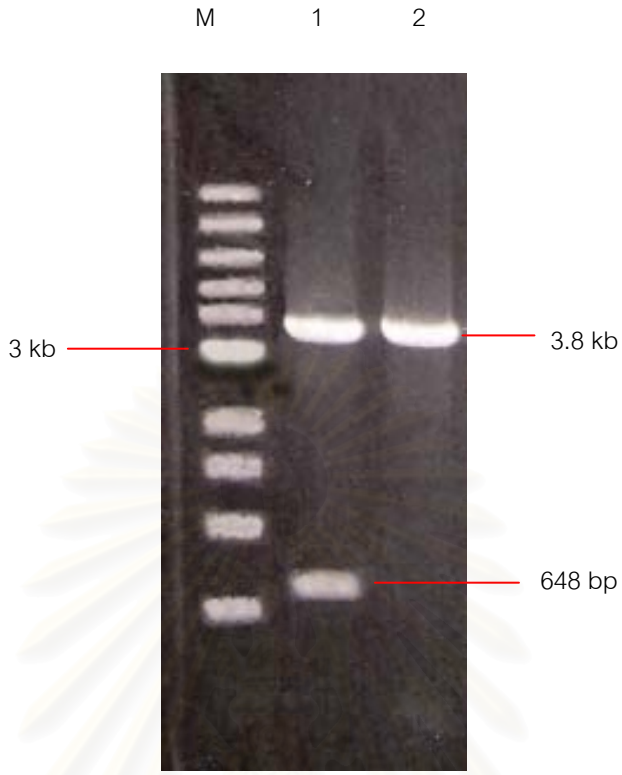

Figure 5-8 Identification of recombinant plasmids, pDrive carrying PCR fragment of *P. falciparum* truncated orotate phosphoribosyltransferase gene. The numbers refer to molecular size. Lane M is molecular size marker. Lane 1 and 2 are cut construct plasmid. Interestingly, the plasmid in lane 1 had insert but lane 2 didn't have, so lane1 is the positive clone.

4. Analysis of the DNA sequence by the BLAST program

Plasmid DNA from one positive clone was sequenced using an automated DNA sequencer (Figure 5-9,10,11,12). In this thesis, we have the full-length and N-terminal truncated *P. falciparum* orotate phosphoribosyltransferase enzyme. We used the alignment program to align between these both type of enzymes. Our truncated sequence was identical to the open reading frame of the candidate gene (66 amino acids N-terminal truncated *P. falciparum* orotate phosphoribosyltransferase gene).

5. Expression of the *P. falciparum* orotate phosphoribosyltransferase gene in the bacterial system

5.1 Subcloning of *P. falciparum* orotate phosphoribosyltransferase gene in a pQE30Xa expression vector and appropriated host cells

The DNA insert of orotate phosphoribosytransfrase gene in the positive clone was subclone into a pQE30Xa expression vector (Figure 5-13). The plasmid DNA from the positive clone with pQE30Xa expression vector was transformed into M15 competent cells. The pQE40 plasmid was transformed as a positive control (data not shown). Six colonies were selected for minipreparation of plasmid DNA (data not shown), then all of them were analyzed by the restriction analysis. The plasmids were digested with *Bam*HI and *Hind*III. It was found that all six clones contained the DNA insert of truncated *P. falciparum* orotate phosphoribosyltransferase homolog, as demonstrated by the agarose gel electrophoresis (Figure 5-14)

5.2 Expression of *P. falciparum* orotate phosphoribosyltransferase gene in *E.coli*

One from all positive clones was selected for expression by induction with IPTG, and compared with positive control pQE40 (data not shown).The plasmid DNA of full-length has been induced with IPTG in the same condition, too. The IPTG-induced *E.coli* cells were harvested to detect the recombinant protein expression. The expressed proteins was purified by the Ni-NTA affinity chromatography and then analyzed by 12%

SDS-PAGE (Figure 5-15). The major band of the full-length protein and the truncated protein at approximately 35 kDa and 30 kDa were identified, respectively. In Western blot, It was found that the major band of the full-length protein and the truncated protein at approximately 35 kDa and 30 kDa, too (Figure 5-17).

6. Study on the enzyme orotate phosphoribosyltransferase of *P. falciparum* recombinantly expressed in *E. coli*

The purified expressed proteins were subjected to enzyme assay of orotate phosphoribosyltransferase activity. The results of full-length and truncated orotate phosphoribosytransferase activities in elute are shown in Table 5-1,2 . The specific activity of full-length orotate phosphoribosyltransferase in elute was 11.38 nmol/min/mg protein (n=2) and the specific activity of truncated orotate phosphoribosyltransferase in elute was 1.15 $\pm$ 0.7 nmol/min/mg protein(n=6). The truncated recombinant enzyme purification was about 10-fold less than the full-length specific activity.

Determination of the molecular mass of the full-length and truncated *P. falciparum* orotate phosphoribosyltransferase was performed by using SDS-PAGE analysis. Various molecular mass marker proteins were loaded on a 12% gel of SDS-PAGE, including phosphorylase b (molecular mass = 97.4 kDa), Serum albumin (molecular mass = 66.2 kDa), Ovalbumin (molecular mass = 45.0 kDa),Carbonic anhydrase (molecular mass = 31.0 kDa), Trypsin inhibitor (molecular mass = 21.5 kDa), and Lysozyme (molecular mass = 14.4 kDa). The relative mobilities of proteins were plotted against molecular mass of full-length and truncated *P. falciparum* orotate phosphoribosyltransferase was then calculated from the standard curve. They were approximately 35 kDa and 30 kDa, respectively(Figure 5-18).

