GENE CLONING AND EXPRESSION OF *map30* FROM MARA KHEE NOK *Momordica charantia* Linn. IN *Escherichia coli*

Mr. Kun Silprasit

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Biochemistry
Department of Biochemistry
Faculty of Science
Chulalongkorn University
Academic Year 2004

ISBN 974-53-1598-2

การโคลนและการแสดงออกของยีน *map30* จากมะระขี้นก *Momordica charantia* Linn. ใน *Escherichia coli*.

นาย กัญจน์ ศิลป์ประสิทธิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีวเคมี ภาควิชาชีวเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2547

ISBN 974-53-1598-2

Thesis Title	GENE CLONING AND EXPRESSION OF MAP30 FROM MARA KHEE NOK *Momordica charantia* Linn. IN *Escherichia coli*.
By	Mr. Kun Silprasit
Field of Study	Biochemistry
Thesis Advisor	Assistant Professor Preerada Mongkolkul, Ph.D.
Thesis Co-advisor	Associate Professor Siriporn Sittipraneed, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

….....Dean of the Faculty of Science
(Professor Piamsak Menasveta, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Associate Professor Aran Incharoensakdi, Ph.D.)

.....Thesis Advisor
(Assistant Professor Peerada Mongkolkul, Ph.D.)

.....Thesis Co-Advisor
(Associate Professor Siriporn Sittipraneed, Ph.D.)

.....Member
(Assistant Professor Kanoktip Packdibamrung, Ph.D.)

.....Member
(Teerapong Buaboocha, Ph.D.)

กัญจน์ ศิลป์ประสิทธิ์ : การโคลน และ การแสดงออกของยีน *map30* จากมะระขี้นก *Momordica charantia* Linn. ใน *Escherichia coli.* (GENE CLONING AND EXPRESSION OF MAP30 FROM MARA KHEE NOK *Momordica charantia* Linn. IN *Escherichia coli* .) อาจารย์ที่ปรึกษา: ผศ. ดร. พีรดา มงคลกุล, อาจารย์ที่ปรึกษาร่วม : รศ. ดร. ศิริพร สิทธิประณีต, 127 หน้า, ISBN 974-53-1598-2

MAP30 เป็นโปรตีนขนาด 30 กิโลดาลตันที่พบในมะระ (*Momordica charantia*) จากการศึกษาที่ผ่านมาพบว่า MAP30 มีฤทธิ์ยับยั้งการสังเคราะห์โปรตีนและมีสมบัติคล้าย topoisomerase ซึ่งสมบัติทั้งสองสามารถยับยั้งวงจรชีวิตของไวรัสได้หลายขั้นตอน มะระขี้นกเป็นมะระสายพันธุ์หนึ่งที่พบได้ทั่วไปในประเทศไทย การศึกษานี้ได้ทำการเพิ่มปริมาณยีนของโปรตีน MAP30 จากจีโนมิกดีเอ็นเอของมะระขี้นกโดยการทำ Polymerase Chain Reaction ด้วยไพร์เมอร์ที่จำเพาะ จากนั้นทำการโคลนและหาลำดับนิวคลีโอไทด์ของ *map30* ยีน จากการเปรียบเทียบลำดับนิวคลีโอไทด์กับยีนของ *map30* จากแหล่งอื่นในฐานข้อมูลพบว่ามีความคล้ายคลึงกันมาก (มี 2-8 นิวคลีโอไทด์ และ 1-3 กรดอะมิโน ที่ต่างกัน) เมื่อนำ *map30* ยีนที่ได้ไป subclone เข้าสู่เวคเตอร์ pET19b และเหนี่ยวนำให้เกิดการแสดงออกใน *Eschericia coli* Rosetta DE3 (pLysS) พบว่ารีคอมบิแนนท์ MAP30 อยู่ในรูป soluble protein และ inclusion bodies การนำ inclusion bodies ไปละลายด้วย 8 โมลาร์ ยูเรีย, refold และทำโปรตีนให้บริสุทธิ์ด้วย CM-Sephadex C-50 และ phosphocellulose P11 คอลัมน์โครมาโทกราฟี พบว่าได้ inclusion bodies ที่บริสุทธิ์แต่ไม่มีแอคติวิตี ต่อมาจึงนำโปรตีนจากส่วนที่เป็นสารละลายไปทำให้บริสุทธิ์ด้วยการตกตะกอนใน 30-60 เปอร์เซ็นต์ แอมโมเนียมซัลเฟตอิ่มตัวและ CM-Sephadex C-50 คอลัมน์โครมาโทกราฟี สามารถแยกสารละลายโปรตีนได้สองส่วนคือ CmI และ CmII จากการติดตามโปรตีน MAP30 พบว่าสารละลายโปรตีน CmII มี topoisomerase activity จึงนำสารละลายโปรตีนดังกล่าวไปทำโปรตีนให้บริสุทธิ์ยิ่งขึ้นด้วย phosphocellulose P11 คอลัมน์โครมาโทกราฟีซึ่งตรวจพบ topoisomerase activity ในสารละลายโปรตีนที่เกาะคอลัมน์ เมื่อตรวจความบริสุทธิ์ด้วย SDS-PAGE พบแถบโปรตีนเพียงหนึ่งแถบขนาดประมาณ 30 kDa จากนั้นทำการยืนยันความเป็น MAP30 โปรตีนโดยหาลำดับกรดอะมิโน 20 ตัวแรกด้านปลาย N-terminal จากการหาขนาดโดยใช้โปรแกรมคำนวน โปรตีนที่ได้มีขนาด 29.62 kDa การศึกษา topoisomerase activity ของโปรตีนที่บริสุทธิ์พบว่าที่ความเข้มข้น 0.85 ไมโครโมลาร์ เป็นความเข้มข้นที่น้อยที่สุดที่แสดงแอคติวิตี

ภาควิชา……. ชีวเคมี…… ลายมือชื่อนิสิต.....

สาขาวิชา……ชีวเคมี…… ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา…… 2547……. ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4472210823 : MAJOR BIOCHEMISTRY

KEY WORD : MAP30 / CLONING / TOPOISOMERASE

KUN SILPRASIT: GENE CLONING AND EXPRESSION OF MAP30 FROM MARA KHEE NOK *Momordica charantia* Linn. IN *Escherichia coli*. THESIS ADVISOR : ASSISTANT PROFESSOR PEERADA MONGKOLKUL, Ph.D., THESIS CO-ADVISOR : ASSOCIATE PROFESSOR SIRIPORN SITTIPRANEED, Ph.D., 127 pp. ISBN 974-53-1598-2

MAP30 is a 30 kDa protein that was identified and purified from bitter melon (*Momordica charantia*). MAP30 contains protein synthesis inhibition activity and topoisomerase-like activity, these activities can inhibit HIV virus by capable of acting multiple stages of the vial life cycle. In Thailand, Mara Khee Nok (Thai bitter melon) is wildly distributed and consumed as food. To clone *map30* gene from this local variety, *map30* gene was first amplified from Mara Khee Nok genomic DNA by Polymerase Chain Reaction using specific primers designed from known *map30* sequence. The putative gene was cloned into pUC18 and electrotransformed to *E. coli* JM109. The resulting, the product about 800 bp correlated with mature *map30* in size. It was sequenced and the amino acids were deduced. The sequences were vary similar (2-8 nucleotides and 1-3 amino acids difference) to those reported in the DNA databank. The result implied that MAP30 is a highly conserved protein. Further subcloned to pET19b vector for high expression in *E. coli* Rosetta DE3 (pLysS), the recombinant MAP30 was found as soluble and inclusion bodies forms. The inclusion bodies was solubilized with 8 M urea, refolded and purified by CM-Sephadex C50 and followed with Phosphocellulose P11. A purified protein of 30 kDa was obtained but it did not contain the topoisomerase activity. Purification of soluble MAP30 showed that most of MAP30 could be precipitated with 30-60% saturated ammonium sulfate. Purification by CM-Sephadex C-50 column gave two unbounded protein peaks, CmI and CmII. Topoisomerase activity was found in CmII fraction. The protein was purified to homogeneity from Phosphocellulose P11 column as confirm by SDS-PAGE analysis. The purified recombinant protein was confirmed to be MAP30 by N-terminal amino acids sequencing of the first 20 residues. The estimated molecular weight from computation program was 29.62 kDa. The minimal protein concentration that could show topoisomerase activity was 0.85 μM.

Department……..Biochemistry…… Student's signature…….....

Field of Study….Biochemistry…… Advisor's signature.....

Academic year….2004…….. Co-advisor's signature……....

ACKNOWLEDGEMENT

I would like to thank Assistant Professor Dr. Peerada mongkolkul and Associate Professor Dr.Siriporn Sittipraneed for an excellent graduate advisor and mentor. I also thank hers for her patience, understanding and encouragement though the years. Without her kindness and understanding, this work could not be accomplished.

My gratitude is also to Associate Professor Dr. Aran Incharoensakdi, Associate Professor Dr. Kanoktip and Dr. Teerapong for serving as the members of my thesis committee, for their valuable comments and also for useful suggestions. I am graceful to Associate Professor Dr. Kanoktip for her kindness in providing the Phosphocellulose P11 and help in the amino acid sequencing.

I also wish to thank all staff members and students of the Biochemistry Department for their help in laboratory and discussion. I also thank them for their sincerity and friendships. Special thanks are also extended to all members of 707; Bong, Taum, Ta, Tuun and my fiends; pee Kung, pee Cell, pee Ohm, pee Chai, pee Ake, Bum, New, Put, Ple, Ann, Ying, Noknoi, Gung, Na, Dear and Nok for their kind suggestions and special thanks to Lah for her kindness and helpfulness.

This work was partially supported by grant from the Graduate school, Chulalongkorn University.

Finally, the greatest indebtedness is expressed to my father, my mother and my younger brother for their unlimited love, understanding and encouragement.

CONTENTS

Page

THAI ABSTRACT……………………………………………………… ………..........iv

ENGLISH ABSTRACT……………………………………………………….………v

ACKNOWLEDGMENT…………………………………………………….….........vi

CONTENTS……………………………………………………………….............vii

LIST OF FIGURES………………………………………………………………...xi

LIST OF TABLES………………………………………………………………...xiv

ABBREVIATIONS…………………………………………………………..........xv

CHAPTER I INTRODUCTION

1.1 *Momordica charantia* ..……………………………………………………......1

1.2 Human Immunodeficiency Virus (HIV)…………………………….............4

1.3 MAP30 (Momordica Anti-HIV Protein, 30kDa)………………………………5

1.4 The structure of MAP30 protein……..………………………….................10

1.5 The enzymatic properties of MAP30…………………………………………11

1.5.1 Site-specific RNA glycosidase activity………………………….….........11

1.5.2 DNA glycosylase/AP lyase: DNA topological Inactivation activity……………………...…………………………………….………14

1.6 Role of MAP30 in plants…..………………………………….……..….........15

1.6.1 Defense protein…………………………………………..….…..………..15

1.6.2 Induction of MAP30 function………………………….…….……….16

1.7 Cloning and expression of MAP30……………………………….............17

CHAPTER II MATERIAL AND METHOD……………………………………..21

2.1 Equipments……………………………………………….………….. …….21

2.2 Inventory supplies…………………………………………………….….……..22

2.3 Chemicals…………………………………………………………..............22

2.4 Enzymes…………………………………………………………...…………25

2.5 Plasmid vectors…………………………………………………................25

2.6 Oligonucleotide primers…………………………………………….…........25

2.7 Organisms…………………………………………………………….........25

2.7.1 *Momordica charantia*………………………………………….…...........25

2.7.2 Bacterial strains…………………..……………………….……..………..25

2.8 Cultivation of bacteria………………..……………………….................26

Page

2.9 Sample preparation……..…….....26
2.10 Preparation of *map30*……...……...……..…… ……...26
2.10.1 Chromosomal DNA extraction…….....26
2.10.2 Amplification of map30 gene by PCR technique……..27
2.10.2.1 Primer designed …….....27
2.10.2.2 PCR amplification……...28
2.10.2.3 Analysis of DNA fragment by agarose gel electrophoresis…….....29
2.10.2.4 Extraction of DNA fragments from agarose gel……...……..29
2.11 Cloning of map30 gene into pUC18 vector…….....……...34
2.11.1 Preparation of pUC18 and map30 gene fragment…… ..34
2.11.2 Construction of recombinant pUCmap ……34
2.11.3 Transformation of ligated products into *E. coli* host cells by Electroporation……..34
2.11.3.1 Preparation of competent cells …….....34
2.11.3.2 Electrotransformation ……...….....35
2.11.4 Selection of map30 positive clone ……...……...35
2.11.4.1 blue/white colony selection……...…….....35
2.11.4.2 Plasmid extraction……...……36
2.11.5 Characterization of pUCmap……...…….. ..38
2.11.5.1 Identification of pUCmap by restriction mapping……..38
2.11.5.2 Nucleotide sequencing and data analysis……. …..38
2.12 Expression of map30 gene in *Escherichia coli* JM109 by pUCmap…….……..39
2.12.1 Optimization of induction time……..... ..39
2.12.2 Protein measurement…….....39
2.12.3 SDS-Poly Acrylamide Gel Electrophoresis……..…….....40
2.12.3.1 Preparation of separating gel (12% acrylamide)…….....40
2.12.3.2 Preparation of stacking gel (5% acrylamide)…….....40
2.12.3.3 Sample preparation……..……..41
2.12.3.4 Running the gel…….……...41
2.12.3.5 Protein staining …….…….....41

Page

2.13 High expression of map30 gene using pET vector…….....42
2.13.1 Subcloning of map30 gene to pET19b vector…….....42
2.13.2 Characterization of positive clone…….....42
2.13.3 Cultivation of recombinant clone……..……45
2.13.4 Optimization of gene expression……..…….....45
2.13.4.1 Induction time …….....45
2.13.4.2 IPTG concentration…….....45
2.13.4.3 Temperature for expression…….....46
2.14 MAP30 purification…….....46
2.14.1 Preparation of soluble MAP30……. …..46
2.14.1.1 Preparation of crude protein……...46
2.14.1.2 Ammonium sulfate precipitation …….....47
2.14.2 Preparation of inclusion bodies …….....47
2.14.2.1 Solubilization and refolding of inclusion bodies……...48
2.14.3 Column chromatography ……....48
2.14.3.1 CM-Sephadex C50 column chromatography…….....48
2.14.3.2 Phosphocellulose P11 column chromatography……49
2.15 Characterization of MAP30…….....50
2.15.1 Molecular weight determination…….....50
2.15.2 N-terminal sequencing…….....50
2.15.3 Topoisomerase I activity assay…….....50
CHAPTER III RESULTS
3.1 Cloning map30 gene from *Momordica charantia* using y pUC18 vector……...52
3.1.1 DNA Extraction…….....52
3.1.2 Amplification and characterization of *map30* gene……....52
3.1.3 Characterization of *map30* gene…….....53
3.1.4 Nucleotide sequence of *map30* gene…….....53
3.2 Expression of pUCmap in *E. coli* JM109 ……..65
3.3 Cloning and expression level of *map30* gene using pET vector…….....65
3.3.1 Subcloning of *map*30 gene…….65

