CLONING, EXPRESSION AND CHARACTERIZATION OF SERINE/THREONINE PROTEIN PHOSPHATASE AND KINASES OF *Mycobacterium bovis* BCG (Pasteur 1173P2)

AINOL AZIFA BT HJ MOHD FAIK

PERPUSTAKAAN
UNIVERSITI MALAYSIA SABAH

UMS
UNIVERSITI MALAYSIA SABAH

THESIS SUBMITTED IN PARTIAL FULFILLMENT FOR THE DEGREE OF MASTER OF SCIENCE

SCHOOL OF SCIENCE AND TECHNOLOGY
UNIVERSITI MALAYSIA SABAH
2007

PUMS 99:1

UNIVERSITI MALAYSIA SABAH

BORANG PENGESAHAN STATUS TESIS @

JUDUL: Cloning, Expression and Characterization of Serine/Threonine Protein Phosphatase and Kinases of *Mycobacterium bovis* BCG (Pasteur 1173P2)

SARJANA: Sarjana Sains (Bioteknologi)

SESI PENGAJIAN: 2002 - 2007

PERPUSTAKAAN
UNIVERSITI MALAYSIA SABAH

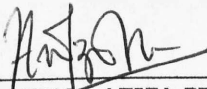
Saya, AINOL AZIFA BT MOHD FAIK mengaku membenarkan tesis Sarjana ini disimpan di Perpustakaan Universiti Malaysia dengan syarat-syarat kegunaan seperti berikut:

1. Tesis adalah hakmilik Universiti Malaysia Sabah.
2. Perpustakaan Universiti Malaysia Sabah dibenarkan membuat salinan untuk tujuan pengajian sahaja.
3. Perpustakaan dibenarkan membuat salinan tesis ini sebagai bahan pertukaran antara institusi pengajian tinggi.
4. TIDAK TERHAD

UMS
UNIVERSITI MALAYSIA SABAH

Disahkan oleh

ANITA BINTI ARSAD
PUSTAKAWAN KANAN
UNIVERSITI MALAYSIA SABAH



(Penulis: AINOL AZIFA BT MOHD FAIK)

(TANDATANGAN PUSTAKAWAN)

Alamat Tetap:

(Penyelia: DR. LEE PING CHIN)

Tarikh: 22 Februari 2008

Tarikh: 30/6/2008

CATATAN: @ tesis dimaksudkan sebagai tesis bagi Ijazah Doktor Falsafah dan Sarjana secara penyelidikan, atau disertasi bagi pengajian secara kerja kursus dan penyelidikan, atau Laporan Projek Sarjana Muda (LPSM).

DECLARATION

The materials in this thesis are original except for quotations, excerpts, summaries and references, which have been duly acknowledged.

22 February 2008

Ainol Azifa Bt Hj Mohd Faik

PS2002-001-493

PERPUSTAKAAN
UNIVERSITI MALAYSIA SABAH

UMS
UNIVERSITI MALAYSIA SABAH

ACKNOWLEDGEMENTS

In the name of Allah, Most Gracious, Most Merciful

Praise and thanks must be first given to Allah for giving me health, patience, and knowledge to complete this study. I would like to express my utmost gratitude to my supervisor Dr. Lee Ping Chin whose guidance, stimulating suggestions and encouragement helped me to complete the writing of this thesis as well as helping me understand the challenging research that lies behind it.

I am deeply indebted to Sanger Institute in Paris and Sabah Health Department for contributing the two important materials that were used in this study; the *Mycobacterium bovis* BCG (Pasteur 1173P2) strain and Lowenstein-Jensen media.

I would also like to acknowledge all the lecturers from the Biotechnology programme, particularly, Prof. Dr. Ho Coy Choke, Dr. Jualang Azlan Gansau, Dr. Zaleha Aziz and Dr. Roziah Kambol, lab assistants and members from the Tissue Culture, Animal Physiology, Biochemical, Biotechnology Research and Postgraduate laboratories for their help and support. I want to thank my colleagues Hartinie Marbawi, Glenda Wong See Sing, Adrian, Awang, Foo Sek Hin, Puah Seok Hwa, Ong Si Mon, Hew Chaw Sen and Ho Wei Loon for their assistance, moral support, interesting discussions and friendship. Last but not least, I owe my deepest gratitude to my parents, Allahyarham Hj. Mohd Faik and Hjh. Norain Abdullah, my siblings, Kak Long, Adli, Syukri and Adik, my husband, Awang Muhd. Sagaf and his family for their infinite patience, understanding, encouragement and unconditional support during the years I have been working with this thesis. Their love has accompanied me all the way in my long struggle and has pulled me through so many hurdles.