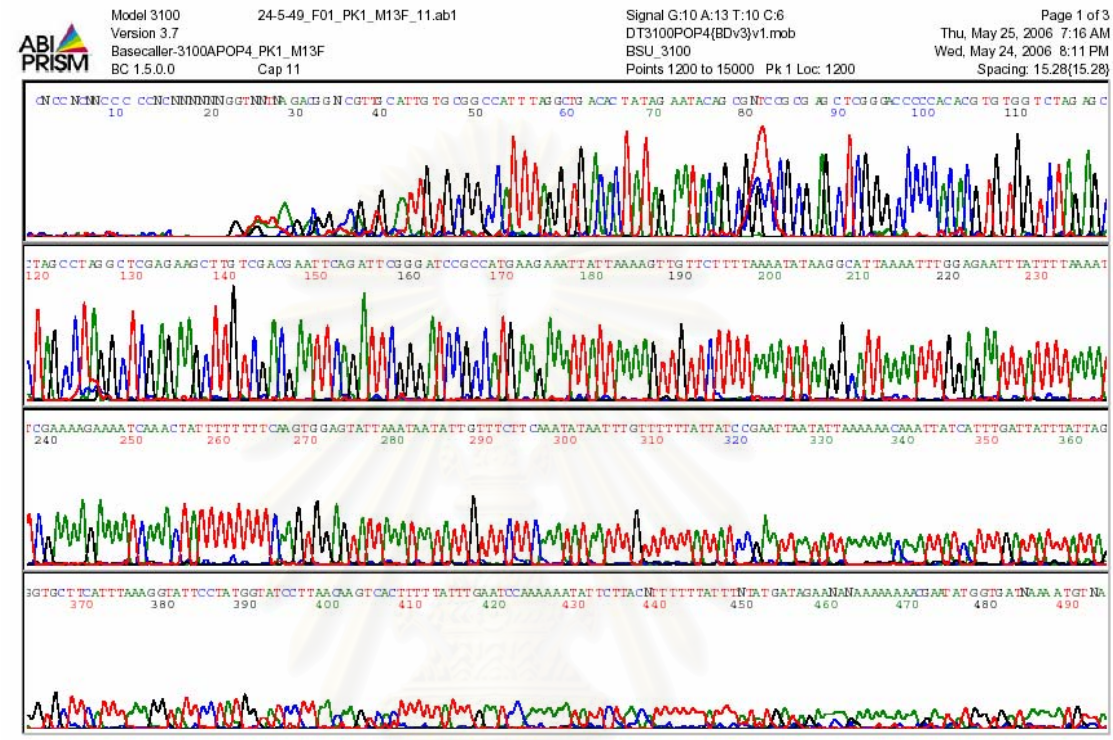

Figure 5-9 Result of forward DNA sequencing by an automated DNA sequencer

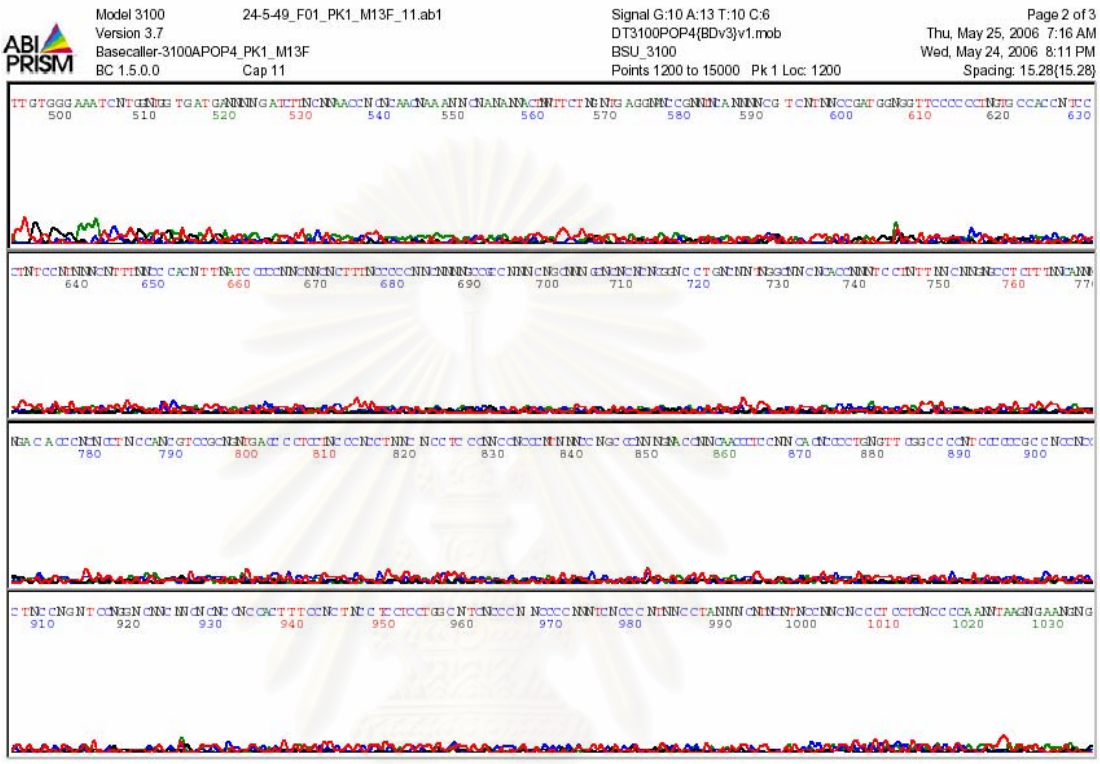

Figure 5-10 Result of forward DNA sequencing by an automated DNA sequencer (cont.)

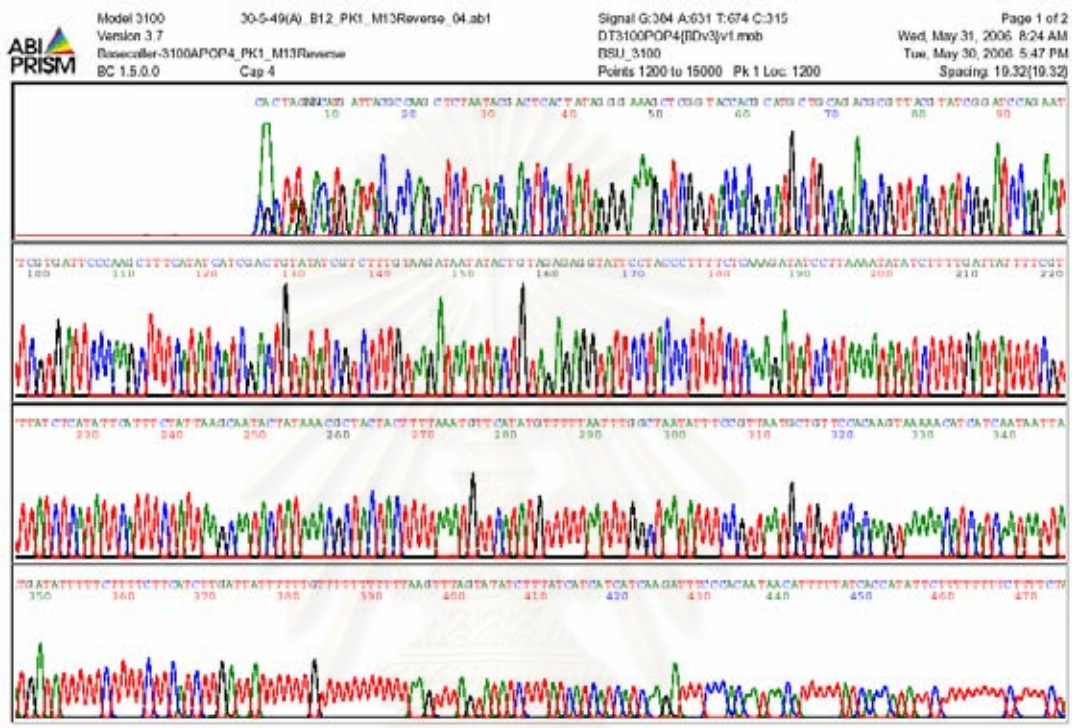

Figure 5-11 Result of reverse DNA sequencing by an automated DNA sequencer

Figure 5-12 Result of reverse DNA sequencing by an automated DNA sequencer (cont.)

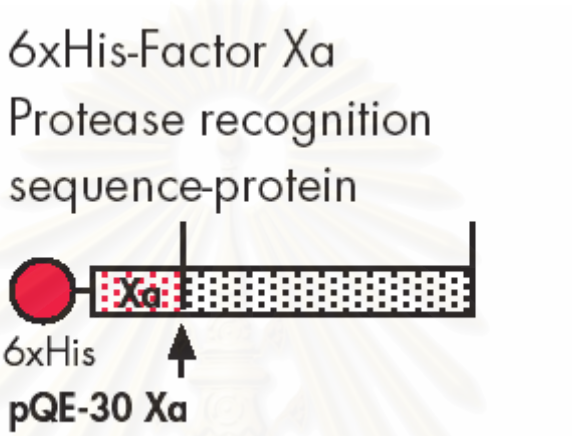

Figure 5-13 Constuction of pQE30Xa expression vector.