Page

3.3.2 Induction of pETmap in *E. coli* Rosetta DE (pLysS)……....68
3.3.3 IPTG concentration for induction…….....68
3.3.4 Temperature for induction…….....69
3.5 Purification and characterization of recombinant MAP30 ……..74
3.5.1 Preparation of MAP30 from inclusion bodies fraction……..74
3.5.1.1 Solubilization and refolding of MAP30 from inclusion bodies……...74
3.5.1.2 Purification of refolded MAP30 by column chromatography….....74
3.5.2 Preparation of crude extract……...83
3.5.3 Purification of soluble protein……...……83
3.5.3.1 Ammonium sulfate precipitation……..……..83
3.5.3.2 CM Sephadex C-50 columns……..……86
3.5.3.3 Cellulose phosphate P11 column……..……..86
3.6 Characterization of MAP30 …….....95
3.6.1 Molecular weight analysis…….95
3.6.2 N-terminal sequence…….....95
3.6.3 Dose response topoisomerase activity of purified MAP30…… ...…95
CHAPTER IV DISCUSSION……..98
CHAPTER V CONCLUSION……....106
REFERENCES……...……107
APPENDIX…….....115
BIOGRAPHY…….128

LIST OF TABLE

Table Page

2.1 Sequences of Oligonucleotide primer designed from the nucleotide sequence of *map30* gene (Lee-Haung, 1995)……31

3.1 Table 3.1 Protein recovery from each purification step……..…….....80

LIST OF FIGURES

Figure Page

1.1 Some characters of bitter melon (*Momordica charantia*)……..……....3

1.2 Composition of HV virus….....6

1.3 Life cycle of HIV virus…… ……..…….....7

1.4 HIV-LTR…… …….…….....8

1.5 Ribbon Diagrams of the Minimized Average…….…….....12

1.6 Secondary structure of MAP30…… ……..……13

1.7 Role of RIPs in plant response……...….....19

1.8 The nucleotides sequence of MAP30……..……....…....20

2.1 The binding position of forward and reverse primers to the *map30* …..…….. …….32

2.2 The cycle program of Polymerase Chain Reaction for amplification *map30* gene from bitter melon genomic DNA …….…….....33

2.3 Diagrammatic presentation of pUCmap construction ……..…….….37

2.4 pETmap construction…….……..44

2.5 The summary of MAP30 purification ……..….....51

3.1 Extraction of genomic DNA from Mara Khee Nok ……....….....55

3.2 PCR product from full length *map30* amplification…….56

3.3 Recombinant plasmid pUCmap in 0.7% agarose gel…….…….. …..57

3.4A Restriction mapping of pUCmap…… ..58

3.4B Restriction mapping of pUCmap……..…...59

3.5 The nucleotide and deduced amino acid sequences of *map30* gene clone from Mara khee Nok……..…….....60

3. 6 Nucleotide sequence alignment of *map30* gene…….……... 61

3.7 Amino acid alignment of MAP30 protein……..…….....63

3.8 Super impose of MAP30 structure from Lee-Haung and Mara Khee Nok…….....64

3.9 Expression of pUCmap in *E. coli* JM109…….……..66

3.10 Restriction analysis of pETmap……...……..…….....67

3.11 Expression of MAP30 in recombinant *E. coli* Rosetta DE3 (pLysE) containing pETmap……..……..70

3.12 Localization of MAP30 from pETmap in *E. coli* Rosetta DE3 (pLysE)…….....71

3.13 Induction of MAP30 expression by various IPTG concentration……...…….....72

3.14 SDS-PAGE analysis of MAP30 localization in cells grown at difference temperature ……..……..……..…..…....73
3.15 SDS-PAGE analysis of inclusion bodies from various treatment…….…….…....76
3.16 Topoisomerase activity of MAP30 from inclusion bodies …….....77
3.17 Purification profiles of refolded recombinant MAP30 by CM Sephadex C 50…….…….....78
3.18 The SDSPAGE of refolded inclusion bodies from CM-Sephadex C50 column ……..……...79
3.19 Topoisomerase activity of reMAP30 from CM Sephadex C50 column……..…….…….. 80
3.20 Purification of recombinant MAP30 by affinity column Phosphocellulose P11 …….…….....81
3.21 The 12%SDS PAGE pattern of proteins from different purification step…...……...82
3.22 Screening of MAP30 from ammonium sulfate precipitates……...84
3.23 Topoisomerase activity of MAP30 from ammonium sulfate precipitations…….……..…….....85
3. 24 Purification profiles of recombinant MAP30 by CM Sephadex C 50……...…. …88
3.25 The SDSPAGE pattern showing profiles of protein purification……..…..89
3.26 Topoisomerase I activity of protein from CM Sephadex C50 column……... …..90
3.27 Purification profiles of recombinant MAP30 by Phosphocellulose P11……..……..91
3. 28 Topoisomerase activity of MAP30 from phosphocellulose P11 column……..92
3.29 SDS-PAGE protein pattern from different step of purification …….…….....93
3.30 Molecular weight determination by 12% SDS PAGE ……..…… ….96
3.31 Dose response activity of MAP30……..…….97

ABBREVIATIONS

A	Absorbance
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumi
°C	Degree Celsius
cm	centrimeter
Da	Dalton
DNA	deoxyribonucleotide
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	kilobase pair in duplex nucleic acid
kDa	kiloDalton
L	Litre
mL	Millilitre
μL	Microlitre
M	Molar
mM	Millimolar
μM	Micromolar
μg	Microgram
PAGE	polyacrylamide gel electrophoresis
rpm	Revolution per minute
SDS	sodium dodecyl sulfate
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

CHAPTER I

INTRODUCTION

1.1 *Momordica charantia*

Momordica charantia, or commonly known as bitter melon and bitter gourd, is an annual climber vine that belong to the Curcubitaceae family. This family also includes the squash, melon and cucumber. The plant is thought to be originated in India and China and is later distributed widely in tropical areas, especially in Asia, Africa, South America and the Caribbeans. The vine is slender with long-stalk palmated leaves. The flowers are yellow solitary male and female (monoecious) borne in the leave axils. The fruits come in different size, shape and color depending on the varieties, but they commonly share ridging surface and have slight to very bitter taste. The young fruit is ranging from creamy beige to dark green color, turning to orange-yellow when ripe. At maturity, the fruit splits revealing the reddish brown seeds attached to arils of scarlet placenta. Seeds are of oral shape with thick, rough etched pericarp. Although wide variation of bitter melon exists as observed from fruit size, shape and left character, bitter melon was divided only into two groups in Thailand. The bigger and longer fruit is called "Chinese bitter melon" and the smaller fruit, usually with dark green prominent ridges, is called "Mara Khee Nok". Morphology of the plant is demonstrated by Figure 1.1.

The young fruit, tender shoots and leaves of bitter gourd are consumed as vegetable. It is rich in Vitamins A and C, folate and many minerals. For centuries, bitter melon was used as an herbal plant for traditional medicine. It has been used as an alternative hypoglycemic agent for type I and II diabetes. Numerous research works suggested that bitter melon extracts contained insulin-like peptide (polypeptide p) and two steroid glycosides charantins which had hypoglycemic properties (Basch *et al*., 2003). There are evidences both in experimental animal and human trials that bitter melon extracts might decrease hepatic gluconeogenesis, increase hepatic glycogen synthesis, enhance glucose uptake, increase pancreatic insulin secretion, increase pancreatic β-cells, increase glucose oxidation

through the G6PDH pathway in erythrocytes and hepatocytes (Shibib *et al*., 1993). Some other medicinal uses of bitter gourd are reduction of total cholesterol and triglycerides in both the presence and absence of dietary cholesterol, antiviral for measles, hepatitis, and relief of feverish conditions. In other research, the seed has demonstrated the ability to induce abortions in rats and mice, the root has been documented with a uterine stimulant effect in animals. Fruit and leaf of bitter melon has demonstrated an *in vivo* antifertility effect in both female and male animals, it was reported to affect the production of sperm negatively (Taylor *et al*., 2002).

Finding during the past decade have placed *M. charantia* in research focus due to its therapeutic potentials as antiviral and anti-tumor agents. These include protease inhibitors (MCTI-I, II and III) from young seeds, several of ribosome inactivating proteins such as MAP30 from ripe seeds, α-and β-momorcharins (from seed), momordin (from seed) and curcubitacin B (from leaf).

A phytochemical GTP-pyrophosphate [cycling] inhibitor (GCI)] is promising for treatment of psoriasis by inhibiting guanylate cyclase which is associated with the pathogenesis of the disease (Clafin *et al*., 1978). Momordin was clinically demonstrated to have cytostatic and cytotoxic activities against Hodgkin's lymphoma, *in vivo* (Talor *et al*., 2002). MAP30 is an anti-HIV agent and also showed anti-tumor activities against many human tumor cell lines (Lee-Haung *et al*., 1995). In rats, extracts from bitter melon showed potency against mammary tumor and prostate carcinoma development. Lectins from bitter melon showed significant antilipolytic and lipogenic activities. It had been shown to reduce total cholesterol and triglycerides in both the presence and absence of dietary cholesterol (Jayasoriya *et al*., 2000).

A

B

C

D

Figure 1.1 Some characters of bitter melon (*Momordica charantia*)

A. fruit shape of Mara Khee Nok

B. fruit shape of Chinese bitter melon

C. bitter melon overripe

D. bitter melon plant

Although bitter melon contains a wide spectrum of bioactive compounds (phytochemicals, peptides, and proteins), more monitoring clinical trials, safety and efficacy assessment are needed since adverse effects from taking them were reported in some cases.

1.2 Human Immunodeficiency Virus (HIV)

HIV is a RNA virus belongs to the class retrovirus. Its morphology is shown in Figure 1.2. HIV contains a nucleoid case composed of two identical single-strand RNA molecules (about 9,500 nucleotide longs) which are associated with basic nucleocapsid protein (NC, p1) and encased in a core-shape capsid made of capsid protein (CA, p24). This viral core also contains *pol* gene products necessary for viral replication, such as protease, reverse transcriptase and integrase and some other components. The capsid is encapsidated by a shell of matrix protein (MA, p17), which is in turn attached to the viral lipoprotein derived from host cell membrane. The viral outside surface contains glycoproteins gp120 and gp41 which enable the virus to bind with the CD4 and CCR5 receptors of the target host cell before fusion and penetration.

The life cycle and infection of HIV is summarized in Figure 1.3 . The target cells of HIV are mainly T-lymphocytes, monocytes and dendritic cells. The provirus contained two long terminal repeats (LTRs) (Figure1.4). The viral transcription is initiated from within the upstream LTR. Drug design against infective cycle, i.e., HIV for treatment of AIDS patients are targeted at many steps of the virus including virus adsorption, fusion, or integration/release of viral DNA, Many anti AIDS drugs act on viral enzyme such as reverse transcriptase, integrase or protease. Combination of drug tonics has been designed to attack HIV at more than one point of its life cycle. The treatment however, is limited by high cost and undesirable side effects. Screening and developing of less toxic but effective drugs are in demand. Today, many proteins/chemicals from medicinal plant have been studied for their potential use.

1.3 MAP30 (Momordica Anti-HIV Protein, 30kDa)

The name MAP30 was derived from Momordica Anti-HIV Protein of 30 kDa. MAP30 gained wide interest when Sylvia Lee Huang and her colleagues at New York University School of Medicine published the anti-HIV activities of MAP30, a protein from ripe seeds of *M. charantia*, in October, 1990. A series of follow-up investigation was done mainly by her group during the past decade. They had cloned, purified and patented the gene. MAP30 is a basic protein of about 30 kDa. It could block the infection of T-lymphocytes and monocytes by HIV-1, the prominent strain HIV in America (Lee-Huang *et al*., 1990). It also inhibited viral replication in HIV-infected cells. The EC_{50} (effective concentration for 50% inhibition) was in subnanomolar range (0.2-0.3 nM) and exhibited dose dependent inhibition. Interestingly, MAP30 showed neither cytotoxic nor cytostatic effects toward normal cells even at 1,000 fold higher than the dose levels (Lee-Haung *et al.,* 1990). Beside HIV, MAP30 was also shown to be effective against Herpes Simplex Virus (HSV) *in vitro*. They reported that the EC_{50} were 0.1 and 0.3 μM for HSV-1 and HSV-II, respectively, which were better than acyclovir, the commonly used anti-HSV drug for the two viruses (EC_{50} 0.2 and 0.7 μM)

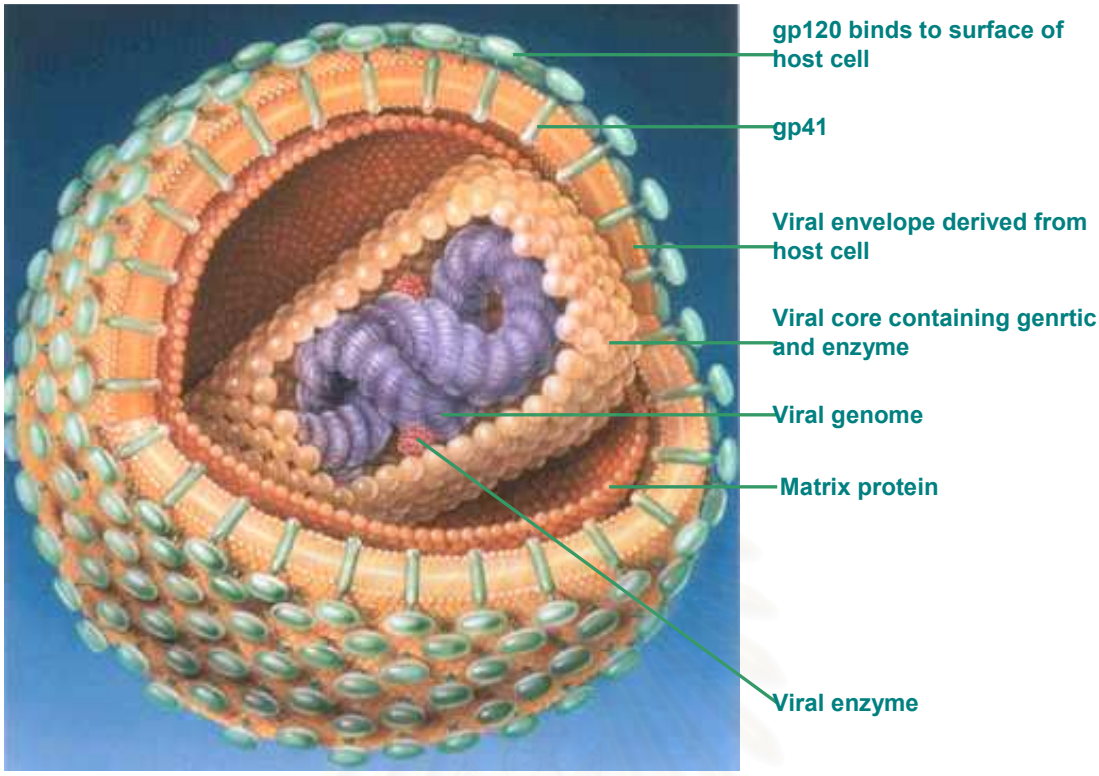


Figure 1.2 Composition of HV virus. The viral RNA genome and viral enzyme were envelope with protein (http://cseserv.engr.scu.edu/StudentWebPages/HHo/ResearchPaper.htm)