ABSTRACT

CLONING, EXPRESSION AND CHARACTERIZATION OF SERINE/THREONINE PROTEIN PHOSPHATASE AND KINASES OF *Mycobacterium bovis* BCG (Pasteur 1173P2)

Pathogenesis of most bacteria is connected to its survival within the host by adaptive regulation of gene expression in response to alterations of the environment. Before completion of the bacterial genome sequencing data, it was thought that protein phosphorylation/dephosphorylation only involves the so-called two-component system consisting of histidine kinase sensors and their associated response regulators. Recent evidence revealed that some prokaryotes contain protein kinases and phosphatases. In this study, three genes with sequence homology to those encoding serine/threonine kinases (pknI, pknK) and serine/threonine phosphatase (ppp) in Mycobacterium tuberculosis $H_{37}Rv$ were cloned from a less pathogenic bacteria, Mycobacterium bovis BCG (Pasteur 1173P2) obtained from Pasteur Institute. The pknI and ppp genes were proposed to be involved in regulation of cell division and elongation while pknK gene might regulate the production of secondary metabolite in Mycobacterium. Amplified ppp, pknI and pknK genes were cloned and expressed as a recombinant proteins in pTrcHis and pET42-a(+). The calculated molecular masses of these proteins designated as Ppp, PknI and PknK were 58.8 kDa, 94.4 kDa and 150.3 kDa respectively. Bioinformatics tools have suggested that PknI and PknK contain 12 Hanks kinase motifs in contrast with Ppp which has 11 motifs that are universally conserved and characteristic of PP2C phosphatases. In addition, PknI and Ppp also revealed the presence of a transmembrane region predicting the location of these proteins in mycobacterial cells. Ppp and PknI were expressed predominantly as inclusion bodies while PknK was found to have partial solubility. Therefore, these proteins were purified as inclusion bodies, solubilized using high concentration of urea and refolded using dialysis by decreasing the urea concentration gradually. Protein concentrations of Ppp, PknI and PknK obtained after refolding were 0.110 mg/mL, 0.246 mg/mL and 0.463mg/mL respectively. Ppp was strictly dependent on Mn^{2+} in vitro and the activity was highest at 55°C. Ppp was not inhibited by okadaic acid, sodium orthovanadate and low concentration of EDTA and NaF but showed a substantially decreased activity when incubated at high concentration of EDTA and NaF. Km and Vmax values of the phosphatase activity using pNPP as a substrate and a fixed amount of Ppp were determined as 0.83 $\pm$ 0.07 mM and 1.49 $\pm$ 0.02 nmol/min/µg respectively. Kinetic analysis of the phosphatase activity of fixed amount of Ppp using threonine phosphopeptide resulted in a Km value of 1.34 $\pm$ 0.704 mM and a Vmax value of 0.206 $\pm$ 0.075 nmol/min/µg. These results show that the Ppp enzyme was biologically active and successfully refolded.

ABSTRAK

Patogenesis kebanyakan bakteria bergantung kepada kebolehan bakteria hidup di dalam perumah dengan beradaptasi terhadap regulasi ekspresi gen yang bertindakbalas terhadap perubahan persekitaran. Sebelum data jujukan genom bakteria lengkap, dipercayai bahawa protein fosforilasi/defosforilasi hanya melibatkan sistem dua-komponen yang terdiri daripada sensor histidine kinase dan regulator respon masing-masing. Terkini, bukti telah menunjukkan bahawa beberapa prokariot turut mengandungi protein kinase dan fosfatase. Dalam kajian ini, tiga gen dengan homologi jujukan yang sama dengan gen yang mengkodkan serine/threonine kinase (*pknI*, *pknK*) dan serine/threonine fosfatase (*ppp*) dalam *Mycobacterium tuberculosis* $H_{37}Rv$ telah diklonkan daripada bakteria yang kurang patogen iaitu *Mycobacterium bovis* BCG (Pasteur 1173P2) yang diperolehi daripada Institut Pasteur. Gen *pknI* dan *ppp* dipercayai terlibat di dalam regulasi sel dan pemanjangan sementara gen *pknK* mungkin meregulasi penghasilan metabolit sekunder di dalam *Mycobacterium*. Gen *ppp*, *pknI* dan *pknK* yang telah diamplifikasi, telah diklon dan diekspres sebagai protein rekombinan di dalam vector pTrcHis dan pET42-a(+). Jisim molekul yang dianggarkan bagi protein Ppp, PknI dan PknK adalah 58.8 kDa, 94.4 kDa dan 150.3 kDa. Analisis bioinformatik telah mencadangkan bahawa PknI dan PknK mengandungi 12 motif Hanks kinase berbanding Ppp yang mengandungi 11 motif yang merupakan ciri bagi fosfatase jenis PP2C. PknI dan Ppp juga menunjukkan kehadiran kawasan transmembran yang menentukan lokasi protein tersebut di dalam sel mikobakteria. Ppp dan PknI diekspres secara dominan sebagai jasad inklusi sementara PknK mempunyai keterlarutan separa. Oleh itu ketiga-tiga protein ditulenkan sebagai jasad inklusi, dilarutkan menggunakan kepekatan urea yang tinggi dan dilipat semula menggunakan kaedah dialisis sambil mengurangkan kepekatan urea secara beransur-ansur. Kepekatan protein yang diperolehi bagi Ppp, PknI dan PknK selepas proses pelipatan semula adalah 0.110 mg/mL, 0.246 mg/mL dan 0.463 mg/mL. Ppp bergantung kepada ion Mn^{2+} untuk aktif secara in vitro dan aktiviti Ppp adalah paling tinggi pada suhu 55°C. Ppp tidak dinyahaktif oleh asid okadaik, sodium ortovanadate dan pada kepekatan rendah EDTA serta NaF tetapi aktivitinya menurun apabila diinkubasi dalam EDTA dan NaF dengan kepekatan tinggi. Nilai Km dan Vmax bagi aktiviti fosfatase menggunakan pNPP dan threonin fosfopeptida sebagai substrat pada suatu amaun Ppp, adalah 0.83 $\pm$ 0.07 mM dan 1.49 $\pm$ 0.02 nmol/min/µg serta 1.34 $\pm$ 0.704 mM dan 0.206 $\pm$ 0.075 nmol/min/µg. Keputusan-keputusan ini menunjukkan bahawa enzim Ppp adalah aktif secara biologikal dan berjaya dilipat semula.