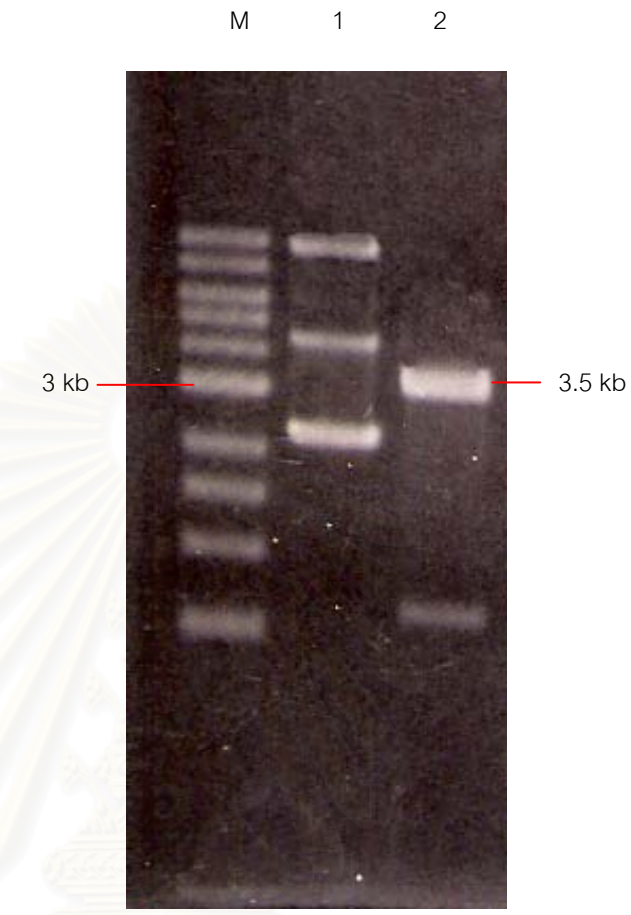

Figure 5-14 Identification of recombinant plasmids, pQE30Xa carrying DNA fragment of *P. falciparum* truncated orotate phosphoribosyltransferase gene. The numbers refer to molecular size. Lane M is molecular size marker. Lane 1 is three forms of uncut construct plasmid. Lane 2 is plasmid cut with *Bam*HI and *Hind*III and showed that it was the positive clone.

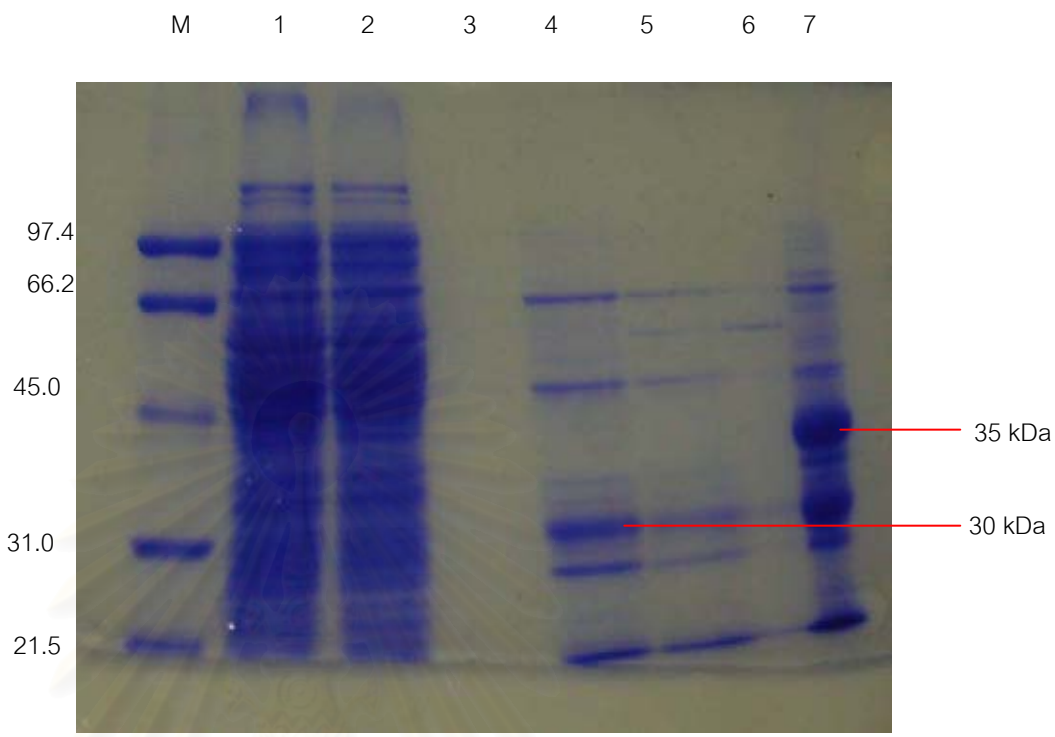

Figure 5-15 SDS-PAGE analysis of recombinantly expressed proteins by Ni-NTA affinity chromatography. Numbers are molecular masses in kiloDalltons (kDa).

Lane M is standard molecular mass marker proteins

Lane 1 is lysate of the truncated enzyme

Lane 2 is supernate of the truncated enzyme

Lane 3 is washing buffer of the truncated enzyme

Lane 4 is 1st elute of the truncated enzyme

Lane 5 is 2nd elute of the truncated enzyme

Lane 6 is 3rd elute of the truncated enzyme

Lane 7 is 1st elute of the full-length enzyme

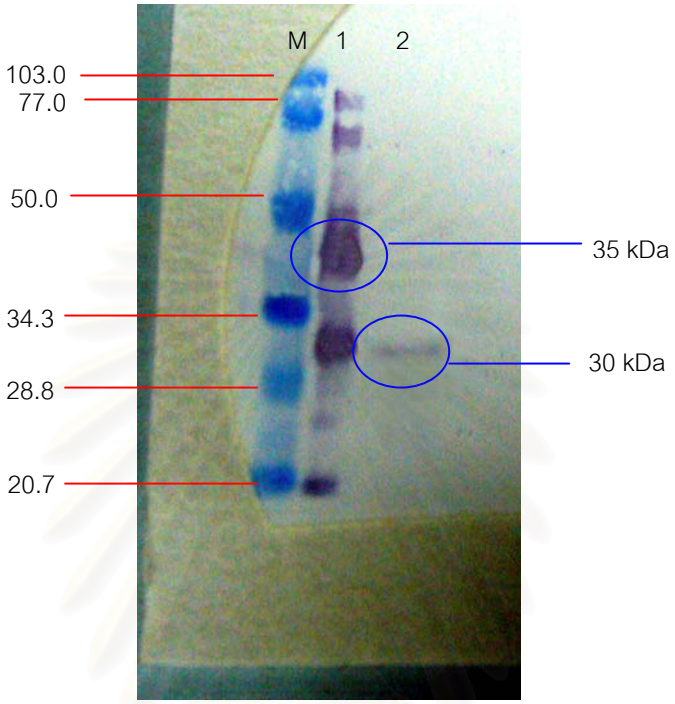

Figure 5-16 The Western blot analysis of the full-length and truncated *P. falciparum* orotate phosphoribosyltransferase enzyme. Numbers are molecular masses in kiloDalltons (kDa). Lane M is standard molecular mass marker proteins (Prestain). Lane 1 is the first elution of the full-length enzyme. Lane 2 is the first elution of the truncated enzyme.

Experiment No.	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)
1	1.349	12.1540	9.010
2	0.847	11.6442	13.7
Average	-	11.8991	11.4

Table 5-1 Results of the full-length *P. falciparum* orotate phosphoribosyltransferase activity after purified by nickel metal-affinity chromatography.

Experiment No.	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)
1	0.602	1.0969	1.82
2	0.614	0.6005	0.936
3	0.582	0.3897	0.670
4	0.591	1.0839	0.659
5	0.602	1.3280	2.21
6	0.574	0.3480	0.606
Mean $\pm$ SD	-	0.81 $\pm$ 0.4	1.2 $\pm$ 0.7

Table 5-2 Results of the truncated orotate phosphoribosyltransferase activity after purified by nickel metal-affinity chromatography.