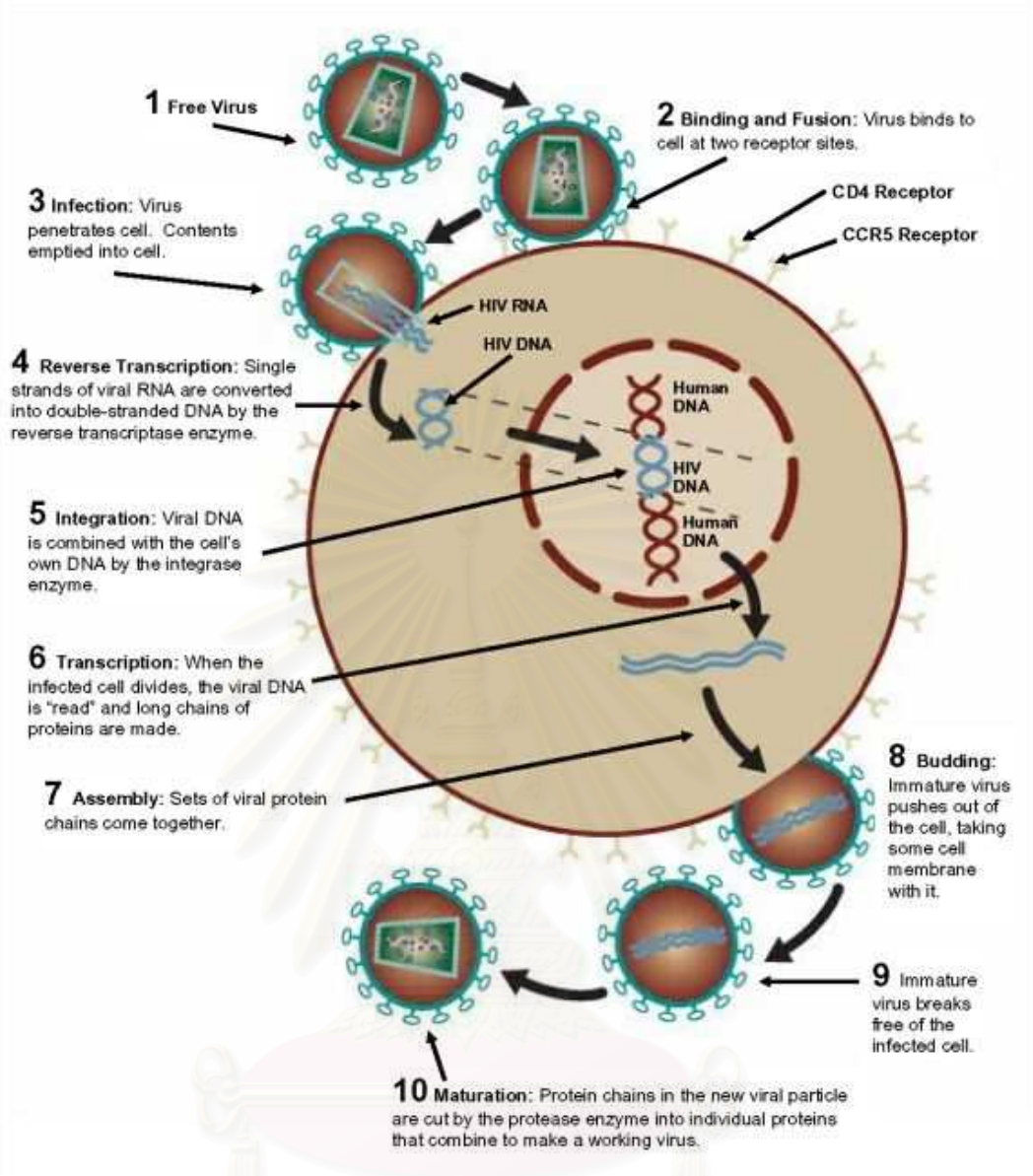


Figure 1.3 Life cycle of HIV virus. The virus infect to host by binding of viral envelope to receptor and then release viral genome into cell, the viral genome is integrated into host chromosome and replicated with host mechanism

(http://www.thebody.com/ nmai/cycle.html)

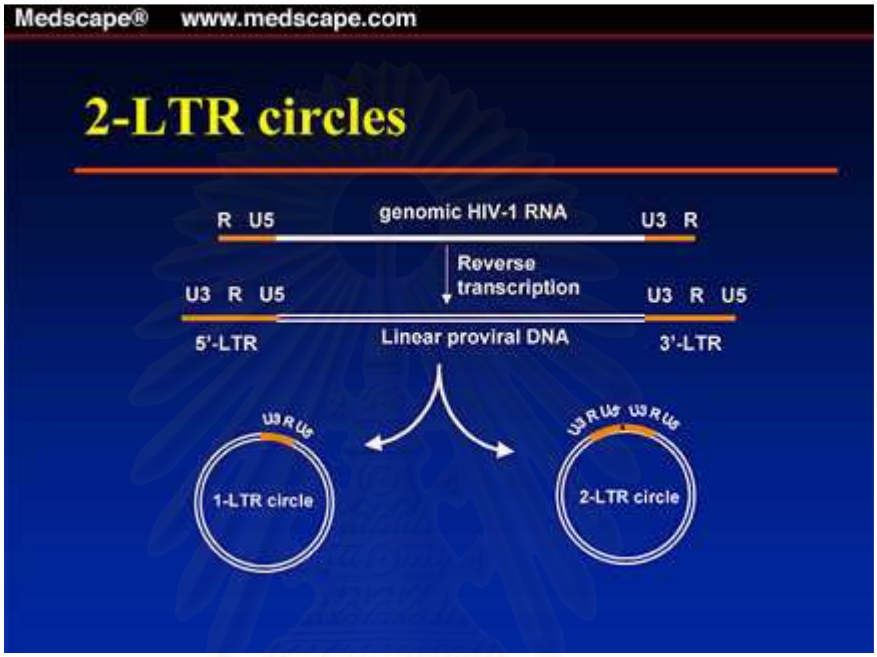


Figure 1.4 HIV-LTR. Firstly they are "sticky ends" (that's a biochemistry term) which the integrase protein uses to insert the HIV genome into host DNA. Secondly, they act as promoter/enhancers - when integrated into the host genome, they influence the cell machinery which transcribes DNA, to alter the amount of transcription which occurs. Protein binding sites in the LTR are involved with RNA initiation. (http://www.medscape.com/ viewprogram/661_pnt)

The anti-HIV and anti-viral activities could be attributed to (1) cell-free ribosome inactivation of a specific N-glycosylase which can terminate protein synthesis , (2) DNA topological inactivation activity and (3) inhibition of each of viral integrase. These activities will be described in details in later text.

In addition to anti-viral activities, MAP30 also exhibited autineoplastic effects. Among these were human cell lines from melanoma, neuroblastoma, AIDS-related lymphoma, myeloma, liver hypatomer, breast carcinoma, small-cell lung cancer and renal cancer (Lee-Haung *et al*., 2001). Prostate carcinoma and epidemoid carcinoma were less responsive to MAP30. Using an AIDS related lymphoma cell line BC-2 which was latently infected with Kaposi's sarcoma-associated herpes virus (KSHV) as the model, Lee-Haung *et al* (2001) showed that MAP30 could modulate a profile of viral and cellular gene expressions. They found that MAP30 down-regulated some viral genes involved in cell cycle regulation, viral pathogenesis, and apoptosis. In addition, MAP30 also down-regulated cellular genes which promote mitogenesis and tumorigenesis and up-regulated pro-apoptotic related genes. In breast cancer cells, MAP30 reduced the expression of tyrosine kinase receptor, encoded by HER2 oncogene, which is a growth factor receptor Lee-Huang *et al*. xenografted these cells to mice, and showed that MAP30 inhibited cancer cell proliferation both *in vitro* and *in vivo*. In 2002, Arazi *et al* studied the effectiveness of MAP30 against a range of Gram-positive and Gram-negative bacteria, yeast and fungi. Inhibition of microbial growth was evaluated by Minimal Inhibitory Concentration (MIC) method. MAP30 was potent against *Candida albicans* (MIC50 0.33 μM), a fatal, and other immunosuppressive patients. It was also effective against *E. coli* (MIC50 0.32 μM) and *S. aureus* (MIC50 0.41μM). Much less inhibition was observed with *A. fumigatus* (MIC50 6.2 μM).

1.4 The structure of MAP30 protein

MAP30 is a single chain protein of 286 amino acids. The first 23 residues serve as leader peptide for protein secretion and are not present in the mature MAP30. Therefore, the

mature protein consists of only 263 residues with a potential N-glycosylation site Asn-Leu-Thr at amino acid position 51-53. It has one each of Trp (190) and Met (254) and does not contain Cys residue. The protein is encoded by a 858 base pair map30 gene (*map30*) which is a single Open Reading Frame without any intron. (Lee-Haung *et al.,*1995). The amino acid and nucleotide sequences are shown in Figure 1.4. The molecule is quite basic, containing 50 basic amino acid residues. Wang and collaborators (Wang *et al.,* 1999) revealed the solution structure of MAP30 using NMR spectroscopy in an attempt to explain for its multifunction.

The ribbon diagram (Figure 1.4) shows that MAP30 exhibits secondary structure similar to the crystal structure of many ribosome inactivating protein (RIP). Most of the β-sheets is in the N-terminal halve of the molecule where as most of the α-helices reside at the c-terminal halve. The N-terminal domain (residues 1- 105) contains an extended mixed sheet of six β-strands. Strand β3 contains the N-linked glycosylation site, found in naturally occurring MAP30. One face of this sheet packs against α helices in the highly helical central domain (residues 108-180) of the molecule. The C-terminal domain (residues 181-263) contains a structure region in which the long bent helix, residues 181-200, packs against an anti parallel two-stranded β-sheet. The 19 residues at the C-terminus (residue 245-263) are highly flexible and disorder. Base on similarity of MAP30 and RIPs secondary structure, binding experiment of MAP30 to HIV-1 LTR DNA, Wang postulated that the DNA binding site reside in a deep pocket between the N-terminal and central domains, which is lined by Tyr70, Tyr109 and Ile158 with Glu158 and Arg16 at the bottom (Figure 1.6). They also suggest that Mn^{+} and Zn^{+} at the molecule surface facilitate DNA binding but were not participate directly in MAP30 catalysis.

1.5 The enzymatic properties of MAP30

1.5.1 Site-specific RNA glycosidase activity

MAP30 is a member of type1 Ribosome-Inactivating Protein (RIP). It can inactivate eukaryotic and prokaryotic ribosome activity and inhibits protein synthesis. RIP contain RNA N-glycosidase activity that recognizes a sarcin/ricin loop that is a highly conserved region in the large 28S rRNA and cleaves a specific N-C glycosidic bond between an adenine (A4324) and the nucleotide on the RNA whereby the adenine residue is removed. Due to removal of this adenine, the deadenylated (or abasic) site becomes unstable and a β-elimination reaction can occur resulting in rRNA cleavage (Peuman *et al.,* 2001). RIP consists mainly of 2 types: type 1 RIP and type II RIP.

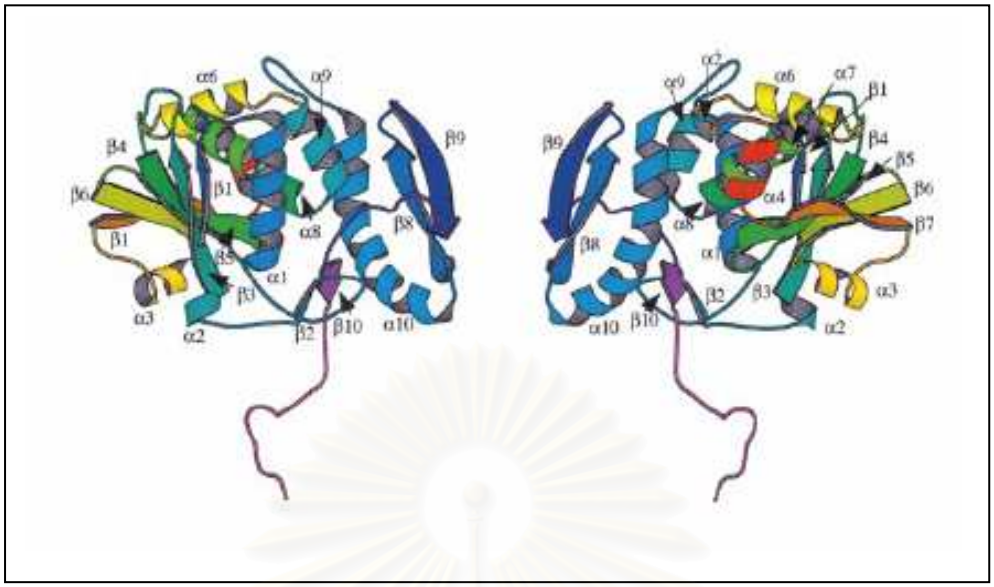


Figure 1.5 Ribbon diagrams of the minimized average structure of MAP30 with secondary structure identified by the program PROCHECK. The left and right views are rotated 1808 relative to one another (Bax *et al*., 1999).