TABLE OF CONTENTS

		PAGE
TITLE		i
DECLARATION		ii
ACKNOWLEDGEMENTS		iii
ABSTRACT		iv
ABSTRAK		v
TABLE OF CONTENTS		vi
LIST OF FIGURES		xiv
LIST OF TABLES		xx
LIST OF ABBREVIATIONS		xxi
LIST OF SYMBOLS		xxiv
CONTENT		
CHAPTER 1	**INTRODUCTION**	1
CHAPTER 2	**LITERATURE REVIEW**	5
2.1	Introduction to the *Mycobacterium* Genus	5
2.1.1	Mycobacteria: Past, Present and Future	5
2.1.2	Classification of Mycobacteria	6
2.1.3	Morphology, Identification and Cell Wall Constituents of *Mycobacterium*	8
2.2	Comparative Genomics of *M. tuberculosis* Complex	11
2.2.1	*M. tuberculosis* complex	11
2.2.2	Methods for Comparative Genomics Studies	11
2.2.3	*M. tuberculosis* H37Rv, *M. bovis* and *M. bovis* BCG Pasteur	13
2.3	Tuberculosis	16
2.3.1	*Mycobacterium tuberculosis* Infectious Process	19

UMS
UNIVERSITI MALAYSIA SABAH

PAGE

2.3.2 Mycobacterial Survival within the Host 21

2.4 Signal Transduction via Protein Phosphorylation 23

2.4.1 Protein Phosphorylation-Dephosphorylation 24

2.4.2 Classification of Protein Kinases 24

2.4.3 Classification of Protein Phosphatases 25

2.5 Phosphorylating Systems in Eukaryotes 29

2.6 Phosphorylating Systems in Prokaryotes 32

2.7 Eukaryotic-like Serine/Threonine Protein Kinases and Phosphatase in *Mycobacterium tuberculosis* 36

2.8 Updates of Findings on Pstp, PknI and Pknk Proteins in *Mycobacterium tuberculosis* Homologous to *Mycobacterium bovis* BCG (Pasteur 1173P2) 37

2.8.1 PstP 37

2.8.2 PknI 38

2.8.3 PknK 39

CHAPTER 3 MATERIALS AND METHODS 40

3.1 Cultivation of *Mycobacterium bovis* BCG (Pasteur 1173P2) on Lowenstein-Jensen Media 40

3.2 Acid-Fast Staining of *Mycobacterium bovis* BCG (Pasteur 1173P2) 41

3.3 High Molecular Weight DNA Extraction of *Mycobacterium bovis* BCG (Pasteur 1173P2) by Salting Out Method 43

3.4 Amplification of *ppp, pknI* and *pknK* Genes by Polymerase Chain Reaction (PCR) 44

UMS UNIVERSITI MALAYSIA SABAH

PAGE

3.5 Construction of Recombinant Plasmid, pUC19 Containing *ppp, pknI* and *pknK* Genes 46

3.5.1 Restriction Enzymes Digestion of *ppp, pknI, pknK* Genes and Vector pUC19 46

3.5.2 Extraction of Digested DNA from Agarose Gel using Qiaquick Gel Extraction Kit 47

3.5.3 Ligation of *ppp, pknI* and *pknK* Genes with Vector pUC19 48

3.6 Transformation of pUC19-*ppp*, pUC19-*pknI* and pUC19-*pknK* into Competent Cells of *E. coli* JM109 49

3.6.1 Preparation of *E.coli* JM109 Competent Cells using Inoue Method 49

3.6.2 Transformation of Recombinant Plasmid in *E. coli* JM109 Competent Cells 50

3.7 Screening of pUC19 Transformants Harboring *ppp*, *pknI* and *pknK* Inserts 50

3.7.1 Plasmid Miniprep using Alkaline Lysis Method 50

3.7.2 Restriction Enzymes Digestion to Confirmed Plasmids Harboring Inserts 51

3.8 DNA Sequencing of Inserts using CEQ 2000XL 52

3.8.1 Purification and Precipitation of DNA 52

3.8.2 CEQ Dye Terminator Cycle Sequencing using Quick Start Kit 52

3.9 Bioinformatics Analysis of Retrieved DNA Sequences 54

3.10 Construction of Plasmid Expression Vector for Expressing Ppp, PknI and PknK Proteins 57

UMS
UNIVERSITI MALAYSIA SABAH

PAGE

3.10.1 Restriction Enzymes Digestion of pUC19 Harboring Positive Inserts and their Expression Plasmid, pTrcHis TOPO and pET42-a(+) 57