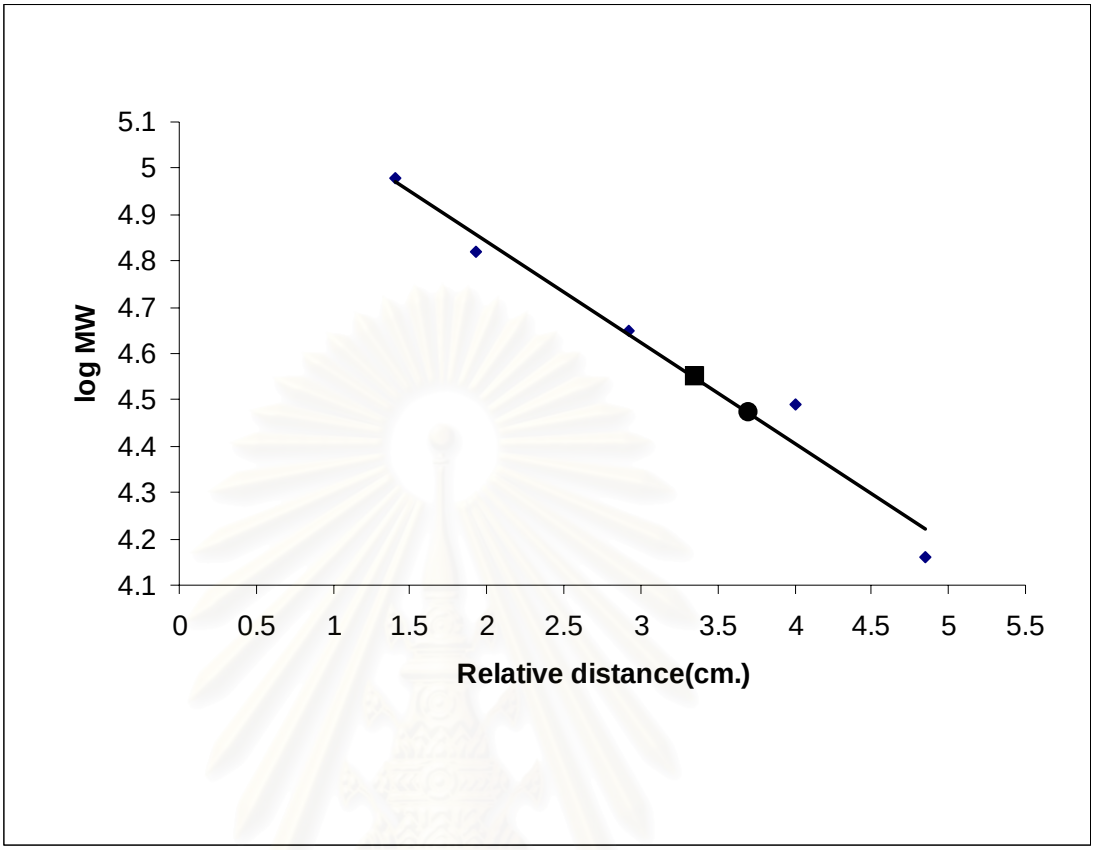

Figure 5-17 Standard curve for protein molecular mass determination. The symbol ● indicate the position of truncated orotate phosphoribosyltransferase and the symbol ■ indicate the position of full-lenght orotate phosphoribosyltransferase.

7. Study on the stability of *P. falciparum* orotate phosphoribosyltransferase enzyme.

We compared the stability of the full-length enzyme and the truncated enzyme by the storage at -20 ^{O}C in presence of 1 mM dithiothreitol and 10% glycerol for about 3 months. The specific activity was determined. It was found that the full-length enzyme could maintain its activity about 3 months, in contrast, the truncated enzyme activity drop rapidly 3 days of the storage, and finally it lost activity in 1 week. Therefore, it was summarized that the truncated *P. falciparum* orotate phosphoribosyltransferase was less stable enzyme when compared with the full-length enzyme.

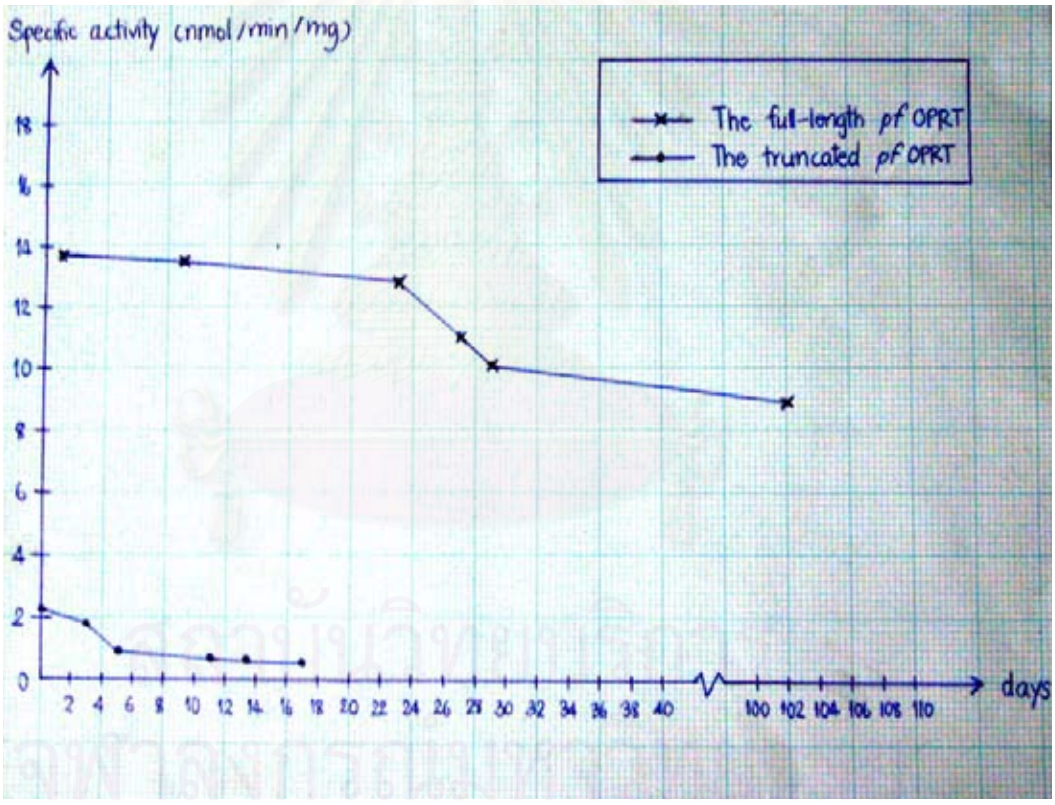

Figure 5-18 The line chart comparing between full-length and truncated orotate phosphoribosyltransferase stability.

CHAPTER VI

DISCUSSION

The need for new antimalarials comes from the widespread resistance to those in current use. The search for such new targets and new drug chemotypes will likely be helped by the advent of functional genomics and structure-based drug design[57].

The enzymes in *de novo* pyrimidine pathway are interesting targets because the malarial parasite has to proliferate in human red blood cell and produce pyrimidine bases for DNA synthesis[58].