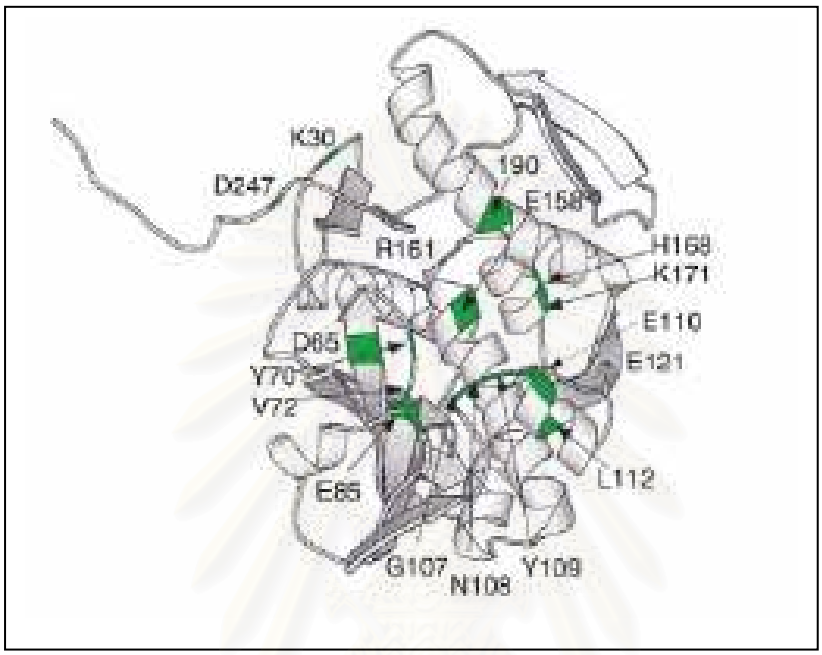


Figure 1.6 Secodary structure of MAP30. Ribbon diagram of MAP30 colored green to indicate residues whose chemical shifts were most sensitive tothe addition of the HIV LTR DNA (Bax *et al*., 1999).

These two types differs in that type I is a single chain protein where as type II. Subsequent work revealed that this particular site-specific RNA N glycosidase activity is a common property of all identified RIPs (Willy *et al.,* 2001). The N-glycosidase activity of MAP0, by sequence and topological homology to other RIPs, is assumed to reside in the highly conserved, deep pocket of MAP30. The residues believed to responsible for N-glycosidase activity are Tyr70, Tyr109 and Glu158 (Wang *et al.,* 2000). The Arg161 stabilizes the adenine binding at this groove.

1.5.2 DNA glycosylase/AP lyase: DNA topological Inactivation activity

In addition to RNA N-glycosidase activity, it was found that MAP30 relax supercoiled DNA irreversibly. It removes an adenine base from DNA via an apurinic/apyrimidic (AP) lyase activity which could result in DNA strand breaking (Putman and Tainer, 2000), making it an unsuitable substrate for either HIV integrase or DNA gyrase. The removal of adenine from DNA interacts with the MAP30's depurination site led us to hypothesis that MAP30 is also a DNA glycosylase which cause DNA damage. Wang *et al* (1999) proposed that this activity may contribute to the observed action of MAP30 in inhibiting viral integrase and DNA gyrase, Thereby exhibiting the anti viral and anti tumor property. Recent studies revealed that all RIPs contain both RNA N glycosidase and DNA N glucosidase activities (Peumans., 2001). An amino acid residue, trp190, which is a highly conserved residue of all RIPs was shown to bind atom the abasic DNA. The abasic DNA comes close to Lys 195 and attacks the C1'of the abasic site.

1.6 Role of MAP30 in plants

1.6.1 Defense protein

MAP30 is a ribosome inactivating protein that could play a role in plant defense. MAP30 as type 1 are not cytotoxic and exhibit no documented oral toxicity toward higher animals or invertebrates. From studying in many RIPs, the reports show type 1 RIPs have a direct effect on plant pathogenic. The antiviral activity of type 1 RIPs is well documented and attempts are under way to exploit RIP gene to protect transgenic plants viral infection and believed to protect the seeds they inhibit against plant-eating organism. The accumulation of MAP30 in storage organs offers an advantage in plant. The seed of mature fruits accumulate large quantities of endosperm or cotyledon specific protein for use as nutrient source during germination. These resources must be protected during grain fill and early stage of seeding growth when the seed is especially vulnerable to both mechanical damage and competition for storage reserves between the embryo and pests and pathogens. (Barbieri *et al*., 1993). Ribosome Inactivating Proteins (RIPs) from many sources have been shown to potently inhibit the infection of test plant with divers plant viruses. All RIPs with proven antiviral activity toward plant viruses are type I RIPs. Basically, three explanations can be put forward. First, RIPs act directly on the virus particles or viral nucleic acid by means of their polynucleotide: adenosine glycosidase activity. Second**,** RIPs selectively gain entrance to the cytosol of infected cells and destroy the protein synthesis machinery so that the virus cannot replicate and infect neighboring cells. In this so-called “local suicide” model, access to the cytosol becomes possible when the integrity of the plasma lemma is breached by a virus vector such as an aphiol or when cells are damaged during mechanical inoculation. Third, RIPs act indirectly though an activation of the plant’s defense (Willy *et al*., 2001)

1.6.2 Induction of MAP30 function

The alls RIP are plant defense protein including MAP30. Some RIP is induced by pathogen attack following the synthesis of signaling molecules such as jasmonic acid. It is in this defense function to biotic stress that RIP are generally seen. In fact, expression of a RIP in transgenic plants increased pathogen and virus resistance (Joerg *et al*., 1997). This figure 1.7 shows working model showing the direct defensive mechanism of RIPs against invasive viral and fungal pathogens. (1) RIPs are compartmentalized into cell walls. (2) Some RIPs possess dual properties, e.g. classical N-glycosidase activity and a novel enzymatic activity. (3) Upon infection, RIPs directly target the pathogens either in cytosolic or extracellular regions, and inhibit the growth of the invading microorganism. (4) RIPs are able to destabilize their own mRNA after its levels are up-regulated, probably by direct depurination of mRNA. (5) The originally proposed suicide defense mechanism of RIPs and (6) enzymatic target site, A4324, of RIPs in the S/R loop of large rRNA. The antiviral working mechanism was the finding that some RIPs (showing antiviral activity and toxicity) cause a constitutive expression of the acidic form of PR-1 (class II pathogenesis related protein PR-1). This acidic PR-1 is normally induced in plant by salicylic acid (SA). PR-1 and class II is form of PR-2, PR-3 and induced by SA and play a role in systemic acquired resistance, whereas class I isoform of pathogenesis-related proteins and induced by ethylene or by stress. It was suggest that RIP acts through the activation of a downstream regulatory signal that is common for both pathways. RIP activate an SA-independent signal transduction pathway reminiscent of the wounding response in plant. The transgenic RIP maybe the one set to a SA-independent stress-associated signal transduction that eventually leads to resistance against virus and fungi (Park *et al*., 2002).

1.7 Cloning and expression of MAP30

Lee-Huang *et al* (1990) isolated MAP30 from mature seed and ripe fruits of

M. charantia and purification to homogeneity by using 30-60% saturated ammonium sulfate, CM-Sephadex CL 6B and Sephadex G-75 (super fine). The molecule weight of the protein determined by SDS-PAGE is 30 kDa and consists of a single chain polypeptide. In 1995, they prepared large scale production of MAP30 in *E. coli* using pRSET, a pUC based vector. The recombinant plasmid of pRSET contains the gene. High expression was possible through the use of Lac promoter. Transcription was induced by adding of IPTG. Employing the advantage of indicating a short leader peptide containing 6 His residues at the N-terminus, simple and one step purification of the re-MAP30 in an affinity column charged with NiCl was achieved. They demonstrated that the re-MAP30 possesses identical activities of native MAP30 (nMAP30) although the protein was not glycosylated (Figure 1.8). The molecular mass of nMAP30 was a little bit higher, 30 kDa as compared to 29 kDa of re-MAP30 (Lee-Haung *et al.,* 1995). Treatment of the protein with 10% endopeptidases showed that the N- and C-terminal were removed whereas the central region of the protein was resistant to proteolysis (Huang *et al.,* 1999). The proteolytic fragment still retained full activity of HIV-integrase inhibition and HIV-LTR topogical inactivation. However, the ribosome inactivation property was loss. They concluded from this study that the anti-viral and anti-tumor activities of MAP30 are independent of ribosome inactivation activity. In 2002, Azari *et al* produced MAP30 from squash using ZYMV-AG II (Zuckini Yellow Mosaic Virus-AGII), a plant vector. The protein was purified using 25-75% saturated ammonium sulfate, DEAE-Sepharose, Sephadex G-75 and CM-cellulose. Their re-MAP30 also exhibits the anti-viral, anti-tumor and anti-microbial activities similar to the native MAP30.

In recognition of the novel activities of MAP30 and its promise as a potential therapeutic for HIV, other viral related diseases, and possible some type of cancerous cells, this study attempted to clone and overexpressed the *map30* gene from Mara Khee Nok, a local accession of *M. charantia*, in *E. coli*. The recombinant protein would be purified to

homogeneity and tested for some of its characters. In addition, MAP30 from Mara Khee Nok would be compared to that reported by Lee-Haung.

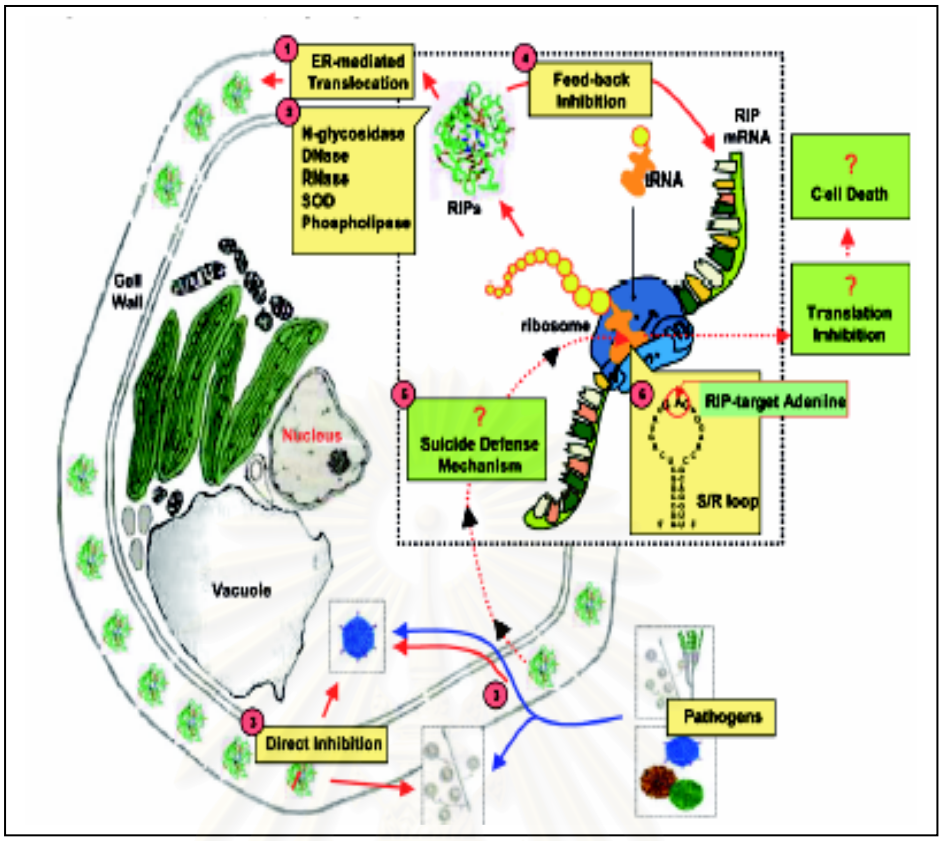


Figure 1.7 Role of RIPs in plant response. The mechanism of RIP response in plant cell when is infected with pathogen (Stirpe *et al.,* 2004)

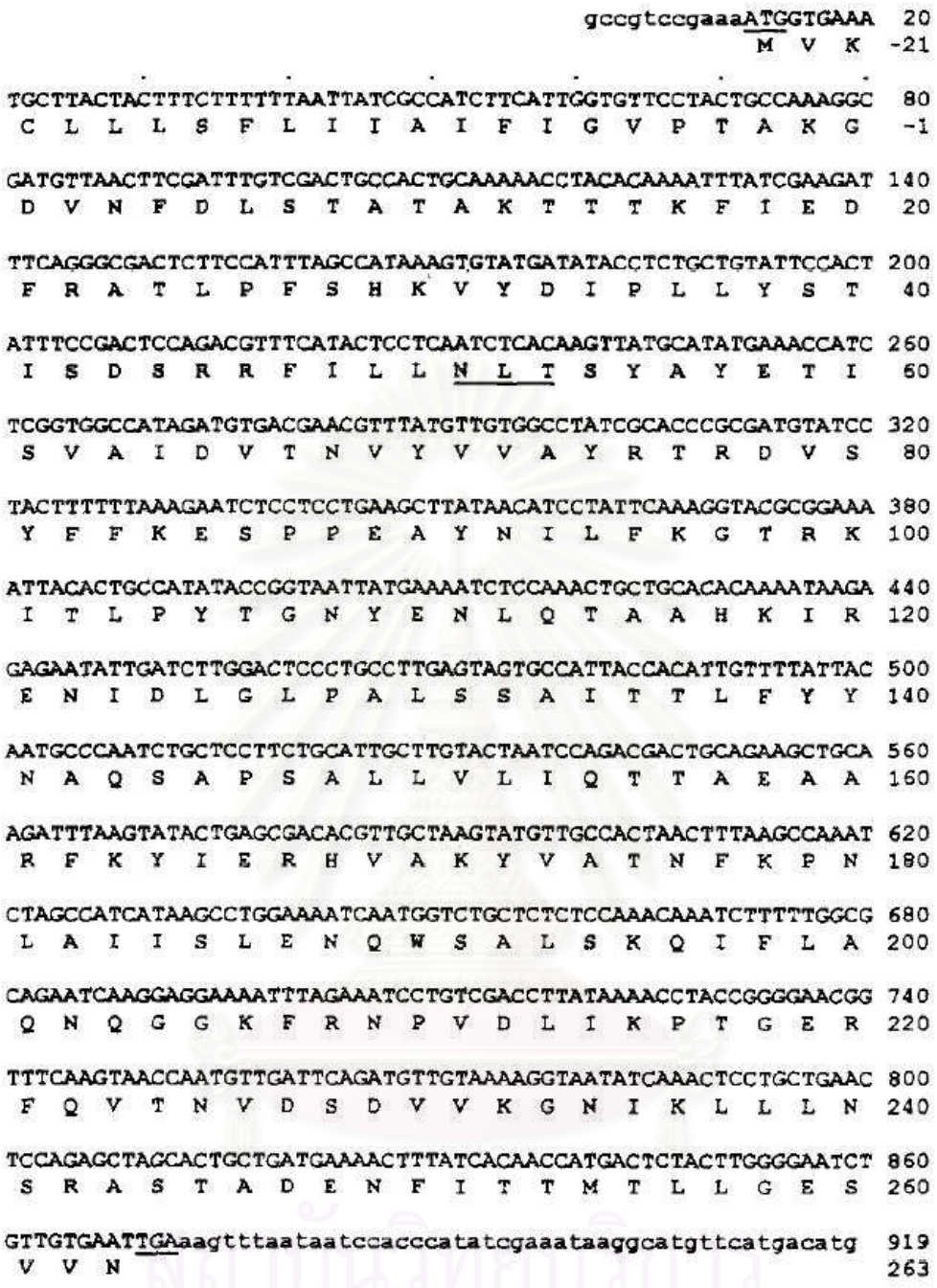


Figure 1.8 The nucleotides sequence of MAP30. The nucleotides sequence of MAP30 and the deduced amino acid sequence. The amino acid sequence in the mature protein is numbered from 1 to 263; the secretory signal sequence is numbered as -23 to -1. A putative N-glycosylation site and the stop codon are underlined.

