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PUMS 99:1

# EXPRESSION AND PURIFICATION OF A PROTEIN KINASE, PknL OF Mycobacterium bovis BCG Pasteur 1173P2

#### LIN LI SHIN

# THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR BACHELOR OF SCIENCE WITH HONOURS

# BIOTECHNOLOGY SCHOOL OF SCIENCE AND TECHNOLOGY UNIVERSITI MALAYSIA SABAH

2005



# DECLARATION

I certify that this thesis does not incorporate any material previously submitted for any bachelor degree in any university, without acknowledgement; and that it does not contain any material previously published where due reference is not made in the text.

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linlishin.

LIN LI SHIN HS2002-3060



#### **ENDORSED BY**

Signature

1. SUPERVISOR

ble (DR. LEE PING CHIN)

2. EXAMINER 1

(DR. ROZIAH HJ. KAMBOL)

3. EXAMINER 2

(MS. TEOH PEIK LIN)

4. EXAMINER 3

(DR. WONG NYET KUI)

5. DEAN

(PROF. MADYA DR. AMRAN AHMED)

CALV July



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#### ABSTRACT

Signal transduction pathways in both eukaryotes and prokaryotes involve specific protein kinases coupled to protein phosphatase. Genome sequence of Mycobacterium tuberculosis H37Rv revealed the existence of eleven eukaryotic Ser/Thr protein kinases (STPK). In this project, pknL, which is one of STPK of Mycobacterium bovis BCG Pasteur 1173P2 was inserted into expression vector, pET-16b which was then transformed into expression host, E.coli BL21 (DE3) pLysS. IPTG was used to induce pknL gene expression followed by recombinant protein purification with Ni-NTA Spin Kit. The molecular weight of purified recombinant PknL was determined using SDS-PAGE. Recombinant PknL has molecular weight of 45 kDa. Most of recombinant protein was purified in first elution while remaining proteins purified in second elution. Full amino acids sequence of *pknL* obtained from database was subjected to bioinformatics analysis. Analysis of the amino acid sequence of PknL with Dense Alignment Surface (DAS) and PHD predictions indicated the presence of transmembrane domain at amino acid 369 - 389 within the helical C-terminal of PknL. Protein kinase domain was found at amino acid 27 - 286 at the N-terminal and conserved kinase domain was showed to be 99% similar to corresponding kinases in Mycobacterium tuberculosis H37Rv and Mycobacterium bovis AF2122/97. Further analysis revealed that the secondary structure of PknL comprised mainly of helices and loops with a helices transmembrane structure and loopy structure at Ser/Thr protein kinase active site. These finding showed that PknL is a membrane spanning protein in Mycobacterium bovis BCG Pasteur 1173P2.



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#### ABSTRAK

Lintasan transduksi isyarat dalam prokariot dan eukariot melibatkan protein kinase khusus berpasangan dengan protein fosfatase. Jujukan genom Mycobacterium tuberculosis H37Ry menunjukkan kewujudan sebelas eukariotik Ser/Thr protein kinase (STPK). Dalam projek ini, pknL, yang merupakan salah satu STPK dalam Mycobacterium bovis BCG Pasteur 1173P2 telah dimasukkan ke dalam vektor pengekspresan, pET-16b dan kemudiannya ditransformasikan ke dalam hos pengekspresan, E. coli BL21 (DE3) pLysS. IPTG digunakan untuk merangsang ekspresi gen pknL seterusnya rekombinan protein ditulenkan dengan Ni-NTA Spin Kit. Berat molekul rekombinan PknL ditentukan dengan SDS-PAGE. Rekombinan PknL memiliki berat molekul sebanyak 45kDa. Kebanyakan rekombinan protein ditulenkan dalam elusi pertama dan selebihnya dalam elusi kedua. Jujukan amino asid penuh PknL dari pangkalan data dianalisis dengan analisis bioinformatik. Analisis jujukan amino asid PknL dengan Dense Alignment Surface (DAS) and PHD predictions menunjukkan kehadiran domain "transmembrane" pada amino asid 369 - 389 dalam helikal C-termini PknL. Domain protein kinase telah dijumpai pada amino asid 27 - 286 di N-termini dan "conserved" domain kinase menunjukkan persamaan sebanyak 99% dengan protein kinase yang sama dalam Mycobacterium tuberculosis H37Rv and Mycobacterium bovis AF2122/97. Analisis seterusnya menunjukkan bahawa struktur sekunder PknL adalah heliks dan gelung dengan struktur helical "transmembrane" dan struktur gelung pada tapak aktif Ser/Thr kinase. Ini menunjukkan PknL adalah protein merentangi membran sel di dalam Mycobacterium bovis BCG Pasteur 1173P2.