At present, A gene encoding orotate phosphoribosyltransferase (OPRT), the fifth enzyme of the de novo pyrimidine pathway has been identified from *P. falciparum* (*pf*OPRT). It was compared with OPRTs from other organisms and found to be most similar to that of *Escherichia* coli [12]. The *pf*OPRT is exceptional in that it contains an amino-terminal extension of 66 amino acids, making the longest amino acid sequence (281 amino acids)[12, 13]. SDS–PAGE revealed that the *pf*OPRT had a molecular mass of 33 kDa and analytical gel-filtration chromatography showed that the enzyme activity eluted at approximately 67 kDa[12]. Using dimethyl suberimidate to cross-link neighboring subunits of the *pf*OPRT, it was confirmed that the native enzyme exists in a dimeric form[12, 56, 59] and is monofunctional [56, 59]. The steady state kinetics of initial velocity and product inhibition studies indicate that the enzyme *pf*OPRT follows a random sequential kinetic mechanism[12]. Although we knew several things about this *pf*OPRT enzyme, it remains something that uncharacterized such as the importance of an amino-terminal extension of 66 amino acids[13]. In this study, we cloned and expressed the full-length and truncated *Plasmodium falciparum* orotate phosphoribosyltransferase gene by using PCR amplification technique with genomic data from bioinformatics underlying NCBI resources. We used SDS-PAGE and Western blot analysis to determine the molecular mass for both types of *pf*OPRT. Then we study

about the specific activity and stability for both types of the enzymes, too. The discussion about the main topics of this thesis is the followings :

1. Gene identification of full-length and truncated *Plasmodium falciparum* orotate phosphoribosyltransferase.

We have used bioinformatics approaches to identify our full-length and truncated *Plasmodium falciparum* orotate phosphoribosyltransferase gene homolog. The full-length *Plasmodium falciparum* orotate phosphoribosyltransferase amino acid sequence has been found on chromosome 5 and aligned with other organisms. It was found that *P. falciparum* orotate phosphoribosyltransferase amino acid sequence had N-terminal extension of 66 amino acids, making the longest amino acid sequence (281 amino acids)[12]. From the bioinformatics underlying NCBI resources, we had selected other *Plasmodium spp.* to study on orotate phosphoribosytransferase amino acid sequences. The enzymes, including *P. falciparum* orotate phosphoribosyltransferase[60], *P. chabaudi* orotate phosphoribosyltransferase[61], *P. berghei* orotate phosphoribosyltransferase[62], and *P. yoelii* orotate phosphoribosyltransferase [63], were identified for their amino acid sequences.

All amino acid sequences was analyzed for sequence homology by the alignment program, ClustralW. *P. berghei* orotate phosphoribosyltranferase amino acid sequences was found to have the most similarity to *P. falciparum* sequence with about 65% homology. This evidence indicated that *P. berghei* orotate phosphoribosyltranferase may resemble to *P. falciparum*, but when we examine the result of alignment between *P. berghei* orotate phosphoribosyltranferase amino acid sequences and *P. falciparum* orotate phosphoribosyltranferase amino acid sequences we found that *P. berghei* orotate phosphoribosyltranferase amino acid sequences didn't have the N-terminal extension as long as *P. falciparum* orotate phosphoribosyltranferase amino acid sequences. Therefore the N-terminal extension is the unique for *P. falciparum* orotate phosphoribosyltranferase.

2. *Plasmodium falciparum* orotate phosphoribosyltransferase cloning and expression.

Using bioinformatics to design primers, we have succeed in DNA amplification by PCR for both full-length and truncated *P. falciparum* orotate phosphoribosyltransferase. The size of the PCR product of the full-length DNA was 846 bp and the truncated DNA was 648 bp. Only truncated PCR product was cloned into a pDrive cloning vector and *E. coli* strain DH5α was uesd.
The full-length gene was cloned directly into pQE30Xa expression vector and *E. coli* strain M15 was used. The restriction analysis with *Bam*HI and *Hind*III was used for truncated *P. falciparum* orotate phosphoribosyltransferase to identify the positive clone for sequencing by an automated DNA sequencer, then the homology of the DNA sequence was analysed by the alignment program. The results of homology analysis showed identical to the 66 amino acids N-terminal truncated *P. falciparum* orotate phosphoribosyltransferase gene.

Studies on heterologous expression system by subcloning the insert to pQE30Xa expression vector in *E. coli* M15 strain. The truncated recombinant protein was expressed after IPTG induction. The expressed protein, as His_6 – tagged at N-terminus, has been purified using the Ni-NTA affinity chromatography. The purified protein is then identified on SDS-PAGE analysis as a major band with a molecular mass of 30 kDa (truncated *pf*OPRT, factor Xa recognition site, and His_6 – tagged) which is very close to the calculated molecular mass of the amino acid sequence of this protein. The full-length protein in pQE30Xa was expressed after IPTG-induction and had His_6 – tagged at N-terminus, too. It has been purified using the Ni-NTA affinity chromatography at the same condition of truncated protein and identified on SDS-PAGE anaysis as a major band with a molecular mass of 34 kDa (full-length *pf*OPRT, factor Xa recognition site, and His_6 – tagged)

From Werstern blot analysis, we can confirm that the purified enzyme was the full-length and truncated enzymes which we expected. We use anti-His tagged to detect these proteins because both types of enzyme has been tagged with histidine 6

residues, and this pattern of histidine couldn't be found in general enzyme. We could examine the size of protein, so we could separate the full-length and truncated enzymes by the sizes of them.

3. The functional properties of *Plasmodium falciparum* orotate phosphoribosyltransferase.

In order to verify the function of the recombinant protein obtained in IPTG-induced *E. coli* harboring the gene inserted in the pQE30Xa expression vector, the activity of the enzyme in the recombinant protein has been determined spectrophotometrically. The specific activity of full-length and truncated orotate phosphoribosyltransferase in the elute after the Ni-NTA affinity chromatography is 11.38 nmol/min/mg protein and 1.15$\pm$ 0.7 nmol/min/mg protein, respectively. These results indicated that the expressed truncated protein had about 10-fold lower activity than full-length protein. The kinetic values of full-length *P. falciparum* orotate phosphoribosyltransferase have been characterized, but we couldn't characterize the kinetic parameters of truncated *P. falciparum* orotate phosphoribosyltransferase because of the low amount protein expression.

For example, the reduction of enzyme activity in truncated form, NAD1-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH) has been found. This enzyme catalyzes the oxidation of the 15(S) hydroxyl group of prostaglandins to a 15-keto group. A significant reduction of the biological activities of prostaglandins was found. The involvement of the C-terminal region in catalytic activity was examined by studies on C-terminally truncated enzymes and on human/rat chimeric enzymes. When three to four amino acids were removed successively from the C-terminal end of human 15-PGDH, the truncated enzymes exhibited decreasing Vmax /Km ratios[64]. This indicated the trend of reducing enzyme activity when it was deleted to be truncated form.

The low enzyme activity which has been found in N-terminal truncated *P. falciparum* orotate phosphoribosyltransferase enzyme indicated that this truncated enzyme didn't do it function extremely. Therefore, the lengthen part of *P. falciparum* orotate phosphoribosyltransferase may play important role in enzyme activity. This may influence to the parasite survival. Furthermore, the stability of the both types of enzyme were compared. From results, the full-length *P. falciparum* orotate phosphoribosyltransferase was more stable than the truncated enzyme, so the N-terminal extension of enzyme may relate to the enzyme stability. We can assume that the N-terminal extension part of *P. falciparum* orotate phosphoribosyltransferase concerned about enzyme activity and stability, the parasite enzyme couldn't work completely and disappeared quickly.

To block the this unique part of *P. falciparum* orotate phosphoribosyltransferase may obstruct the parasite proliferation or kill them. Human orotate phosphoribosyltransferase didn't have this unique N-terminal extension, so the inhibitors or drugs may act only with the parasite enzyme and don't make the side effects for human which is the host. The parasite couldn't survive by limited synthesis of pyrimidine bases.

For further study, the amount of protein expression should increase for characterization of kinetic parameters such as V_{max}, K_m, and K_{cat},, and for more purification. The study about how the N-terminal extension part of *P. falciparum* orotate phosphoribosyltransferase related to the enzyme activity and stability is needed. The *P. falciparum* orotate phosphoribosyltransferase crystal structure should be studied for structural-based inhibitors or drug synthesis.