3.10.2 Ligation of *ppp, pknI* and *pknK* Genes into Expression Vector 58

3.11 Transformation of pTrcHis-p*pp,* pET42a(+)-p*knI* and pET42a(+)-*pknK* into their Respective Hosts, *E. coli* Top 10 or *E. coli* BL21 (DE3) 59

3.12 Screening of Expression Vectors Harboring *ppp*, *pknI* and *pknK* Inserts 60

3.13 Sequencing of N-terminal of pTrcHis-*ppp*, pET42a(+)-*pknI* and pET 42a(+)-*pknK* Recombinant Plasmid 60

3.14 Protein Expression, Analysis of Expression Level and Solubility of Ppp, PknI and PknK Proteins 61

3.14.1 Culture Growth of Ppp, PknI and PknK Proteins 61

3.14.2 Determination of Target Protein Solubility 62

3.15 Isolation, Purification, Refolding and Quantification of Ppp, PknI and PknK Proteins 63

3.15.1 Isolation and Purification of Ppp, PknI and PknK Proteins as Inclusion Bodies 63

3.15.2 Refolding of Ppp, PknI and PknK Proteins using Decreasing Urea Concentration 64

3.15.3 Determination of Ppp, PknI and PknK Refolded Proteins Concentrations using Bradford Method 65

3.16 Protein Phosphatase Assay 65

3.17 Protein Kinase Assay 67

UMS UNIVERSITI MALAYSIA SABAH

PAGE

CHAPTER 4 RESULTS 69

4.1 Culturing, Acid-Fast Staining and Extracting Genomic DNA of *Mycobacterium bovis* BCG (Pasteur 1173P2) 69

4.1.1 Cultivation of *M. bovis* BCG (Pasteur 1173P2) on Lowenstein-Jensen Medium (LJ medium) 69

4.1.2 Ziehl-Neelsen Staining to Identify Acid-Fast Bacillus 70

4.1.3 Genomic DNA Extraction of *M. bovis* BCG (Pasteur 1173P2) 71

4.2 Result for PCR Amplification of *M. bovis* BCG (Pasteur 1173P2) *ppp, pknI* and p*knK* Genes 72

4.3 Purification and Identification of Plasmid pUC19 Harboring *ppp*, *pknI* and *pknK* Genes 73

4.4 Sequencing Result for *ppp, pknI* and *pknK* Genes 75

4.4.1 Edited DNA Sequences of *ppp* Gene 75

4.4.2 Edited DNA Sequences of *pknI* Gene 76

4.4.3 Edited DNA Sequences of *pknK* Gene 76

4.5 The DNA Sequences Aligned with the Complete Genome Database of *M. bovis* BCG (Pasteur 1173P2) 78

4.5.1 The *ppp* DNA Sequences Aligned with the *M. bovis* BCG (Pasteur 1173P2) Genome Database. 78

4.5.2 The *pknI* DNA Sequences Aligned with the *M. bovis* BCG (Pasteur 1173P2) Genome Database. 80

4.5.3 The *pknK* DNA Sequences Aligned with the *M. bovis* BCG (Pasteur 1173P2) Genome Database. 82

4.6 BLAST Search Result for *ppp, pknI* and *pknK* Genes 85

4.6.1 BLAST Search Result from NCBI Server for *ppp* 85

UMS UNIVERSITI MALAYSIA SABAH

PAGE

4.6.2 BLAST Search Result from NCBI Server for *pknI* 87

4.6.3 BLAST Search Result from NCBI Server for *pknK* 88

4.7 Genbank Accession Number 90

4.8 *In Silico* Analysis of *ppp, pknI* and *pknK* Genes 90

4.8.1 Analyzing DNA Sequences of the *ppp, pknI* and *pknK* Genes 90

4.8.2 Primary Structure Analysis 95

4.8.3 Multiple Sequence Alignment 100

4.8.4 Phylogenetic Tree Analysis 106

4.8.5 Secondary and 3D-Structure Prediction 109

4.9 Cloning of *ppp, pknI* and *pknK* Genes into Expression Vector 116

4.10 Expression of the Recombinant Proteins 117

4.10.1 Analysis of Protein Expression and Solubility of Ppp Protein 117

4.10.2 Analysis of Protein Expression and Solubility of PknI 118

4.10.3 Analysis of Protein Expression and Solubility of PknI 120

4.11 Purification and Refolding of Recombinant Ppp, PknI and PknK Proteins 121

4.12 Determination of Protein Concentration for Ppp, PknI and PknK After Refolding 123

4.13 Biochemical Characterization of Ppp by Phosphatase Assay 125

4.13.1 Effect of pH, Cations and Temperature towards Ppp Activity using pNPP as Substrate 126

4.13.2 Effect of Various Inhibitors on Ppp Activity using pNPP as a Substrate 129

4.13.3 Determination of Enzyme Kinetics of Ppp using pNPP and Threonine Phosphopeptide as Substrate 130

UMS UNIVERSITI MALAYSIA SABAH

PAGE

4.14 Kinase Assay Result 134

CHAPTER 5 DISCUSSION 135

5.1 *Mycobacterium bovis* BCG (Pasteur 1173P2) Culture and Acid-Fast Staining 135