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# LIST OF SYMBOLS

М	Molar
mM	Milimolar
mm	Milimeter
cm	Centimeter
μg	Microgram
mg	Miligram
g	Gram
ng	Nanogram
μL	Microliter
mL	Mililiter
L	Liter
rpm	Revolutions per minute
v	Volt
°C	Degree Celsius
%	Percentage
[]	Concentration
bp	Base pair
kb	Kilo base
α	Alpha
β	Beta
dH <sub>2</sub> O	Distilled water



DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
EDTA	Ethylenediamidetetraacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TE	Tris-EDTA
RNase A	Ribonuclease A
TAE	Tris acetate EDTA buffer
TBE	Tris borate EDTA buffer
UV	Ultraviolet
EtBr	Ethidium bromide
PCR	Polymerase chain reaction
APS	Ammonium persulfate
DMSO	Dimethyl sulfoxide
LB	Luria-Bertani
IPTG	Isopropyl-
Ni-NTA	Nickel-nitrilotriacetic acid
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
STPK	Serine/threonine protein kinase
STPP	Serine/threonine protein phosphatase



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# **CHAPTER 1**

#### INTRODUCTION

#### **1.1 Introduction**

Tuberculosis is the leading cause of mortality worldwide with 3 million death reported annually with 1.7 billion people (one-third of the world population) are estimated to harbor latent *Mycobacterium tuberculosis* infection (Parrish *et al.*, 1998; Manabe and Bishai, 2000). Even with the available of well developed diagnostic test, antibiotics and BCG vaccine, possible eradication of tuberculosis from human kind remains as elusive as ever. The rising number of deaths is mainly caused by the emergence of drug resistant strains and concurrent Human Immunodeficiency Virus (HIV) epidemic.

The causative agent of human tuberculosis is *Mycobacterium tuberculosis*, an obligate aerobic bacterium which requires oxygen to survive. However, *Mycobacterium tuberculosis* can adapt to microaerophilic environment in granuloma where they continue to survive by entering into a prolonged dormant state. One of the proposed mechanisms



that mycobacteria employ during its survival in macrophage and persistence in host involves the protein phosphorylation mechanism.

Signal transduction in eukaryotes and prokaryotes involves protein phosphorylation where extracellular signals are translated into cellular responses catalyze by specific protein kinases coupled to protein phosphatase. In prokaryote, phosphorylation involves mainly the two-component system while Hanks-type protein kinases are prominent in eukaryotes and thought to be exclusive only to eukaryotes. However, studies based on a combination of the polymerase chain reaction (PCR), X-ray crystallography and genomic sequencing had shown that Serine/Threonine protein kinases (STPKs) are present in bacteria. STPKs in bacteria have been shown to be involved in three different processes namely regulation of development, stress responses and pathogenicity (Av-Gay and Everett, 2000).

In recent year, genome sequence of *Mycobacterium tuberculosis* H37Rv revealed the existence of eleven putative eukaryotic Ser/Thr protein kinases (STPK). Since then, numerous studies have been carried out to investigate the biochemical characteristic and functional roles of *Mycobacterium tuberculosis* STPKs, especially in the pathogen-host relationship. This project focuses on one of these STPKs that is the PknL. PknL is chosen because it has neither been characterized nor localized yet in *Mycobacterium tuberculosis, Myocobacterium bovis* and *Myocobacterium bovis* BCG. However, because of the highly pathogenic nature of the *Mycobacterium tuberculosis, Myocobacterium bovis* BCG Pasteur 1173P2 is used instead as it is the attenuated strain and genome



sequence analysis showed a very high homology, more than 99.9 % identity at DNA level with *Mycobacterium tuberculosis*.

In this project, the main focus is to clone and transform the plasmid with *pknL* insert into expression host for protein expression follow by protein purification. This work may assist in the subsequent study of the characteristic and functional roles of STPKs in *Mycobacterium tuberculosis*.

#### **1.2 Research Objectives**

The objectives of this research are as following:

- To clone the *pknL* gene from pUC19 (cloning vector) into pET16 (expression vector).
- To transform the expression vector carrying *pknL* into *E.coli* BL21 (DE3) pLysS (expression host).
- To induce protein expression of PknL in *E.coli* BL21 (DE3) pLysS (expression host).
- 4) To purify the PknL protein expressed.



- 5) To verify molecular weight of PknL.
- 6) To perform bioinformatics analysis in order to facilitate purification of PknL.



# **CHAPTER 2**

#### LITERATURE REVIEW

# 2.1 Mycobacterium tuberculosis: Etiologic Agent of Tuberculosis

Mycobacterium tuberculosis is a slender, straight, or slightly curved rod with rounded ends. The organisms vary in width from 0.3 to 0.6 µm and in length from 1 to 4 µm. The bacilli are obligate aerobe, acid-fast, nonsporogenous, slow growing and caused Tuberculosis (TB) in humans. The name "tuberculosis" is derived from the formation by the body of characteristic cellular structures called tubercles, in which the bacilli are trapped and walled off (Glickman and Jacobs, 2001).