CHAPTER VII

CONCLUSION

1. *P. falciparum* orotate phosphoribosyltransferase amino acid sequence had N-terminal extension of 66 amino acids, making the longest amino acid sequence (281 amino acids).
2. *P. berghei* orotate phosphoribosyltranferase amino acid sequences was found to have the most homology to *P. falciparum* sequence with about 65% similarity.
3. The PCR products of full-length *P. falciparum* orotate phosphoribosyltransferase gene were 846 bp and The PCR products of truncated *P. falciparum* orotate phosphoribosyltransferase gene was 648 bp.
4. The *P. falciparum* orotate phosphoribosyltransferase amino acid sequence was identical to the open reading frame of the candidate gene (66 amino acids N-terminal deleted *P. falciparum* orotate phosphoribosyltransferase gene).
5. From SDS-PAGE and Western blot analysis, the major band of the full-length protein and the truncated protein at approximately 35 kDa and 30 kDa was identified, respectively.
6. The specific activity of full-length orotate phosphoribosyltransferase in elute was 11.38 nmol/min/mg protein (n=2) and the specific activity of truncated orotate phosphoribosyltransferase in elute was 1.15 $\pm$ 0.7 nmol/min/mg protein (n=6).
7. The truncated recombinant enzyme purification was about 10 -fold less than the full-length specific activity.
8. The full-length enzyme could maintain its activity for all 3 months, in contrast, the truncated enzyme activity drop rapidly about 3 days of the storage. So, the truncated *P. falciparum* orotate phosphoribosyltransferase had slight or none stability when compared with the full-length enzyme.

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APPENDICES

APPENDIX A

BUFFER AND REAGENTS

1. 10% Sodium dodecyl sulfate(SDS)

SDS	100.00	g
dH_2O	870.00	ml

adjust pH to 7.2 with conc. HCL

adjust volume to 1.0 litre with dH_2O

2. SolutionI

50 mM glucose

25 mM Tris·CI(pH 8.0)

10 mM EDTA(pH 8.0)

3. SolutionII

0.2 N NaOH

1% SDS

4. SolutionIII

5 M Sodium acetate	60.00	ml
Glacial acetic acid	11.50	ml
dH_2O	28.50	ml

5. 3 M Sodium acetate(pH 5.0)

Sodium acetate	40.82	g
dH_2O	80.00	ml

adjust pH to 5.0 with conc. HCL

adjust volume to 100 ml with dH_2O and sterilize by autoclaving

6. TE buffer

10 mM Tris-HCL(pH 7.4)

1 mM EDTA

7. 10x Phospate buffer(pH 8.0)

Sodium phosphate	34.50	g
Sodium chloride	87.80	g
dH_2O	300.00	ml

adjust pH to 8.0 with 10 N NaOH

adjust volume to 500 ml with dH_2O

8. 10x Ficoll loading buffer

Ficoll	25.00	g
Bromphenol blue	0.025	g
0.5 M EDTA(pH 8.0)	0.2	ml

adjust volume to 10 ml with dH_2O

store at -20°C

9. Phosphate buffered saline (PBS)

Sodium chloride	8.00	g
Potassium chloride	0.20	g
Sodium phosphate	1.40	g
Potassium phosphate	0.24	g
dH_2O	800.00	ml

adjust pH to 7.4 with conc. HCL

adjust volume to 1.0 litre with dH_2O

10. IPTG stock solution (100 mM)

IPTG	238.30	mg
dH_2O	10.00	ml

filter-sterilize and store in aliquot at -20°C

11. Ampicillin stock solution (50mg/ml)

Ampicillin sodium salt	1.25	g
dH_2O	25.00	ml

filter-sterilize and store in aliquot at 4 ^{o}C

12. Kanamycin stock solution (25 mg/ml)

Kanamycin monosulfate salt	1.00	g
dH_2O	40.00	ml

filter-sterilize and store in aliquot at 4 ^{o}C

13. 10x TAE buffer (pH 8.0)

Tris Hcl	48.40	g
EDTA	3.72	g
dH_2O	500.00	ml

adjust pH to 8.0 with acetic aic

adjust volume to 1.0 litre with dH_2O

14. 1% Agarose gel (w/v)

Agrarose	2.00	g
1x TAE buffer	200.00	ml

dissolve by heating until homogeneous

15. LB medium (Luria-Bertani medium)

Tryptone	10.00	g
Yeast extract	5.00	g
NaCL	10.00	g

Adjust volume to 1.0 litre with sterile water

Sterilize by autoclaving at 120^{o}C for 25 min

Cool to 50^{o}C or below

LB-ampicillin medium was made by addition of ampicillin to a final concentration of 100 μg/ml

16. LB Agar plate

Tryptone	10.00	g
Yeast extract	5.00	g
NaCL	10.00	g
Agar	15.00	g

Adjust volume to 1.0 litre with sterile water

Sterilize by autoclaving at 120°C for 25 min

Cool to 50°C or below

LB-ampicillin agar was made by addition of ampicillin to a final concentration of 100 μg/ml, pour into petridishes and allowed to harden at RT, then keep the plate at 4°C in an inverted position

17. Acrylamide/Bis acrylamide

Acrylamide	29.20	g
Bis acrylamide	0.80	g
ddH_2O	100.00	ml

store in brown bottle at 4°C

18. Seperating gel for 12% acrylamide gel

Acrylamide/Bis acrylamide	3.00	ml
1M Tris (pH 8.8)	2.80	ml
ddH_2O	1.67	ml
10% SDS	0.075	ml
10% APS	25.00	μl
TEMED	10.00	μl

19. Stacking gel for 12% acrylamide gel

Acrylamide/Bis acrylamide	0.84	ml
1M Tris (pH 6.8)	0.63	ml
ddH_2O	3.50	ml
10% SDS	50.00	μl
10% APS	30.00	μl
TEMED	10.00	μl

20. Running buffer for SDS-PAGE

Tris·base	3.03	g
Glycine	14.42	g
dH_2O	500.00	g
10% SDS	10.00	ml

adjust volume to 1.0 with dH_2O

21. 2x Sample buffer

1M Tris (pH 6.8)	1.25	ml
Glycerol	2.00	ml
10% SDS	4.00	ml
Mercaptoethanol	1.00	ml
0.1% BPB	0.50	ml

the solution was mixed with 5 μl of sample and boiled for 6 min before loading

22. Coomassie Blue R staining

Coomassie Brilliant Blue R250	0.10	g
Glacial acetic acid	50.00	ml
ddH2O	50.00	ml

after electrophoresis, the gel was stained with Coomassie Blue for 30 min

23. Destraining solution

Absolute methanol	50.00	ml
Glacial acetic acid	50.00	ml
ddH2O	400.00	ml

the stained gel was subsequently destained with he destaining solution until the gel was clear