5.2 High Molecular Weight DNA Extraction of *M. bovis* BCG (Pasteur 1173P2) 137

5.3 PCR Products and Plasmid pUC19 Harboring *ppp*, *pknI* and *pknK* Genes of *M. bovis* BCG (Pasteur 1173P2) 137

5.4 Bioinformatics and Mechanistic Aspects of Ppp 138

5.5 Bioinformatics and Mechanistic Aspects of PknI 141

5.5.1 Conserved Protein Kinase Subdomain: Mechanistic Aspect 141

5.6 Bioinformatics and Mechanistic Aspects of PknK 145

5.6.1 Conserved Protein Kinase Subdomain of PknK: Mechanistic Aspect 145

5.6.2 Motifs in the C-terminal Region of PknK 147

5.7 Cloning and Expression of the Ppp, PknI And PknK Recombinant Proteins 150

5.8 Purification and Refolding of Ppp, PknI And PknK Proteins 151

5.9 Enzymatic Properties of Ppp 152

5.10 Kinase Assay 155

5.11 Possible Role of Ppp 155

5.12 Functional Prediction of PknI 157

5.13 Functional Prediction of PknK 159

CHAPTER 6 CONCLUSION 160

REFERENCES 164

UMS UNIVERSITI MALAYSIA SABAH

PAGE

Appendix 1	Circle Map and Multiple Cloning Sites for pUC19	189
Appendix 2	Circle Map and Vector Cloning Region of pTrcHis-TOPO	190
Appendix 3	Circle Map and Vector Cloning Region of pET42-a(+)	191
Appendix 4	Solutions and Media for Bacterial Culture	192
Appendix 5	Solutions for Ziehl-Neelsen Staining Procedure	193
Appendix 6	Solutions for DNA Gel Electrophoresis	194
Appendix 7	Solutions for Protein Gel Electrophoresis	195
Appendix 8	Solutions for Genomic DNA Extraction of *Mycobacterium bovis* BCG (Pasteur 1173P2) and General DNA Precipitation	197
Appendix 9	Solutions for Competent Cells Preparation and Plasmid Extraction using Alkaline Lysis Method	198
Appendix 10	Solutions for DNA Sequencing using CEQ 2000XL Machine	199
Appendix 11	Solutions for Inclusion Bodies Isolation	200
Appendix 12	Solutions used in Refolding Procedure	201
Appendix 13	Stock Solutions for Phosphatase Assay	203
Appendix 14	Concentration of BSA and their Corresponding Absorbance at 595 nm.	205
Appendix 15	Data of pNPP Hydrolysis Monitored at 405 nm over a Period of 150 Minutes	206
Appendix 16	Data of Ppp Activity Influenced by pH (A) and Temperature (B).	207
Appendix 17	Data of Ppp Activity Influenced by Different Cations (A) and Various Concentration of $MnCl_2$ (B)	208
Appendix 18	Data of Ppp Activity Measured by pNPP Hydrolysis at 405 nm in the Absence or Presence of Various Inhibitors	209
Appendix 19	Data of Potassium Dihydrogen Phosphate (KH_2PO_4) containing 0 to 1500 pmol Free Phosphate (A) for used as a Standard Curve (B).	210
Appendix 20	Data of Ppp Activity in Various Substrate Concentrations of pNPP (A) and Threonine Phosphopeptide (B).	211

UMS
UNIVERSITI MALAYSIA SABAH

LIST OF FIGURES

FIGURE		PAGE
2.1	Scientific classification of the *Mycobacterium* genus.	6
2.2	Mycobacterial speciation derived from a common ancestor *Mycobacterium*.	7
2.3	Mycobacterial, indicating the compact, wrinkled appearance of the colony.	9
2.4	Model of the mycobacterial cell wall consisting of the cytoplasmic membrane and the cell wall components (peptidoglycan, arabinogalactan, and mycolic acids).	10
2.5 (A)	Total TB cases in Malaysia and Sabah, 2004.	18
2.5 (B)	Proportion of TB cases among the states in Malaysia, 2004 (Dony, 2006).	18
2.6	Model of a granuloma.	20
2.7	Protein kinases catalyze the transfer of a phosphate group from ATP to the side chains of serine/threonine or tyrosine (protein-serine/threonine or tyrosine kinases) residues.	26
2.8	Activation of the ERK MAP kinases (Cooper, 1997).	30
2.9	Phosphorelay that governs osmoregulation in *S. cerevisiae*.	31
2.10	The basic two-component system consists of a sensor histidine kinase and a response regulator that are involved in the phosphotransfer reaction.	34
3.1	Culture of *M. bovis* BCG (Pasteur 1173P2) after 4 weeks.	41
3.2	Steps in acid-fast staining using Ziehl-Neelsen method	42
4.1	Black arrow showing a breadcrumb like colony of *M. bovis* BCG (Pasteur 1173P2) on Lowenstein-Jensen medium.	69
4.2 (A)	Acid-fast staining of *M. bovis* BCG (Pasteur 1173P2) using Ziehl-Neelsen procedure.	70
4.2 (B)	Acid-fast staining of *M. tuberculosis* using Ziehl-Neelsen procedure.	70
4.3	Agarose gel electrophoresis of DNA isolated from *M. bovis* BCG (Pasteur 1173P2) cells.	71