(Lee-Haung *et al*., 1995)

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Autoclave: Model HA-30, Hirayama Manufacturing Corperation, Japan.

Autopipette: Pipetman, Gilson, France.

Centrifuge: Refrigerate centrifuge: Model J-21C, Beckman Instrument Inc., U.S.A.

Centrifuge High Speed: Microcentrifuged High Speed : Model 1110 Mikro 22R, Hettich Zentrifugen, Germany.

Electrophoresis Unit: Model Mini-protein II Cell, BioRad, U.S.A.

Gel documentation: SYNGEND, English

Gel dryer: Model 583, BIORAD, U.S.A

Fraction collector: Redifrac, Pharmacia Biotech, Italy

Incubator: Model OB-28L Fisher Scientific Inc., U.S.A.

Incubator shaker: Innova^{TM}4080, New Brunwick Scienfic, U.S.A.

Lumina flow: HT123, ISSCO, U.S.A.

Lyophilize: Flexi-dry™ MP, FTS system, U.S.A.

Orbital shaker: Gallenkamp, Germany.

Power supply for electrophoresis: Model EC 135-90, E-C Apparatus Corperation

Magnetic stirrer and heate : Type Mini MR standard, IKA® - WERKE, GMBH and CO. KG, Germany

Microwave oven: Model TRX1500, Turbora International Co. Ltd., Korea

Sequencer: Model CEQTM8000 Genetic Analysis system, Beckman Coulter, U.S.A.

Sonicator: SONOPLUS Ultrasonic homogenizers, BANDELN, Germany

Spectrophotometer: Jenway 6400, England.

Transformation apparatus: Gene pulser™, BioRad, U.S.A.

Thermal cycle:Gene Amp PCR system 2400, Perkin Elmer Cetus, U.S.A.

U.V. transilluminator: 2011 MA Crovue, San Gabrial, U.S.A.

Vortex: Model K 550-GE, Scientific Industries, U.S.A.

Water bath: Charles Hearson Co. Ltd., England.

.

2.2 Inventory supplies

Dialysis bags: tubing, Sigma, U.S.A.

Filter paper: Whatman 3 mm No 1, Whatman International Ltd., England

Microcentrifuge tubes 0.2, 0.5 and 1.5 ml: Axygen Hayward, U.S.A.

Pipette tips 10, 200 and 1,000 µl: Axygen Hayward, U.S.A.

Centricon: Amicon® centicon® centrifugalfilter, Millipore, U.S.A

Qiaquick Gel Extraction Kit: Qiagen, Germany

2.3 Chemicals

Acrylamide: Merck, Germany.

Agarose: SEAKEM LE Agarose, FMC Bioproducts, U.S.A.

Ammonium hydroxide: Merck, Germany.

Ammonium persulfate: Sigma, U.S.A.

Ammonium sulfate: Sigma, U.S.A.

Amplicilin: Biobasic Inc, Thailand.

Bacto-Agar: DIFCO, U.S.A.

Boric acid: Merck, Germany.

Bovine Serum Albumin (BSA): Sigma, U.S.A.

Bromophenol blue: Merck, Germany.

5-bromo-4-chloro-3-indoyl-b-D-galactopyranoside (X-gal) : Sigma, U.S.A.

Calcium chloride: Merck, Germany.

Carboxymethyl-Sephadex C50: Sigma, U.S.A.

Chloroform: Sigma, U.S.A.

Citric acid: Sigma, U.S.A.

Coomassie® brilliant blue R: Acros organics, Belgium.

Coomassie® brilliant blue G 250: Fluka, Switzerland.

Deoxyribonucleic acid (dATP, dTTP, dGTP and dCTP) : Promega, Co. Ltd., U.S

Di-potassium hydrogen phosphate anhydrous: Carlo Erba Reagenti, Italy.

Di-Sodium hydrogen phosphate: Fluka, Switzerland.

DNA marker: *Hin*d III digest of λ- DNA: BioLabs,Inc.,U.S.A.

100 basepair DNA ladder:: Promega Co.,U.S.A.

Ethidium bromide: Sigma, U.S.A.

Ethylenediamine tetraacetic acid (EDTA): Fluka, Switzerland.

Ethyl alcohol absolute: Carlo Erba Reagenti, Italy.

Ficoll type 400: Sigma, U.S.A.

Glacial acetic acid: BDH, England.

Glycerol: Scharlau, Spain.

Glycine: Sigma, U.S.A.

Hexadecyltrimethylammonium bromide (CTAB): Sigma, U.S.A.

Hydrochloric acid: Lab Scan, Ireland.

Isopropyl-β-D-thiogalactopyranoside (IPTG): Serva, Heidelberg, Germany.

Isoamyl alcohol: Merck, Germany.

Low molecular weight protein marker: Page ruler^{TM} protein ladder: Fermentas, U.S.A.

Magnesium sulfate 7-hydrate: BDH, England.

Methanol: Scharlau, Spain.

β-Mercaptoethanol: Fluka, Switzerland

N, N'-methyl-bis-acrylamide: Sigma, U.S.A.

NNN'N'-tetramethyl-1, 2-diaminoethane (TEMED): Carlo Erba Reagenti, Italy.

Phenol: BDH, England.

85%Phosphoric acid: Lab Scan, Ireland.

Phosphocellulose P11: Whatman® BioStstems Ltd., U.S.A.

Potassium acetate: Merck, Germany.

Potassism chloride: Sigma, U.S.A.

Potassium phosphate monobasic: Carlo Erba Reagenti, Itly.

Sodium azide: BDH, England.

Sodium chloride: Univar, Australia.

Sodium dihydrogen orthophosphate: Carlo Erba, Italy.

Sodium dodecyl sulfate: Sigma, U.S.A.

Sodium hydroxide: Carlo Erba, Italy.

Silver nitrate: Baker Analyed, Germany.

Tris-base: USB, U.S.A.

Triton x-100: Merck, Germany.

Tryptone: Scharlau, Spain.

Xylene cyanol FF: Sigma, U.S.A.

Yeast extract: Scharlau, Spain.

2.4 Enzymes

Restriction endonuclease

: *Bam*HI, *Pst*I, *Nco*I, *Nde*I and *Sal*I were purchased from New England, Biolabs Inc, U.S.A.

: *EcoR*I, *Hind*III were purchased from GIBCOBRL, U.S.A.

Lysozyme was purchased from Sigma, U.S.A.

Pfu DNA polymerase was purchased from Promega, Co. Ltd., U.S.

RNaseA was purchased from Sigma, U.S.A.

T_4 DNA ligase was purchased from Promega, Co. Ltd., U.S.A.

2.5 Plasmid vectors

pUC18 was used as vector for *map30* (map30 gene) cloning and DNA sequencing (Appendix G)

pET19b was used as overexpression vector for *map3*0. (Appendix H)

2.6 Oligonucleotide primers

Primers: All primers were designed from *map30* Lee-haung *et a*l., (1995), mapFE, mapRB and mapFN were synthesized by Sigma, ASO2 and SO2: U.S.A. and BSU: Thailand

2.7 Organisms

2.7.1 *Momordica charantia*

Strains were Mara Khee Nok commercially available at local market.

2.7.2 Bacterial strains

: *Escherichia coli* JM109, genotype: F/ [*tra*D36 *pro*AB$^+$ *lac*I^q *lac*ZΔM15] *recA*1 *endA*1 *gyrA*96 *hsdR*17 *supE*44 *relA*1 *thi* Δ(*lac-proAB*) was used as

a host for cloning *map30.*

: *Escherichia coli* Rosetta (DE3) pLysS, genotype: F^- [*omp*T *hsdS* (r_B^- m_B^-) *gal dcm lac*Y1] (DE3) pLysS RARE (Cm^R) which provided rare codon tRNAs was used as high-stringency expression host.

2.8 Cultivation of bacteria

Luria-Bertani broth (LB medium)

LB broth consists of 1% (w/v) Bacto tryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl. The medium was adjusted to pH 7.0 with NaOH. For solid medium, 1.5% (w/v) Bacto agar was added and was supplemented with appropriate antibiotics when used as selection media.

2.9 Sample preparation

Young leaves of bitter melon which were freed of infection were collected and washed with appropriate amount of distilled water. The sample could be used freshly or stored at -80 ^oC until used.

2.10 Preparation of *map30*

2.10.1 Chromosomal DNA extraction

Genomic DNA from leaf tissue of freshly grown bitter melon (Mara Khee Nok) was extracted in CTAB isolation buffer according to the protocol of Doyle and Doyle (1987). One gram of leaf tissue was washed in appropriate volume of distill water. The leaves were grounded at 4 ^oC in mortar with 500 µl of 60 ^oC preheated CTAB buffer (2% CTAB, 1.4 M NaCl, 0.2% β-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0). Then 200 µl of CTAB buffer was added again into the sample homogenate before it was transferred to a new 1.5 ml Eppendorf tube and incubated at 60 ^oC for 45 minutes with occasional gentle swirling.

To remove protein and undesirable compounds, the extract was added with 700 µl of chroloform-isoamylalcohol (24:1) and gently mixed for 10 minutes. Then the mixture was centrifuged for 15 minutes at 12,000 rpm at 4 ^{o}C, two separated phases would appear. Six hundred microlitres of the upper phase was pipetted to a new 1.5 ml Eppendorf tube, added 2/3 volume of cold isopropanol, gently mixed and left stand at -20 ^{o}C for at least 1 hour for nucleic acid precipitation. The precipitate was collected by centrifugation for 15 minutes at 6,000 rpm, 4 ^{o}C and the supernatant was removed. The pellet was washed one with 70% ethanol, discarded the alcohol solution carefully and allowed the tube to air dry briefly at room temperature, followed by resuspending the precipitate in 400µl TE buffer (10mM Tris-HCl, 1 mM EDTA, pH 7.4). At this stage, the nucleic acid preparation was composed of both DNA and RNA. To remove RNA, RNaseA solution was added to make a final concentration of 10µg/µl and incubated the mixture for 30 minutes at 37 ^{o}C. Residual protein was removed by adding 1 volume of phenol (saturated in TE buffer)/chroloform/isoamylalcohol (24:1:1) and gently mixed for 10 minutes until the solution became welly mixed. The two phases was separated by centrifugation at 12,000 rpm for 15 minutes and 500µl of the upper phase (DNA fraction) was transferred to a new 1.5 ml Eppendorf tube. The DNA solution was diluted by adding a half volume of 7.5 M ammonium acetate buffer pH 7.0. DNA was reprecipitated by adding 2.5 volumes of cold absolute ethanol with gently mixing, left at -20 ^{o}C for 30 minutes. The DNA was recovered by centrifugation at 10,000 rpm 4 ^{o}C for 10 minutes and alcohol was removed by air dried at room temperature. Finally, the DNA pellet was resuspened in appropriate amount of TE buffer and incubated at 37 ^{o}C, 30 minutes until completely dissolve. DNA quality was determined using the ratio of A_{260}/ A_{280} and investigation by 1% agarose gel electrophoresis. The DNA preparation would be kept at 4 ^{o}C until further used.

2.10.2 Amplification of map30 gene by PCR technique

2.10.2.1 Primer designed

The purified genomic DNA from bitter melon leaves was used for amplification of *map30* using appropriate primers based on gene sequence reported by Lee-Haung (1995). The forward primer (mapFE) was designed from 5'-nucleotide sequence of *map30* and was further modified by adding of *Eco*R I restriction site before the full length gene. The reverse primer (mapRB) was designed from 3'-end of *map30* by adding of *Bam*H I site enzyme restriction following the stop codon (TGA) of the gene. For high expression, the forward primer mapRB was used in which the Shine-Dalgarno sequence of expression vector pTrc99 was added to mapFE before the start codon (ATG) of matured *map30* (excluded the leader sequence). Full length, map30 gene was amplified by polymerase chain reaction (PCR) using *pfu* DNA polymerase and the PCR product was used for cloning and gene expression in *Escherichia coli*. The properties of these primers were summarized in Table 2.1. The binding location to the *map30* was shown in Figure 2.1. The primer pair of ASO2 and SO2 was used for sequencing of internal *map30* gene.

2.10.2.2 PCR amplification

The Polymerase Chain Reaction (PCR) was carried out in a Thermal Cycler (Gene Amp PCR system 9600, Perkin-Elmer, USA.) using a three-temperature program for 30 cycles (Figure2.2). The condition was modified from that reported by Lee-Haung (1995), consisted of pre-heat the DNA at 94 ^{o}C for 5 minutes, started the cycle with denaturation at 94 ^{o}C for 30 seconds, annealing at 60 ^{o}C for 45 seconds and extension at 72 ^{o}C for 45 seconds, After completion of 30 cycles, the extension phase was prolonged to 10 minutes at 72 ^{o}C for complete amplification. The reaction mixture was carried out in a final volume of 100 µl containing 0.5-1.0 µg bitter melon genomic DNA, 0.2 mM of each dNTPs (dATP, dTTP, dCTP and dGTP), 50 pmol each of appropriate forward and reverse primers, 2.5 units of *pfu* DNA polymerase and 1x PCR reaction buffer (2.5 mM Mg_2Cl, 10 mM Tris-

Cl, 50 mM KCl, pH8.3). The size of PCR product was analyzed by comparing with the relative electrophoretic mobility of λ/*Hind* III DNA marker and 100 bp DNA ladder on 1.5% agarose gel.