24. Blotting buffer

25 mM Tris base

150 mM glycine

10% methanol

25. TBS buffer

10 mM Tris-Cl,pH 7.5

150 mM NaCl

26. TBS-Tween/Triton buffer

20 mM Tris-Cl,pH 7.5

500 mM NaCl

0.05% (v/v) Tween 20

0.2% (v/v) Triton X-100

27. Blocking buffer

0.25 g of blocking reagent in 50 m of 1X blocking reagent buffer

APPENDIX B

Plasmodium spp. OROTATE PHOSPHORIBOSYLTRANSFERASE AMINO ACID SEQUENCES

1. *Plasmodium yoelii* orotate phosphoribosytransferase

```
LOCUS       XP_724927                214 aa            linear   INV 21-APR-2006
DEFINITION  hypothetical protein PY04605 [Plasmodium yoelii yoelii str. 17XNL].
ACCESSION   XP_724927
VERSION     XP_724927.1  GI:82541363
DBSOURCE    REFSEQ: accession XM_719834.1
KEYWORDS    .
SOURCE      Plasmodium yoelii yoelii str. 17XNL
  ORGANISM  Plasmodium yoelii yoelii str. 17XNL
            Eukaryota; Alveolata; Apicomplexa; Aconoidasida; Haemosporida;
            Plasmodium; Plasmodium (Vinckeia).
REFERENCE   1  (residues 1 to 214)
  AUTHORS   Carlton,J.M., Angiuoli,S.V., Suh,B.B., Kooij,T.W., Pertea,M.,
            Silva,J.C., Ermolaeva,M.D., Allen,J.E., Selengut,J.D., Koo,H.L.,
            Peterson,J.D., Pop,M., Kosack,D.S., Shumway,M.F., Bidwell,S.L.,
            Shallom,S.J., van Aken,S.E., Riedmuller,S.B., Feldblyum,T.V.,
            Cho,J.K., Quackenbush,J., Sedegah,M., Shoaibi,A., Cummings,L.M.,
            Florens,L., Yates,F.R. III, Raine,J.D., Sinden,R.E., Harris,M.A.,
            Cunningham,D.A., Preiser,P.R., Bergman,L.W., Vaidya,A.B., van
            Lin,L.H., Janse,C.J., Waters,A.P., Smith,H.O., White,O.R.,
            Salzberg,S.L., Venter,J.C., Fraser,C.M., Hoffman,S.L., Gardner,M.J.
            and Carucci,D.J.
  TITLE     Genome sequence and comparative analysis of the model rodent
            malaria parasite Plasmodium yoelii yoelii
  JOURNAL   Nature 419 (6906), 512-519 (2002)
   PUBMED   12368865
REFERENCE   2  (residues 1 to 214)
  AUTHORS   Carlton,J.M., Suh,B.B., Fraser,C.M. and Gardner,M.J.
  TITLE     Direct Submission
  JOURNAL   Submitted (25-SEP-2002) The Institute for Genomic Research, 9712
            Medical Center Dr, Rockville, MD 20850, USA
COMMENT     PROVISIONAL REFSEQ: This record has not yet been subject to final
            NCBI review. The reference sequence was derived from EAA16492.
            Method: conceptual translation.
FEATURES             Location/Qualifiers
     source          1..214
                     /organism="Plasmodium yoelii yoelii str. 17XNL"
                     /strain="17XNL"
                     /isolate="17XNL"
                     /sub_species="yoelii"
                     /db_xref="taxon:352914"
     Protein         1..214
                     /product="hypothetical protein"
                     /calculated_mol_wt=24675
     Region          40..>163
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                     /note="Orotate phosphoribosyltransferase [Nucleotide
                     transport and metabolism]; COG0461"
                     /db_xref="CDD:30809"
     CDS             1..214
                     /locus_tag="PY04605"
                     /coded_by="XM_719834.1:1..645"
                     /db_xref="GeneID:3790262"
ORIGIN
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       61 ilkskrksky fvstgflnna isaniisfli snlilsknih fdylfgasyk gipivsltsh
      121 fllntnkfhn ifylydrkek keygdktiiv gnikendqdc vinnnccnpq fekknkkkvi
      181 iiddvftcgt alteifnkmk yypdftsvac isss
```

2. *Plasmodium falciparum* orotate phosphoribosytransferase

```
LOCUS       CAD51491                 281 aa            linear   INV 16-APR-2005
DEFINITION  orotate phosphoribosyltransferase, putative [Plasmodium falciparum
            3D7].
ACCESSION   CAD51491
VERSION     CAD51491.1  GI:23504612
DBSOURCE    embl accession AL929352.1
KEYWORDS    .
SOURCE      Plasmodium falciparum 3D7
  ORGANISM  Plasmodium falciparum 3D7
            Eukaryota; Alveolata; Apicomplexa; Aconoidasida; Haemosporida;
            Plasmodium; Plasmodium (Laverania).
REFERENCE   1
  AUTHORS   Hall,N., Pain,A., Berriman,M., Churcher,C., Harris,B., Harris,D.,
            Mungall,K., Bowman,S., Atkin,R., Baker,S., Barron,A., Brooks,K.,
            Buckee,C.O., Burrows,C., Cherevach,I., Chillingworth,C.,
            Chillingworth,T., Christodoulou,Z., Clark,L., Clark,R., Corton,C.,
            Cronin,A., Davies,R., Davis,P., Dear,P., Dearden,F., Doggett,J.,
            Feltwell,T., Goble,A., Goodhead,I., Gwilliam,R., Hamlin,N.,
            Hance,Z., Harper,D., Hauser,H., Hornsby,T., Holroyd,S.,
            Horrocks,P., Humphray,S., Jagels,K., James,K.D., Johnson,D.,
            Kerhornou,A., Knights,A., Konfortov,B., Kyes,S., Larke,N.,
            Lawson,D., Lennard,N., Line,A., Maddison,M., McLean,J., Mooney,P.,
            Moule,S., Murphy,L., Oliver,K., Ormond,D., Price,C., Quail,M.A.,
            Rabbinowitsch,E., Rajandream,M.A., Rutter,S., Rutherford,K.M.,
            Sanders,M., Simmonds,M., Seeger,K., Sharp,S., Smith,R., Squares,R.,
            Squares,S., Stevens,K., Taylor,K., Tivey,A., Unwin,L.,
            Whitehead,S., Woodward,J., Sulston,J.E., Craig,A., Newbold,C. and
            Barrell,B.G.
  TITLE     Sequence of Plasmodium falciparum chromosomes 1, 3-9 and 13
  JOURNAL   Nature 419 (6906), 527-531 (2002)
   PUBMED   12368867
REFERENCE   2  (residues 1 to 281)
  AUTHORS   Devlin,K., Baker,S., Davies,P., Mungal,K., Berriman,M., Pain,A.,
            Hall,N., Bowman,S., Churcher,C., Quail,M. and Barrell,B.
  TITLE     Direct Submission
  JOURNAL   Submitted (20-SEP-2002) P.falciparum Genome Sequencing Consortium,
            The Welcome Trust Sanger Institute, Wellcome Trust Genome Campus,
            Hinxton, Cambridge CB10 1SA, UK
COMMENT     For more information about this sequence or the Malaria Project,
            see http://www.sanger.ac.uk/Projects/P_falciparum.
FEATURES             Location/Qualifiers
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                     /isolate="3D7"
                     /db_xref="taxon:36329"
                     /chromosome="5"
     Protein         1..281
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                     /EC_number="2.4.2.10"
     Region          83..275
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                     /note="Orotate phosphoribosyltransferase [Nucleotide
                     transport and metabolism]; COG0461"
                     /db_xref="CDD:30809"
     CDS             1..281
                     /gene="PFE0630c"
                     /coded_by="complement(AL929352.1:199732..200577)"
                     /db_xref="GOA:Q8I3Y0"
                     /db_xref="HSSP:P08870"
                     /db_xref="InterPro:IPR000836"
                     /db_xref="UniProtKB/TrEMBL:Q8I3Y0"
ORIGIN
        1 mttikenefl cdeeiyksfv hlkdkiceer kkkelvnnni dnvnfndddd nnydddgnsy
       61 ssyikemkkl lkvvllkyka lkfgefilks krksnyffss gvlnnivssn iicfllseli
      121 lknklsfdyl lgasykgipm vsltshflfe skkysnifyl ydrkekkeyg dknvivgnld
      181 dddkdilnlk kktknnqdee kkniiiiddv ftcgtaltei laklktyehl kvvafivlln
      241 rneyeinenn qkiyfkdife krvgiplysi lsykddiqsm i
```