UNIVERSITI MALAYSIA SABAH

LIST OF FIGURES

FIGURE **PAGE**

4.4 PCR products of *ppp, pknI* and *pknK* genes of *M. bovis* BCG (Pasteur 1173P2) at approximately 1.5, 1.7 and 3.3 kb respectively. 72

4.5 Plasmid miniprep of potential plasmid harboring *ppp* digested with *Eco*R I and *Hin*d III. 73

4.6 Plasmid miniprep of potential plasmid harboring *pknI* digested with *Eco*R I and *Hin*d III. 74

4.7 Plasmid miniprep of potential plasmid harboring *pknK* digested with *Kpn* I and *Hin*d III. 74

4.8 Edited DNA sequences of *ppp* gene in 5' to 3' direction. 75

4.9 Edited DNA sequences of *pknI* gene in 5' to 3' direction. 76

4.10 Edited DNA sequences of *pknK* gene in 5' to 3' direction. 77

4.11 The alignment result of *ppp* gene with *M. bovis* BCG (Pasteur 1173P2) genome database 79

4.12 The alignment result of *pknI* gene with *M. bovis* BCG (Pasteur 1173P2) genome database 81

4.13 The alignment result of *pknK* gene with *M. bovis* BCG (Pasteur 1173P2) genome database. 85

4.14 Result of the BLAST search for *ppp* gene using blastp programme with other non-redundant GenBank coding sequence. 86

4.15 BLAST search result from NCBI server for *pknI* gene using blastp programme with other non-redundant GenBank coding sequence. 88

4.16 BLAST search result of *pknK* gene using blastp programme with other non-redundant GenBank coding sequence. 89

4.17(A) Complete nucleotide and amino acid sequence of putative protein phosphatase (Ppp) of *M. bovis* BCG (Pasteur 1173P2). 91

4.17(B) Complete nucleotide and amino acid sequence of PknI of *M. bovis* BCG (Pasteur 1173P2). 92

4.17(C) Complete nucleotide and amino acid sequence of putative serine/threonine protein kinase (PknK) of *M. bovis* BCG (Pasteur 1173P2). 94

UMS UNIVERSITI MALAYSIA SABAH

LIST OF FIGURES

FIGURE		PAGE
4.18(A)	Structural states defined for amino acids of Ppp.	95
4.18(B)	Structural states defined for amino acids of PknI.	96
4.19(A)	Schematic presentation of the membrane topology of Ppp.	97
4.19(B)	Schematic presentation of the membrane topology of PknI.	97
4.20(A)	Schematic presentation of the main structural components of Ppp	98
4.20(B)	Schematic presentation of the main structural components of PknI.	99
4.20(C)	Schematic presentation of the main structural components of PknK.	100
4.21(A)	Comparison of Ppp *M. bovis* BCG (Pasteur 1173P2) with ser/thr phosphatases of PP2C family of other organisms.	102
4.21(B)	The amino acid sequence alignment of the N-terminal catalytic domain of PknI and other putative eukaryotic ser/thr protein kinases.	104
4.21(C)	The amino acid sequence alignment of the N-terminal catalytic domain of PknK and other putative eukaryotic ser/thr protein kinases.	106
4.22(A)	Neighbour joining tree based on the bacterial PP2C family.	107
4.22(B)	Neighbour joining tree shows the relative positioning of PknI (cyan box) with respect to other prokaryotic kinases.	108
4.22(C)	Neighbour joining tree shows the relative positioning of PknK (yellow box) with respect to other prokaryotic kinases.	109
4.23(A)	ß-strands (arrows), α-helices (cylinders) and random coil (yellow C) indicated below the amino acids sequences are the three conformational states of secondary structure predicted in Ppp.	110
4.23(B)	ß-strands (arrows), α-helices (cylinders) and random coil (yellow C) indicated below the amino acids sequences are the three conformational states of secondary structure predicted in PknI by Porter server.	111
4.23(C)	ß-strands (arrows), α-helices (cylinders) and random coil (yellow C) indicated below the amino acids sequences are the three conformational states of secondary structure predicted in PknK by Porter server.	112