2.10.2.3 Analysis of DNA fragment by agarose gel electrophoresis

The size, concentration and quality of DNA were determined in 0.7-1.5% agarose gel. Appropriated amount of agarose powder was added to 100 ml electrophoresis buffer TBE (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0) in an Erlenmeyer flask and heated in microwave oven until completly solubilized. The agarose solution was cooled down to below 60°C and air bubbles were completely eliminated. The solution was then poured into an electrophoresis mould. After the gel was completely set, the comb and seal of the mould was carefully removed. When ready, the DNA samples (~1 μg) was mixed with 1x tracking dye (0.025% bromophenol blue, 0.025% xylenecyanol FF, 1.5% ficoll400 and 5% SDS) were loaded into the wells. The gels were run at 100 volts (10 volts/cm) for 1 hour, or until bromophenol blue almost reached the bottom of the gel. After electrophoresis was completed, the gel was stained with ethidium bromide solution (2.5 μg/ml) for 2-5 minutes, destained with 1xTBE buffer for 10 minutes and the DNA bands were visualized under UV light (302nm) from a UV transilluminator. The gels were photographed through the Syngene gel documentation. The molecular weight of DNA fragment was determined by comparing its relative electrophoretic mobility with those of the standard DNA (λ/*Hind*III and 100 bp DNA ladder).

2.10.2.4 Extraction of DNA fragments from agarose gel

QIAquick gel extraction kit was used for extracting DNA from agarose gel, and performed according to the kit protocol. Briefly, gel containing the desired DNA

band was sliced from the agarose gel weighed and placed in a new 1.5 ml Eppendorf tube. Three volumes (3µl/µg) of QG buffer was added and incubated for 10 minutes at 50 °C. After the gel slice had dissolved completely, the sample was applied to the QIAquick spin column and centrifuged at 10,000 rpm for 1 minute. At this step, the DNA was bound to the membrane. The flow-through was discarded. The column containing the DNA was washed twice with PE buffer and centrifuged at 10,000 g for 1 minute. Finally, the EB elution buffer was added to the center of the QIAquick membrane to elute the DNA. The column was left stand for 1 minute, and then centrifuged at 10,000 rpm for 1 minute. The recovered DNA was kept at 4 ^{o}C until used.

Table 2.1 Sequences of Oligonucleotide primer designed from the nucleotide sequence of *map30* gene (Lee-Haung, 1995)

Primers name	sequence	Tm (°C)	Primer length (bp)	application
mapFE	*EcoRI* Shine Dalgarno sequence start 5'-cg gaattc cg aggaaagagacc atg gat gtt aac ttc gat ttg tcg act gcc-3'	70 °C	52	5' *map30* primer for cloning into pUC vector
mapFN	*NcoI* start 5'-catg ccatgg at tca aac ttc gat ttg tcg act gcc ac-3'	72 °C	38	5' *map30* primer for cloning into pET vector
mapRB	*BamHI* stop 5'-cg ggatcc a tca attc aca aca gct tcc cca agt aga gtc a-3'	73 °C	41	3' *map30* primer for cloning into pUC and pET vector
AS02	5'-catctcggtggccatagatgtgac-3'	60 °C	24	Internal amplification for *map30* sequencing
S02	5'-tctgctgacgtcttcgacgt-3'	60 °C	24	Internal amplification for *map30* sequencing

Figure 2.1 The binding position of forward and reverse primers to the *map30*. The gene sequence was reported by Lee-Haung (1995) and red arrow shows primer direction (⟶)

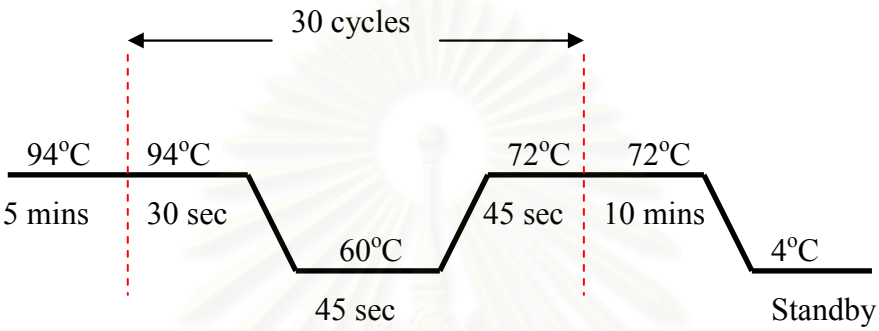


Figure 2.2 The cycle program of Polymerase Chain Reaction for amplification *map30* gene from bitter melon genomic DNA.

2.11 Cloning of *map30* gene into pUC18 vector

2.11.1 Preparation of pUC18 and *map30* gene fragment

Both pUC18 vector and the PCR product from 2.10.2.4 were completely digested with *Nco*I and *BamH*I. The 100µl reaction mixture containing 5 µg of the DNA, 1x *Nco*I reaction buffer (50mM Tris-HCl, pH 8.0, 10 mM Mg_2Cl and 100 mM NaCl), 10 U of *Nco*I and 10 U *Bam*HI was incubated at 37 ^{o}C, overnight. The DNA digest was electrophoresed in 0.7 % agarose gel in 1xTBE buffer. The linear plasmid and putative *map30* fragment were analyzed and compared with DNA marker (λ/*Hind* III DNA marker and 100 bp DNA ladder). The desired DNA fragment (~800) was harvested from agarose gel by method as described in 2.10.2.4.

2.11.2 Construction of recombinant pUCmap

The *map30* and pUC18 fragment from 2.11.1 were ligated in 20 µl reaction volume containing 50 ng of pUC18, 250 ng of *map30* gene fragment, 1x ligation buffer (66 mM Tris-HCl, pH 7.6, 6.6 mM Mg_2Cl, 10mM DTT and 66 µM ATP) and T_4 DNA ligase (Tested User Friendly, USA). The reaction mixture was incubated at 14 ^{o}C for 16 hours. The ligation products were used for transformation.

2.11.3 Transformation of ligation products into *E. coli* host cells by electroporation

2.11.3.1 Preparation of competent cells

A single colony of *E. coli* strain JM109 or Rosetta (pLysS) was cultured overnight as a starter in 2 ml LB-broth at 37 °C with 250 rpm shaking. The starter was made to 200 ml with LB-broth and the culture was incubated at the same condition until the optical density of the cells at 600 nm reached 0.5-0.6 (~3-4 hours). Then the culture was chilled in ice for 15 minutes and the cells were harvested by centrifugation at 5,000 rpm for

15 minutes at 4 °C. The cell pellet was washed and suspended twice with 1 volume and 0.5 volume of cold sterile ultrapure water, respectively. The suspended cell was centrifuged at 6,000 rpm for 15 minutes at 4°C and the supernatant was discarded. The pellet was collected and washed with 10 ml of ice cold sterile 10% (v/v) glycerol, and finally resuspended again in 1-2 ml of ice cold sterile 10% glycerol. The resulting cell suspension was divided into 40 µl aliquots and store at -80°C until used.

2.11.3.2 Electrotransformation

The competent cells from 2.11.3.1 were thawed on ice before used. Forty microliters of the cell suspension were mixed with 1-2 µL(1µg) of the ligation product from 2.11.2, gently mixed, and placed on ice for 1 minute. The mixture in a cold 0.2 cm cuvette was electroporated by one short pulse with the apparatus setting of Gene Pulser, (Biorad) as follows : 2.5 mF, 200 Ω of the pulse controller unit and 2.50 kV. The electroporated mixture was immediately added with 1 ml LB medium and incubated with 250 rpm shaking at 37^{o}C for 1 hour. Approximately100 µl of this cell suspension was then spreaded on a selective LB agar plate containing 50 µg/ml amplicilin, 40µl (20mg/ml) X-gal and 35 µl (40 mg/ml) IPTG. The plate was incubated at 37°C overnight to select for *map30*-containing colonies.

2.11.4 Selection of map30 positive clone

2.11.4.1 blue/white colony selection

Cells containing pUC18 plasmid that has amplicilin resistant gene can grow on amplicilin selection plate. The β-galactosidase gene of pUC can be induced by IPTG to produce β-galactosidase that can cleave X-gal, resulting in the appearance of blue colonies. Upon insertion of DNA fragment into the β-galactosidase gene locus, the β-

galactosidase in the recombinant plasmid becomes inactivate and the cell loss ability to cleave X-gal which is colorless. Therefore the cell which contains the recombinant plasmid will grow as white-colony. Thus, white-colony clones were selected and preceded for recombinant plasmid characterization. The recombinant pUC18 plasmid harboring *map30* would be named pUCmap.

2.11.4.2 Plasmid extraction

Plasmid was extracted by the alkaline lysis method of Sambrook and Russell, (2001). Transformed white colony was randomly picked to culture in a test tube containing 1.5 ml LB broth at 37 ^{o}C for 1 hour and harvested by (transferring the cell culture to a new 1.5 ml Eppendorf tube) centrifugation at 4°C, 5,000 rpm for 3 minutes. The packed cells were resuspended in 100 µl of Solution I (25 mM Tris-HCl, pH 8.0, 10 mM Na_2EDTA and 50 mM glucose), mixed well by vortexing and kept on ice for 15 minutes. A 200 µl of freshly prepared Solution II (1% SDS and 0.2 N NaOH) was then added, mixed by inversion and kept on ice for another 5 minutes. The mixture was neutralized by adding 150 µl of Solution III (3 M sodium acetate, pH 4.8), mixed by inversion and kept on ice for 30 minutes. After centrifugation at 12,000 rpm, 4 ^{o}C for 10 minutes, the supernatant was transfered to a new 1.5 ml Eppendorf tube and extracted with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). The two phases was separated by centrifugation at 12,000 rpm for 15 minutes and 500µl of the upper phase (DNA fraction) was transferred to a new 1.5 ml Eppendorf tube. Two volumes of absolute ethanol was added, mixed and placed the tube at –20 °C for 30 minutes. The plasmid was pelleted by centrifugation at 12,000 rpm for 10 minutes, washed with 70% ethanol and air dried. The plasmid was finally dissolved in TE buffer containing 20 µg/ml DNase-free RnaseA and stored at 4 ^{o}C until used.

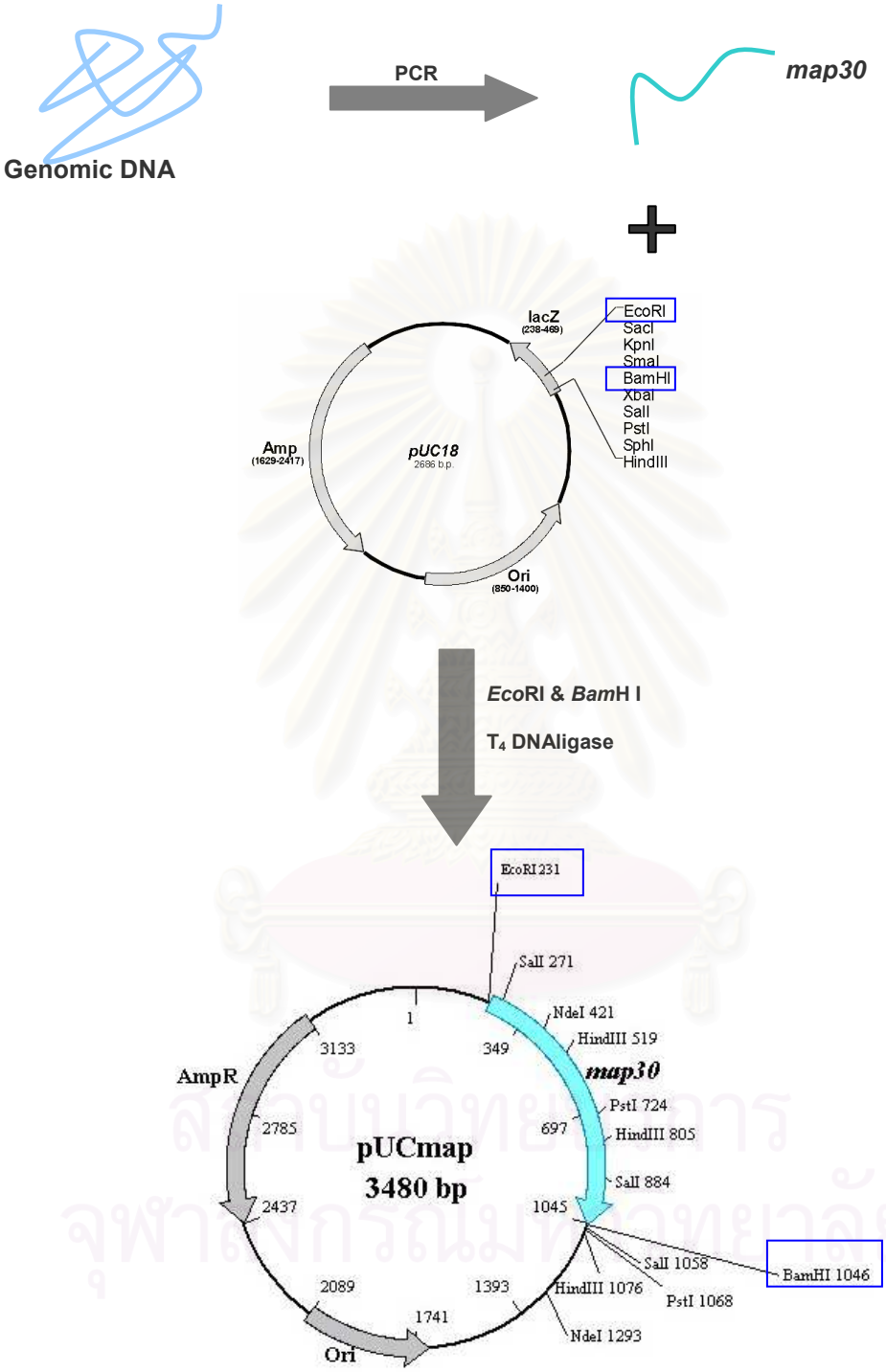


Figure 2.3 Diagrammatic presentation of pUCmap construction.