3. *Plasmodium berghei* orotate phosphoribosytransferase

```
LOCUS       CAI00059                160 aa            linear   INV 16-NOV-2004
DEFINITION  orotate phosphoribosyltransferase, putative [Plasmodium berghei].
ACCESSION   CAI00059
VERSION     CAI00059.1  GI:56482315
DBSOURCE    embl accession CAAI01003134.1
KEYWORDS    .
SOURCE      Plasmodium berghei
  ORGANISM  Plasmodium berghei
            Eukaryota; Alveolata; Apicomplexa; Haemosporida; Plasmodium.
REFERENCE   1
  AUTHORS   Hall,N., Karras,M., Raine,J.D., Carlton,J.M., Kooij T.W.A.
            Berriman,M., Florens,L., Janssen,C.S., Pain,A., Christophides,G.K.,
            James,K., Rutherford,K., Harris,B., Harris,D., Churcher,C.,
            Quail,M.A., Ormond,D., Doggett,J., Trueman,H.E., Mendoza,J.,
            Bidwell,S.L., Rajandream,M.A., Carucci,D.J., Yates,J.R.,
            Kafatos,F.C., Janse,C.J., Barrell,B., Turner,C.M.R., Waters,A.P.
            and Sinden,R.S.
  TITLE     A global survey of the molecular life-strategies of malaria
            parasites by integrated genomic, transcriptomic and proteomic
            analyses of both vertebrate and mosquito stages
  JOURNAL   Unpublished
REFERENCE   2  (residues 1 to 160)
  AUTHORS   Hall,N.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-SEP-2004) Pathogen Sequencing Unit, The Sanger
            Institute, The Wellcome Trust Genome Campus, Cambridge CB10 1SA
COMMENT     THIS IS A LOW COVERAGE GENOME ASSEMBLY CONTAINING AUTOMATED
            ANNOTATION THERE MAY BE MISTAKES IN THE ANNOTATION AS WELL AS THE
            UNDERLYING SEQUENCE AND ASSEMBLY. MANY GENE PREDICTIOS ARE PARTIAL
            AND WILL NOT CONTAIN START OR STOP CODONS. SOME GENE PREDICTIONS
            HAVE NON-STANDARD SPLICING OR IN FRAME STOP CODONS.THE SEQUENCE HAS
            BEEN RELEASED AND PUBLISHED IN THIS CONDITION BY THE SANGER
            INSTITUTE FOR THE BENEFIT OF THE SCIENTIFIC COMMUNITY. THE SANGER
            INSTITUTE IS COMMITTED TO TRYING TO OBTAIN FUNDS TO COMPLETE THE
            GENOME AND CURATE THE ANNOTATION.
FEATURES             Location/Qualifiers
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                     /db_xref="taxon:5821"
                     /clone_lib="pUC18"
     Protein         1..160
                     /product="orotate phosphoribosyltransferase, putative"
     Region          <3..>75
                     /region_name="PyrE"
                     /note="Orotate phosphoribosyltransferase [Nucleotide
                     transport and metabolism]; COG0461"
                     /db_xref="CDD:30809"
     CDS             1..160
                     /locus_tag="PB000705.03.0"
                     /coded_by="CAAI01003134.1:<4..>483"
ORIGIN
        1 isanivsfli snlilsknia fdylfgasyk gipivsltsh fllntnkfhn ifylydrkek
       61 keygdktiiv gnikessqdc vinscnpqfe kkkkviiidd vftcgtalte ifnkmkayey
      121 lqvvacivll nrneheinen nekvyfkdlf eqkynipiyt
```

4. *Plasmodium chabaudi* orotate phosphoribosytransferase

```
LOCUS       CAH81477                 251 aa            linear   INV 16-NOV-2004
DEFINITION  orotate phosphoribosyltransferase, putative [Plasmodium chabaudi].
ACCESSION   CAH81477
VERSION     CAH81477.1  GI:56523923
DBSOURCE    embl accession CAAJ01004379.1
KEYWORDS    .
SOURCE      Plasmodium chabaudi
  ORGANISM  Plasmodium chabaudi
            Eukaryota; Alveolata; Apicomplexa; Haemosporida; Plasmodium.
REFERENCE   1
  AUTHORS   Hall,N., Karras,M., Raine,J.D., Carlton,J.M., Kooij,T.W.A.,
            Berriman,M., Florens,L., Janssen,C.S., Pain,A., Christophides,G.K.,
            James,K., Rutherford,K., Harris,B., Harris,D., Churcher,C.,
            Quail,M.A., Ormond,D., Doggett,J., Trueman,H.E., Mendoza,J.,
            Bidwell,S.L., Rajandream,M.A., Carucci,D.J., Yates,J.R.,
            Kafatos,F.C., Janse,C.J., Barrell,B., Turner,C.M.R., Waters,A.P.
            and Sinden,R.S.
  TITLE     A global survey of the molecular life-strategies of malaria
            parasites by integrated genomic, transcriptomic and proteomic
            analyses of both vertebrate and mosquito stages
  JOURNAL   Unpublished
REFERENCE   2  (residues 1 to 251)
  AUTHORS   Hall,N.
  TITLE     Direct Submission
  JOURNAL   Submitted (01-SEP-2004) Pathogen Sequencing Unit, The Sanger
            Institute, The Wellcome Trust Genome Campus, Cambridge CB10 1SA
COMMENT     THIS IS A LOW COVERAGE GENOME ASSEMBLY CONTAINING AUTOMATED
            ANNOTATION THERE MAY BE MISTAKES IN THE ANNOTATION AS WELL AS THE
            UNDERLYING SEQUENCE AND ASSEMBLY. MANY GENE PREDICTIOS ARE PARTIAL
            AND WILL NOT CONTAIN START OR STOP CODONS. SOME GENE PREDICTIONS
            HAVE NON-STANDARD SPLICING OR IN FRAME STOP CODONS.THE SEQUENCE HAS
            BEEN RELEASED AND PUBLISHED IN THIS CONDITION BY THE SANGER
            INSTITUTE FOR THE BENEFIT OF THE SCIENTIFIC COMMUNITY. THE SANGER
            INSTITUTE IS COMMITTED TO TRYING TO OBTAIN FUNDS TO COMPLETE THE
            GENOME AND CURATE THE ANNOTATION.
FEATURES             Location/Qualifiers
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     Protein         1..251
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     Region          41..>157
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                     transport and metabolism]; COG0461"
                     /db_xref="CDD:30809"
     CDS             1..251
                     /locus_tag="PC000646.04.0"
                     /coded_by="CAAJ01004379.1:2702..3457"
ORIGIN
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       61 fvlkskktsh yyvstgflnn aissnivsfl isnlilsknl sfdylfgasy kgipivtlts
      121 hfllntnkfh nvfylydrke kkdygdasvi ignleenhig saqvekktdk kkvividdvf
      181 sygtaltdif nkikafdyle ivacivilnr neheinekne kiyfkdkfeq khnipvysvi
      241 synddishli k
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BIOGRAPHY

Miss Sawirasagee Pongsabut was born on April 1,1983 in Pathumthani, Thailand. She received her Bachelor degree of Science (Medical Technology) in 2004 from Department of Medical Technology, Faculty of Allied Health Science, Chulalongkorn University, Bangkok Thailand with the second honour degree. She was an apprentince at Bangkok Metropolitan Administration General Hospital and Nonthavej Hospital. She has enrolled Chulalongkorn University in graduated program for Master degree of Science (Medical Biochemistry) since 2005.