UNIVERSITI MALAYSIA SABAH

LIST OF FIGURES

FIGURE		PAGE
4.24(A)	Sequence alignment of phosphatase catalytic domain of Ppp and PstP (1TXO chain A).	113
4.24(B)	Sequence alignment of catalytic domain of PknI and PknB (1O6Y)	113
4.24(C)	Sequence alignment of the catalytic domain of PknK and PknB (1MRU).	114
4.25(A)	View into the active site of the PstP phosphatase domain.	114
4.25(B)	Tertiary structure of the catalytic domain of PknB (1O6Y) consists of two lobes: an N-terminal subdomain represented as N (A) includes the ß-strands and long α-helices (Helix C) while the C-terminal represented as C (A) essentially composed of α-helices.	115
4.25(C)	Tertiary structure of the catalytic domain of PknB (1MRU).	115
4.26	Agarose gel electrophoretic analysis of digested pTrcHis-*ppp*, pET-42a(+)-*pknI* and pET-42a(+)-*pknK* showing successful cloning of these gene fragments into their expression vectors.	116
4.27(A)	SDS-PAGE analysis of Ppp expression in *E.coli* Top 10.	117
4.27(B)	SDS-PAGE analysis of Ppp solubility in *E.coli* Top 10.	118
4.28(A)	SDS-PAGE analysis of PknI expression in *E. coli* BL21 DE3.	119
4.28(B)	SDS-PAGE analysis of PknI solubility in *E. coli* BL21 DE3.	119
4.29(A)	SDS-PAGE analysis of PknK expression in *E. coli* BL21 DE3.	120
4.29(B)	SDS-PAGE analysis of PknK solubility in *E. coli* BL21 DE3.	120
4.30(A)	SDS-PAGE analysis of purified Ppp.	121
4.30(B)	SDS-PAGE analysis of purified PknI.	122
4.30(C)	SDS-PAGE analysis of purified PknK.	122
4.31	Standard curve of protein microassay.	123
4.32	Purified pNPP (0.1 μg) was incubated with 10 mM pNPP in a phosphatase buffer at room temperature over a period of 150 minutes.	125
4.33	Color development during incubation of pNPP substrate with Ppp.	126

UMS UNIVERSITI MALAYSIA SABAH

LIST OF FIGURES

FIGURE		PAGE
4.34(A)	Influence of pH on Ppp activity.	127
4.34(B)	Effect of manganese ion concentration on Ppp activity.	128
4.35	Effects of divalent cations on the Ppp activity as determined by pNPP hydrolysis.	128
4.36	Effects of temperature on the activity of Ppp.	129
4.37(A)	Kinetics of pNPP hydrolysis by Ppp was measured at different substrate concentrations over a period of 30 minutes. The graph shows a curve of initial velocity vs. substrate concentration referred to as Michaelis-Menten plot.	131
4.37(B)	The graph shows a kinetic analysis of the data in the form of a Lineweaver-Burk plot with a determined Km of 0.83 $\pm$ 0.07 mM and a Vmax of 1.49 $\pm$ 0.02 unit/µg.	131
4.38(A)	Kinetics of threonine phosphopeptide hydrolysis by Ppp was measured at different substrate concentrations over a period of 30 minutes. The graph shows a curve of initial velocity vs. substrate concentration referred to as Michaelis-Menten plot.	132
4.38(B)	The graph shows a kinetic analysis of the data in the form of a Lineweaver-Burk plot with a determined Km of 1.34 $\pm$ 0.704 mM and a Vmax of 0.206 $\pm$ 0.075 unit/µg.	133
4.39	Phosphatase assay using threonine phosphopeptide as a substrate.	134
5.1(A)	The 3D structure showing the residues and side-chain positions in the two-metal centers, as well as Asp118, Asp191 and Ser160 involved in coordinating Mn3 in PstP (Pullen *et al.*, 2004).	140
5.1(B)	The schematic figure showing the residues and side-chain positions in the two-metal centers, as well as Asp118, Asp191 and Ser160 involved in coordinating Mn3 in PstP (Pullen *et al.*, 2004).	140
5.2	Serine/threonine protein kinase catalytic domain of PknI.	142
5.3	Serine/threonine protein kinase catalytic domain of PknK.	146
5.4	Alignment of the A- and B-type ATP/GTP binding motifs of several LAL bacterial transactivator families.	149
5.5	Schematic map describing the chromosomal location of the *ppp* open reading frame (yellow box) and its nearby genes (Cole *et al.*, 1998).	156

UMS UNIVERSITI MALAYSIA SABAH

LIST OF FIGURES

FIGURE		PAGE
5.6	Schematic map describing the chromosomal location of the *pknI* open reading frame (chocolate box) and its nearby genes (Cole *et al.,* 1998).	158
5.7	Schematic map describing the chromosomal location of the *pknK* open reading frame (green box) and its nearby genes (Cole *et al.* 1998).	160

UMS
UNIVERSITI MALAYSIA SABAH

LIST OF TABLES

TABLE		PAGE
2.1	Genomic regions that differ between *M. tuberculosis* H37Rv and *M. bovis* BCG Pasteur[a,b]	14
2.2	Medically certified and inspected deaths by specific cause in Malaysia, 1998 (Department of Statistics, Malaysia).	17
2.3	Criteria used for distinguishing protein phosphatases (Cohen, 1989).	28
2.4	Summary of the *Mycobacterium tuberculosis* serine/threonine protein kinases and phosphatase properties (Av-Gay & Everett, 2000).	36
3.1	List of primers used in amplification of *ppp, pknI* and *pknK* genes	44
3.2	The final concentration of the components and primers for PCR reaction	45
3.3	The final concentration of the components used in restriction enzyme digestion.	46
3.4	The final concentration of the components required in ligation reaction	48
3.5	The final concentration of the components required for CEQ cycle sequencing.	53
3.6	List of primers used for DNA sequencing of inserts in pUC19.	53
3.7	The final concentration of the components required for digestion of pUC19, *ppp* and *pknI*	58
3.8	The final concentration of the components required for digestion of pET-42a(+) and *pknK*	58
3.9	The final concentration of the components needed for ligation into expression vector	59
3.10	List of primers used in N-terminal sequencing	61
4.1	Protein concentration of Ppp, PknI and PknK samples before and after refolding.	124
4.2	Effect of various inhibitors on the catalytic activity of Ppp	130