2.11.5 Characterization of pUCmap

2.11.5.1 Identification of pUCmap by restriction mapping

For detection of recombinant cells harboring pUCmap, the extracted plasmids from white colonies were identified by cutting with both *EcoR*I and *Bam*HI. The digested products were electrophoresed through 1.5% agarose gel as described in 2.6.1 and estimated for their sizes by comparing with DNA marker (λ/*Hind* III and 100 bp DNA ladder) and linearized pUC18. Plasmid which contains *map30* should give product of ~800 bp in size, together with a fragment size of linear pUC18. Such recombinant plasmid would be used for *map30* sequencing and for subcloning the *map30* gene into pET vector for high expression of the gene. In addition, the location of the *map30* gene was confirmed by restriction map using NEBcutter program (http://tools.neb.com/NEBcutter2/index.php) . The restriction mapping was done by digesting the plasmid with *BamH*I, *Nco*I, *Pst*I, *Nde*I, *Hin*dIII and *Sal*I. The cutted products were electrophoresed through 1.5% agarose gel and compared with 100 bp DNA ladder and λ/*Hin*dIII

2.11.5.2 Nucleotide sequencing and data analysis

pUCmap was extracted and *map30* gene was sequenced by Bio Service Unit, Thailand. The obtained nucleotide sequence was analyzed by Genetyx version 3.1 program for open reading frame prediction, deduced amino acid sequence, estimation of molecular weight and isoelectric point. To compare the *map30* gene of Thai bitter melon with that of Lee-Haung and other RIPs deposited in the GenBank database, the nucleotide sequence and deduced amino acid sequence were analyzed by BLAST search (www.ncbi.nlm.nih.gov/) and the most homologous sequence was

aligned by ClustalW program. Homology modeling of the structure of this protein was accomplished by SWISS MODEL version 36.002 (http://swissmodel.expasy.org/).

2.12 Expression of *map30* gene in *Escherichia coli* JM109 by pUCmap

2.12.1 Optimization of induction time

After obtaining the putative *map30* recombinant clones, a single recombinant colony was picked from a freshly streaked nutrient agar plate and inoculated into 50 ml LB broth containing 100 µg/ml amplicilin in a 250 ml Erlenmeyer flask. The culture was incubated with shaking at 37 ^{o}C until A_{600} reached 0.6. Then 0.5 ml was taken to serve as uninduced control. The remainder suspension was added with IPTG from 0.5 M stock to make a final concentration of 1 mM and continued the incubation for another 6 hours. At time intervals (0, 0.5, 1, 2, 3, 4 and 6 hour), 0.5 ml of culture medium was collected. To determine for protein expression, the cell pellet was harvested by centrifugation at 5,000 rpm for 10 min at 4 ^{o}C and washed with the 50 mM phosphate buffer pH 6.0. The cell was completely resuspended in 500 µl of the same buffer and lysed by pulses of sonication. The protein concentration of cell lysate was measured by using Bradford method. For protein pattern analysis and determination of 30 kDa protein, 50 µl (5-10 µg protein) of the lysate was mixed with 50 µl 2x sample buffer, heated for 5 minutes in boiling water and subjected to SDS-PAGE analysis.

2.12.2 Protein measurement

Protein concentration was determined by the method of Bradford (1976). Various concentrations of protein solution (maximum 100 µl) were pipetted into each

test tube. Appropriate volume of 50 mM phosphate buffer was added to make a total volume of 100 µl. Then 1 ml of Bradford working buffer (Appendix A) was added and mixed. The solution was incubated at room temperature for at least 2 minutes but not more 1 hour. A_{595} was read against blank and the protein concentration was calculated from the standard curve using bovine serum albumin as standard (Appendix B).

2.12.3 SDS-Poly Acrylamide Gel Electrophoresis

2.12.3.1 Preparation of separating gel (12% acrylamide)

The gel sandwich was assembled according to the manufacturer's instruction. For 2 slab gels, 2.33 ml of Solution A (30%(w/v) acrylamide, 0.8%(w/v) bis-acrylamide and 2.5 ml of Solution B (1.5 M Tris-HCl, pH 8.8) were mixed with 5.17 ml of distilled water in a 25 ml Erlenmeyer flask and mixed. Then 50 µl of 10% ammonium persulfate and 10 µl of TEMED were added and mixed rapidly by swirling the flask gently. The solution was carefully introduced into the gel sandwich by using a Pasteur pipette. After the appropriate amount of separating gel solution was added, gently layered about 1 cm height of water on top of the separating gel solution. The gel was allowed to polymerize, distinguished by a clear interface between the separating gel and the water. The water was then poured off.

2.12.3.2 Preparation of stacking gel (5% acrylamide)

The stacking gel was prepared by mixing 0.67 ml of Solution A [30%(w/v) acrylamide, 0.8%(w/v) bis-acrylamide] with 1.0 ml of Solution C (0.5 M Tris-HCl, pH 6.8) and 2.3 ml distilled water in a 25 ml Erlenmeyer flask and

mixed. Subsequently, 50 µl of 10% ammonium persulfate and 10 µl of TEMED were added and mixed rapidly. This stacking solution was loaded on to the separating gel layer until solution reached the top of the short plate. Then the comb was carefully inserted into gel sandwich. After the stacking gel was polymerized, the comb was removed carefully. The gel was then placed into an electrophoresis chamber which was filled with electrophoresis buffer (25 mM Tris and 192 mM Glycine, pH 8.8) in the inner and outer reservoir.

2.12.3.3 Sample preparation

The protein sample was mixed with 5x sample buffer (0.3 mM Tris-HCl, 50% glycerol, 25% β-mercaptoethanol and 0.05% bromophenol blue) to make a final concentration of 1x in a 1.5 ml Eppendorf tube and mixed. The mixture was heated for 5 minutes in boiling water before it was introduced into the well by mean of an autopipette.

2.12.3.4 Running the gel

Electrode plugs were attached to electrodes in which the current was flowed towards the anode for pH 8.8 gel. The power supply was turned on at constant current (30 mA). Electrophoresis was continued until the dye front almost reached the bottom of the gel. Power supply was then turned off and the electrode plugs were removed. The gel plate was removed from the electrophoresis chamber, and the gel slab was removed from glass plates carefully using a scrapple before it was transferred to a small plastic box for protein staining.

2.12.3.5 Protein staining

The gel slab was stained with Coomassie solution (1% Coomassie Blue R-250, 45% methanol and 10% glacial acetic acid). The gel was agitated gently for 20-30 minutes on a shaker. After completion, the staining solution was poured out and replaced with destaining solution (10% methanol and 10% glacial acetic acid). The gel was again agitated slowly. To completely destained, the destaining solution was changed many times during agitation overnight or until clear blue bands of protein could be visualized.

2.13 High expression of *map30* gene using pET vector

2.13.1 Subcloning of *map30* gene to pET19b vector

The pET19b vector was prepared from the pET19b harboring Escherichia *coli* JM109 cell. The cell was culture in LB broth containing 100 µg/ml amphicilin with constant shaking at 37 ^{o}C for 18 hours. Cells were harvested by centrifugation at 5,000 rpm for 10 minutes and used for plasmid extraction (see 2.6.4.2). The plasmid was double digested with *Nco*I and *Bam*HI and the resulting product were separated by electrophoresis in 1% agarose gel. The linear pET19b fragment was extracted and purified by gel extraction kit as described in 2.10.2.4 and used for subcloning. To subclone, the recombinant plasmid pUCmap was used as template for *map30* gene amplification using specific primers mapFN and mapRB (primer described in 2.10.2.2.). The ~800 bp PCR products was isolated, purified and

ligated with the linearized pET19b by the same method described in 2.10.2.4. This recombinant plasmid was name pETmap. The pETmap was electrotransformed into *Escherichia coli* Rossetta DE3 (pLysS) by the same method described in 2.11.3.2**.** After transformation, cells were spreaded on selection agar plate containing 36 μg/ml chloramphenicol and 100 μg/ml amphicilin for selection of positive colony. The positive clone was colony which has the characteristic chloramphenicol resistance gene of host plasmid and amphicilin resistance gene of pETmap. The selected recombinant colony containing the plasmid pETmap was then maintained by streaking in nutrient agar plate. The figure 2.4 shows a brief outline of plasmid pETmap construction.

2.13.2 Characterization of positive clone

The plasmid was extracted as described in 2.6.4.2. For characterization of pETmap, the extracted plasmids were restriction cutted by both *Nco*I and *Bam*HI. The cutted products were electrophoresed through 1.5% agarose gel and estimated for their sizes by comparing with DNA marker (λ/*Hind* III and 100 bp DNA ladder). The recombinant plasmid containing the *map30* gene should give a product of ~800 bp in size. Then, characterization of the recombinant plasmid was done by enzyme-restriction mapping as described in 2.11.5.1

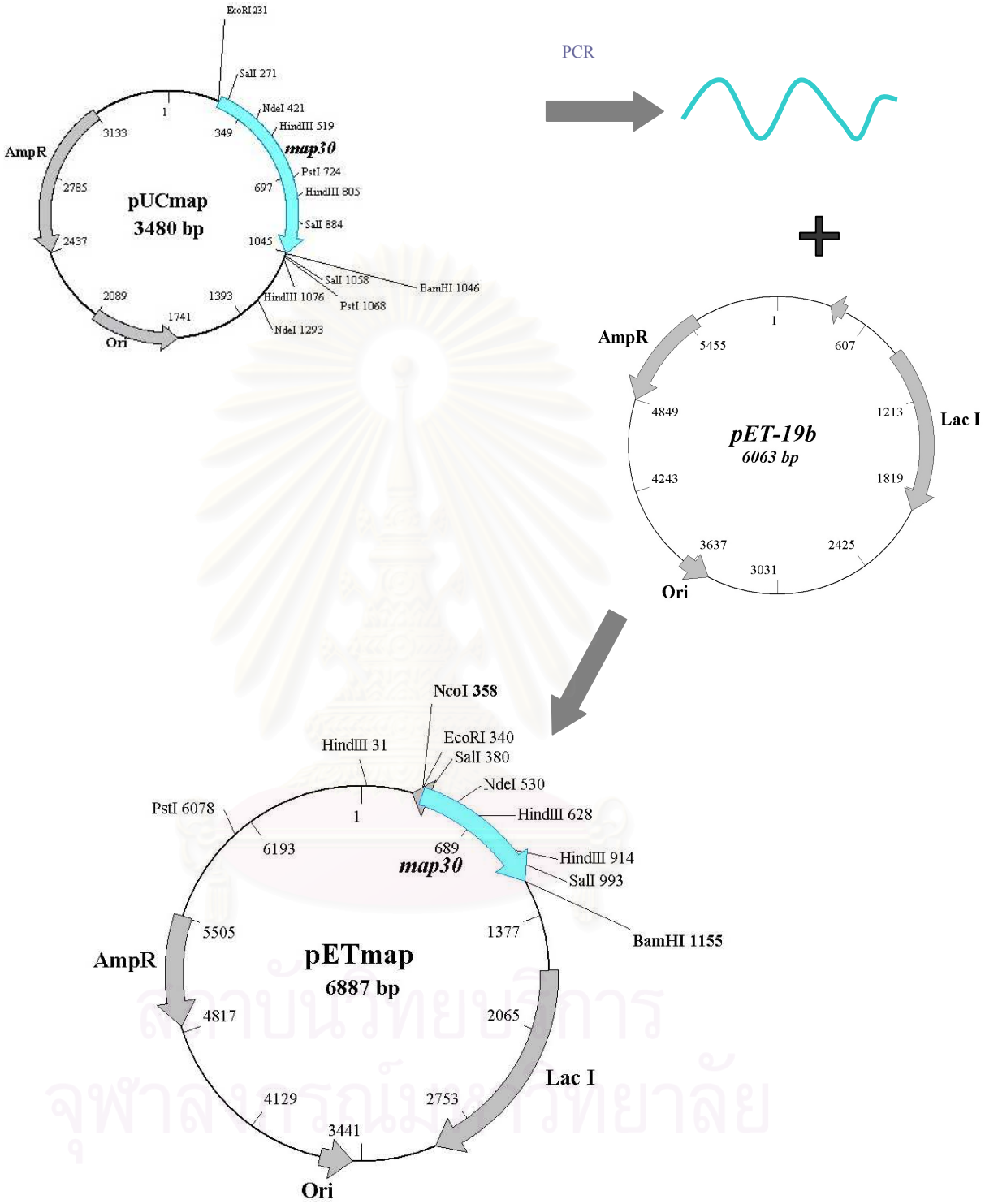


Figure 2.4 pETmap constructions.

2.13.3 Cultivation of recombinant clone

A single colony of *E. coli* strain Rosetta DE3 (pLysS) harboring the expression plasmid pETmap was inoculated into 5 ml LB medium containing 36 μg/ml chloramphenicol and 100 μg/ml amphicilin and incubated with shaking (about 200 rpm) at 37 °C until a A_{600} value of 0.6 – 0.7 was reached. The cells were collected by centrifugation at 5,000 rpm for 5 minutes, resuspended in 5 ml of fresh LB medium and inoculated into a flask containing 500 ml LB medium and cultivated as described previously.

Recombinant cell harboring pETmap was cultured in LB medium as described above. When the A_{600} value reached 0.6, IPTG was added to make a final concentration of 1 mM for expression induction. Five milliliter of the cell culture was drawn after induction for 0.5, 1, 2, 3, 4 or 6 hrs. At each period, collected cells were washed and resuspended in 500 μl of 50 mM phosphate buffer, pH 6.0 and lysed by sonication on ice with half second pulse and half second stop interval for 10 minutes by Sonic Dismembrator (3 mm diameter-stepped microtip, 10 amplitude). The protein concentration of cell lysate was measured by Bradford method. For screening of 30 kDa inducible protein 50 μl (5-10 μg protein) of the lysated was mixed with 50 μl 2x sample buffer. The mixture was heated for 5 minutes in boiling water and subjected to SDS-PAGE analysis as described in 2.12.3**.** Sample from uninduced cells was served as control. The appropriated induction time would be used in future experiments.

2.13.4 Optimization of gene expression

2.13.4.1 Induction time

To also investigate MAP30 localization in the induced cell, 5 ml of cell culture was collected at each induction period. Cells were treated as before mentioned. Proteins from soluble and insoluble fractions were used to screen for 30 kDa inducible protein by SDS-PAGE analysis.

2.13.4.2 IPTG concentration

The recombinant cell was cultured and induced with IPTG as described previously. In this experiment, the concentration of IPTG was varied to a final concentration of 0.1, 0.5 and 1 mM, respectively, using the appropriate induction time from 2.13.4.1.After centrifugation at 5,000 rpm 10 minutes, cells were harvested. There after, cells were lysed and screened for MAP30 as described in 2.13.4.1. The appropriate concentration of IPTG would be used in future experiments.

2.13.4.3 Temperature for expression

The recombinant cell was cultured and induced with IPTG but varying the culturing temperature at 25, 30 and 37 °C, respectively. Cells were again harvested, lysated and investigated for MAP30 by SDS-PAGE analysis. .