Tuberculosis infection is acquired primarily by the inhalation of dried droplets containing tubercle bacilli that have been expelled into the air by coughing, sneezing or talking. These droplet nuclei remain suspended in the air for prolonged periods and particles with a diameter of 1 to  $5\mu$ m are sufficiently to reach the alveoli and initiate infection. At this primary infection stage, healthy individual with strong immune system



will be able to kill the bacilli in the lung from developing into further threat. But this may not be the case for certain individual as once in the lung, the bacilli are phagocytes by alveolar macrophages and reside in a specialized phagosome, which traffics along the endosomal-lysosomal pathway. Instead of destroying tubercle bacillus, the macrophages provide a supportive environment for the replication of *M. tuberculosis* (Schluger and Rom, 1998). The bacterium survives and multiplies within the macrophage using a variety of mechanisms including inhibition of phagosome-lysosome fusion, inhibition of the acidification of phagosomes, resistances to killing by reactive oxygen intermediates and reactive nitrogen intermediates, recruitment and retention of trytophan/aspartatecontaining coat protein (TACO) (Basu, 2004; Dubnau and Smith, 2003; McDonough *et al.*, 1993).

At 4 to 6 weeks after the initial contact, cell-mediated immunity and hypersensitivity to tuberculin will develop and the classic lesions, tubercles will develop at the site of bacterial multiplication. Activated macrophage by immunocompetent lymphocytes will surround the infected area and with their increase activity against bacteria ingested, bacteria multiplication became slow or stops (Glickman and Jacobs, 2001). The tubercule is a granuloma consists of some multinucleated giant cells formed by fusion of several macrophages, surrounded by activated macrophages and lymphocytes. Often, there is caseous necrosis with semisolid and cheesy characteristic in the center of the granuloma. This caseation necrosis is the characteristic hallmark of tuberculosis. The lesion caused by caseation necrosis may later calcify and referred as the Ghon complex (Bentrup and Russell, 2001).



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In the majority of the patients (about 90 %), the primary lesion heals completely, leaving no clinical evidence of prior infection other than hypersensivity to tuberculin at which this stage is known as the latent tuberculosis. Reactivation of infection may occur when the host becomes immunnocompromised due to aging or immunodeficiency diseases such as acquired immune deficiency syndrome (AIDS). In some patients, however, the primary infection progress directly and spread to other organ of the body leading to chronic pulmonary tuberculosis. At this late-stage disease, serious cough occurs and bacteria are transmitted to others through aerosol transmission.

# 2.2 Latency of Mycobacterium tuberculosis

In latent tuberculosis, *M. tuberculosis* continues to survive by enters into a pro-longed dormant state inside the tubercle lesions. The physiological state in which *M. tuberculosis* survives in lesions is unknown. In the literature, this stage of the disease has been referred to as latency or dormancy. Dormancy has also been used to describe the physiological state in which the bacteria exist. In bacteria physiology, the term dormancy is defined as a reversible state of low metabolic activity, in which cells can persist for extended periods without division (Smeulders *et al.*, 1998).

The entry of a bacterial population into stationary phase can be affected by several ways, including accumulation of toxic by-products and environment stresses such as low temperatures, acidicity and high osmorality. Other factor such as nutrient limitation has also been linked to the survival of persisting mycobacterium. However, the exact limiting



nutrient that keeps *M. tuberculosis* in stationary phase during the latent phase of the disease remains to be elucidated (Nyka, 1974).

Wayne has proposed that within calcified lung lesions, oxygen is the growthlimiting factor and has developed an oxygen-limited model for mycobacterial persistence (Wayne, 1994). Together with co-worker, Wayne showed that although mycobacteria are obligate aerobes, cultures can still survive stationary or microaerophilic phase by upregulating enzymes (isocitrate lyase and malate synthase) involved in the glyoxylate shunt. Consequently, the glyoxylate bypass allows *M. tuberculosis* to synthesize carbohydrates from fatty acids, as well as supplying intermediates to support the Krebs cycle. Low oxygen tension had also been shown to result in cell wall thickening during the stationary phase of mycobacteria (Yuan *et al.*, 1996). Therefore, it has been suggested that reduced oxygen tension might trigger latency and lead to the controlled shutdown of active gene.

A number of genes that are expressed or required during the mycobacterial persistence have been identified. The products of these genes are divided into three basic categories: respiratory proteins, stress-response and general metabolic proteins and proteins involved in fatty acids metabolism (Bentrup and Russell, 2001; Kinger and Tyagi, 1993). The regulation of gene expression during mycobacterial persistence remains to be unknown.



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