UMS UNIVERSITI MALAYSIA SABAH

LIST OF ABBREVIATIONS

aa	amino acid
AFB	acid-fast bacillus
ATP	adenosine triphosphate
N-terminal	amino terminal
amp	ampicillin
APS	ammonium persulfate
BCG	bacillus Calmette-Guérin
BLAST	basic local alignment search tool
BAC	bacterial artificial chromosome
bp	base pair
BRNN	Bidirectional Recurrent Neural Networks
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
C-terminal	carboxyl terminal
CD	conserved domain
CBB R-250	Coomassie Brilliant Blue R-250
CAPS	3-(cyclohexylamino)-1-propanesulfonic acid
ddNTP	dideoxynucleotide triphosphate
DAS	Dense Alignment Surface
dNTP	deoxynucleoside-5'-triphosphate
DNA	deoxyribonucleic acid
DMSO	dimethylsulfoxide
DOTS	directly observed treatment/therapy short course
DTT	dithiotreitol
DU	duplicated
ESAT	early secreted antigenic target
EtBr	ethidium bromide
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol-bis (2-aminoethylether)-N,N,N',N' tetraacetic acid
EBI	European Bioinformatics Institute
ERK	extracellular signal regulated kinase
FMN	flavin mononucleotide
GTP	guanosine 5'-triphosphate
HK	histidine kinase
HTH	helix-turn-helix
HIV	human immunodeficiency virus
HCl	hydrochloric acid
Tris	hydroxymethyl aminomethane
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
I-1	inhibitor 1
Pi	inorganic phosphate
IL-12	interleukin 12
IPTG	isopropyl-ß-D-thiogalactopyranosid
kan	kanamycin
LAL	Large ATP-binding regulators of the LuxR family
LJ	Lowenstein-Jensen
LB	Lennox broth
$MgCl_2$	magnesium chloride
$MnCl_2$	manganese chloride

UMS
UNIVERSITI MALAYSIA SABAH

MEK	MAPK/ERK kinase
β-ME	ß-mercaptoethanol
MAPK	mitogen-activated protein kinase
MWCO	molecular weight cutt off
MES	morpholinoethane sulfonic acid
MDR	multidrug resistant
NCBI	National Center for Biotechnology Information
NF-κB	nuclear factor kappa beta
RB	refolding buffer
SLS	sample loading solution
SDSC	San Diego Supercomputer Center
SEDS	shape, elongation, division, and sporulation
SRP	signal recognition particle
NaCl	sodium chloride
NaF	sodium fluoride
NaOH	sodium hydroxide
Na_3VO_4	sodium orthovanadate
OD	optical density
ORF	open reading frame
pNPP	para nitrophenyl pyrophosphate
pNP	para nitrophenol
PBP	penicillin binding protein
PMSF	phenylmethanesulfonyl fluoride
PIM	phosphatidylinositol mannosides
PEP	phosphoenolpyruvate
PTS	phosphoenolpyruvate-dependent phosphotransferase
PPP	phosphoprotein phosphatase
PPM	phosphoprotein phosphatase Mg^{2+} dependent
PHYLIP	Phylogeny Inference Package
PIPES	piperazine-1,4-bis(2-ethanesulfonic acid)
PCR	polymerase chain reaction
PE	proline-glutamic acid
PPE	proline-proline-glutamic acid
PDB	Protein Data Bank
PP1	protein phosphatase 1
PTPs	protein tyrosine phosphatases
RD	regions of difference
RR	response regulator
RNase A	ribonuclease A
rRNA	ribosomal ribonucleic acid
RT	room temperature
SS	secondary structure
STPKs	serine/threonine protein kinases
STPPs	serine/threonine protein phosphatases
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
NaF	sodium fluoride
dH_2O	sterile distilled water
TPR	tetratricopeptide repeat
3D	three dimensional
TEMED	N,N,N′,N′-tetramethylethylenediamine
TLR2	toll-like receptor 2

UMS UNIVERSITI MALAYSIA SABAH

TMHMM	transmembrane based on a hidden Markov model
TAE buffer	Tris-acetate-EDTA buffer
TE buffer	Tris-EDTA buffer
TACO	tryptophan/aspartate-containing coat protein
2DE	two dimensional electrophoresis
TB	tuberculosis
TNFα	tumor necrosis factor alpha
UPGMA	Unweighted Pair Group Method with Arithmeric mean
UV	ultra violet
WHO	World Health Organization
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside
$ZnCl_2$	zinc chloride

UMS
UNIVERSITI MALAYSIA SABAH