2.14 MAP30 purification

Since MAP30 was found as soluble protein as well as inclusion bodies, both forms would be fractionated purified and tested for topoisomerase activity. The highly purified concentrated inclusion bodies would only be submitted to purification by CM-Sephadex C50 and phosphorcellulose P11 chromatography. A summary of the purification steps is presented in Figure 2.5.

2.14.1 Preparation of soluble MAP30

2.14.1.1 Preparation of crude protein

Five hundred milliliters of induced cell culture was centrifuged at 3,000 g for 10 minutes at 4 °C. The cell pellet was washed twice with appropriate volume of 50 mM buffer containing 50 mM Tris-HCl, 1 mM EDTA, 1 mM β-mercaptoethanol and 1 mM PMSF,

pH 7.5 and resuspended in 50 ml of the same buffer. The cells were sonicated on ice until the solution became clear and not viscous. After centrifugation at 12,000 rpm for 30 minutes, the supernatant (crude extract) and pellet were stored in 4 ^{o}C for further analysis and purification.

2.14.1.2 Ammonium sulfate precipitation

All steps were carried out at 4 ^{o}C. The crude protein was slowly added with solid ammonium sulfate to 30% saturation with gentle stirring by a magnetic stirrer. After let stand for 30 minutes in ice, the supernatant was collected by centrifugation at 12,000 rpm for 30 minutes and then brought to final concentration of 60% saturation with solid ammonium sulfate. The solution was left for another 30 minutes on ice with continuous stirring and was subsequently centrifuged at 12,000 rpm for 30 minutes. The precipitate was dissolved in appropriate amount of 50 mM phosphate buffer, and the protein solution was exhaustively dialyzed against the same buffer for 3 times (at least 4 hours). The dialysate was concentrated by showering with aquasorb powder on to the dialysis bag in a plastic box. This was done until only half volume of dialysate remained. The protein solution was determined for topoisomerase activity, protein pattern analysis by SDS-PAGE, and protein measurement by Bradford method. The active fraction was purified further by column chromatography.

2.14.2 Preparation of inclusion bodies

The pellet containing the inclusion bodies from 2.13.4.1 was washed twice with 50 ml of Wash buffer I (0.5 M NaCl, 2% Triton X-100), 50 ml of Wash buffer II (0.5 M NaCl) and 50 ml distilled water. The pellet was collected by centrifugation at 12,000 rpm for 30 minutes. The MAP30 inclusion bodies were stored at 4 °C until further use.

2.14.2.1 Solubilization and refolding of inclusion bodies.

Following the method of Rajamohan *et al* (1999), the inclusion bodies were solubilized in 50 mM Tris–HCl (pH 8.0) containing 8 M urea in a final volume of 10 ml. It was incubated at room temperature with shaking for 10 minutes and any insoluble proteins were removed by centrifugation at 15,000 rpm for 5 minutes. The remaining clear solution was renatured by slowly adding 20% glycerol (final concentration) and dialyzed very slowly in 4 liters of 50 mM phosphate buffer, pH 6.0, overnight. The dialysate was purified by column chromatography using CM Sephadex C50 column and phosphocellulose P11 column.

2.14.3 Column chromatography

Both soluble and inclusion bodies fraction are subjected to ion-exchange and affinity column chromatography.

2.14.3.1 CM-Sephadex C50 column chromatography

CM-Sephadex bead was prewashed with excess volume of deionized water for 2-3 times and resuspended in appropriate amount of 50 mM phosphate buffer, pH 6.0. The bead was packed into a 2.0 x 45 cm column followed by equilibration with the same phosphate buffer for 5-10 column volume at flow rate of 0.5 ml / minute. The purification was performed at 4 ^{o}C, as follows: 5 ml of the pooled active fractions were applied to the equilibrated column and ran at 0.5ml/minute flow rate. The unbound was removed by keep washing the column with buffer until the absorbance at 280 nm of the eluant decrease to about zero. The bounded proteins were eluted from the column with linear salt gradient of 0-0.2 M NaCl in the same buffer. Fractions of 3 ml were collected using a automated fraction collector. The elution profile was monitored for protein by measuring the absorbance at 280 nm and the sodium chloride concentration was obtained by measuring the conductivity. The topoisomerase activity was

determined to detect the active fraction (MAP30). They were pooled, exhaustive dialyzed against the 50 mM phosphate buffer for 3 times at least 4 hours each, and concentrated with aquasorb to reduce the sample volume for further purification step.

2.14.3.2 Phosphocellulose P11 column chromatography

Five milligrams of phosphocellulose P11 was activated by washing and gentle swirling with 0.05 N NaOH for 5 minutes and let stand for 5 minutes to removed contaminated matter. The liquid was decanted off and the cellulose was washed again with 0.05 N NaOH until the pH of the solution was 11.0 or below. Then stirred the cellulose phosphate into 25 volumes of 0.05 N HCl and left stand for 5 minutes. The supernatant was decanted off and the cellulose was washed again with the same concentration of HCl until the pH of the supernatant was 3.0. Finally, the activated cellulose phosphate was washed with 50 mM phosphate buffer pH 6.0 until the mixture pH reached pH 6.0. The cellulose was then packed into a 2.0 x 20 cm column followed by equilibration with 5-10 column volume of the same phosphate buffer at flow rate of 0.5 ml /minute. Ten milliters of the pooled active fractions from CM column were applied to the equilibrated column and ran at 0.5ml/minute flow rate. The bound protein was eluted from the column with 0-0.2 M NaCl linear gradient. The unbound and the bound fraction were collected as mention above. The elution profile was monitored for protein by measuring the absorbance at 280 nm. Sodium chloride concentration was obtained by measuring the conductivity. MAP30 fraction was identified by topoisomerase activity. These active fractions were pooled, dialyzed against 50 mM phosphate buffer pH6 and concentrated with aquasorb. The purity of MAP30 preparation was investigated by SDS-PAGE analysis.

2.15 Characterization of MAP30

2.15.1 Molecular weight determination

SDS-PAGE was performed with a Mini Protein II apparatus (Bio-Rad) according to 2.12.3. The apparent molecular mass of the purified recombinant MAP30 protein was determined by comparing its electrophoretic mobility with those of standard protein markers.

2.15.2 N-terminal sequencing

The purified MAP30 protein solution was dialyzed against deionized water at 4 ^{o}C (approximately 4 hours for 3 times). The protein concentration was measured by Bradford method (Bradford, 1976). Then, the protein solution was lyophilized to protein powder. The powder was automatically sequenced for N-terminus residues using Edman degradation method (Cohen *et al*., 1993) at Department of Bioresources Science, Faculty of Agriculture, Kochi University, Japan.

2.15.3 Topoisomerase I activity assay

The method was done following the method of Muller *et al* (1998). Supercoiled pGEM as substrate was incubated with 0.5, 1 or 2 μg of MAP30 protein sample in buffer (40 mm Tris-HCl, pH 7.5, 100 mM KCl, 10 mM, $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, and 30 mg/ml BSA) at 37 ^{o}C for 15 minutes. The reaction was stopped by adding 1/5 volume of 5x SDS loading buffer (10% SDS, 10 mM dithiothreitol, 50% glycerol, and 0.01% bromophenol blue). The products from this reaction were analyzed on a 0.8% agarose gel, and visualized by ethidium bromide staining. Sample treated with 1.0 U of recombinant human DNA topoisomerase I (Fermentas, U.S.A) was used as positive control and sample without MAP30 protein served as negative control.

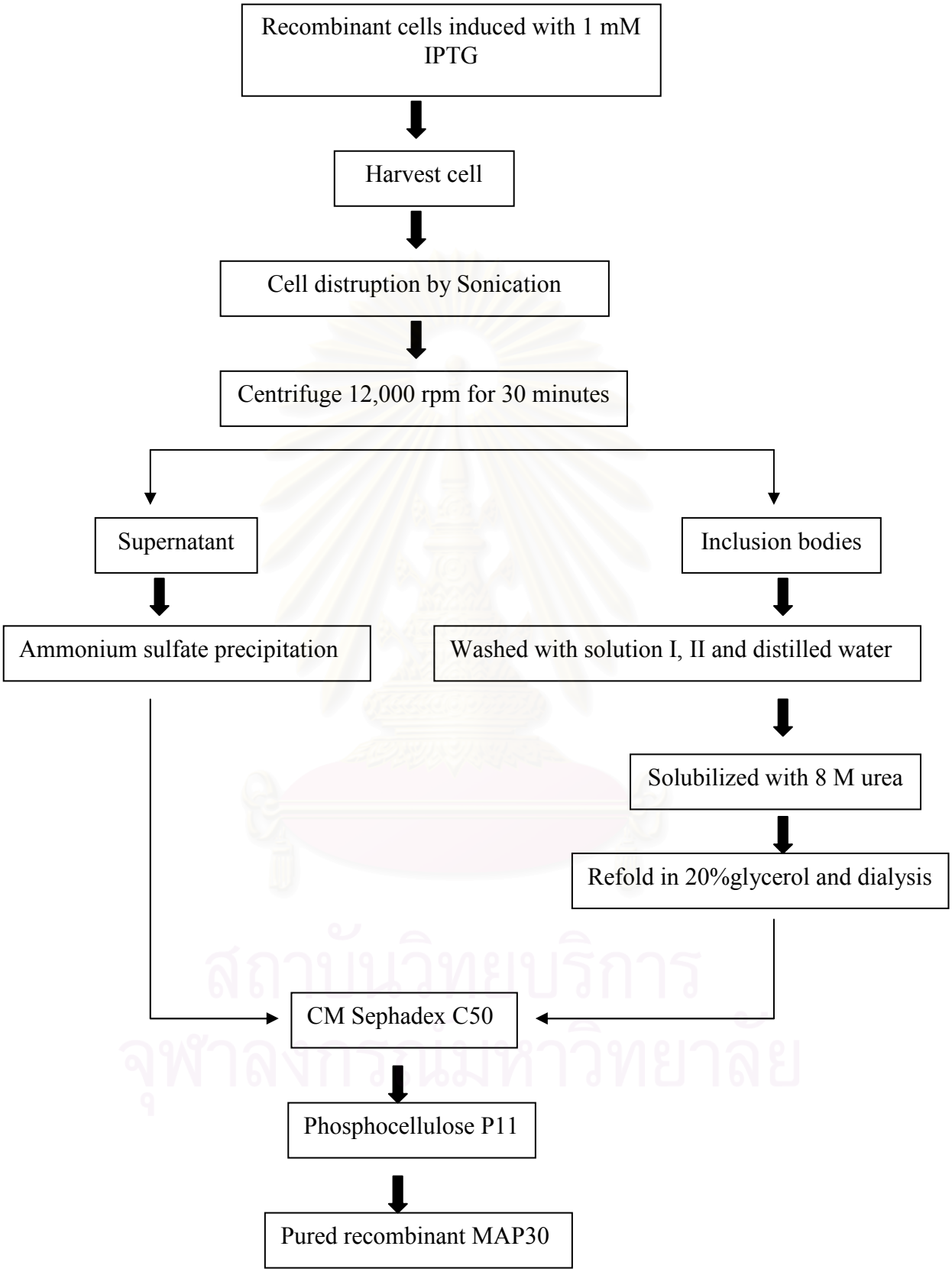


Figure 2.5 The summary of MAP30 purification.

CHARTER III

RESULTS

3.1 Cloning map30 gene from *Momordica charantia* using y pUC18 vector

3.1.1 DNA Extraction

Total DNA was extracted from young leaf of Mara Khee Nok by CTAB method (Bekesiova *et al.,* 1999). Extracted DNA was determined for its quality by 0.7% agarose gel electrophoresis as describe in section 2.10.1. High molecular weight DNA larger than 23.1 kb was obtained as shown in Figure 3.1. The A260/A280 ratio was in the range between 1.8-2.0, indicated high DNA purity. The DNA concentration was about 0.4-1.0 μg/g leaf. Thus the quality of DNA was suitable for molecular procedure such as restriction digestion and PCR amplification.

3.1.2 Amplification and characterization of *map30* gene

PCR- Amplification of *map30* from genomic DNA of bitter gourd was performed by using primers mapFE and mapRB which were designed based on the reported *map30* sequence by Lee-Huang (1995). The sequences of the primers are shown in Table 2.1. PCR products of ~800 bp generated from those sets of primer are shown in Figure 3.2. The forward-primer contains *Eco*R I restriction site, a Shine-Dalgano sequence of pTrc99 and start codon (ATG) at 5'end. The reverse primer contains 3'end of map30 gene, the TAA translational terminator signal followed by the restriction site for *Bam*H I at 3'end. The optimization of PCR was done optimized annealing temperature by varying at 50, 55, 60 and 65 ^{o}C. The optimal annealing temperature was 60 ^{o}C, while raising the temperature to 65 ^{o}C did not yield any PCR

product and non-specific amplified was observed in reaction of 50 and 55 ^{o}C (data not shown). The amplification of this primer pair in optimized condition consistently gave a product of ~800 bp in size which is corresponded with the size of reported matured *map30* gene (Figure 3.2).

3.1.3 Characterization of *map30* gene

The purified PCR product was ligated to *Eco*RI and *Bam*HI digested pUC18 as descripted in section 2.10.2.4 and transform into *E. coli* JM109 by electroporation. White colonies on selected plate containing amplicilin, X-gal and IPTG which were assumed to contain the putative recombinant plasmids were randomly picked for plasmid extraction and digestion with *Eco*R I–*Bam*H I. The results show two strong bands of supercoiled and relaxed forms that had higher molecular weight than wild type pUC18 (Figure 3.3). Moreover, it could be double digested by *Eco*R I-*Bam*H I to yield linear pUC18 and another fragment of ~800 bp (Figure 3.4, Lane 1). To confirm the inserted DNA was *map30* gene, restriction mapping was performed. Briefly, the recombinant plasmid which was name pUCmap was digested with restriction enzyme *Eco*R I, *Bam*H I, *Pst*I, *Sal*I, *Nde*I and *Hin*dIII and analyzed by 1% agarose gel electrophoresis using λDNA/*Hin*dIII and standard DNA ladder (100 bp) as markers The result was shown in the Figure 3.4. Comparision of the resulting restriction map with the restriction map of *map30* as applied from a Bioedit Program demonstrated that the clone contains a correct DNA insert fragment of expected full length *map30* gene.

3.1.4 Nucleotide sequence of *map30* gene

For further verification, the recombinant plasmid pUCmap was extracted and purified the positive clone. The DNA insert of purified plasmid was sequenced by

BSU (Bio Service Unit) using M13-forward and reverse primer system. The amplified fragment contained 819 nucleotides composing of 5'-*Eco*RI restriction site, Shine Dalgarno sequence of pTrc99, ATG, full mature *map30* gene and 3'-*Bam*HI restriction site, The Open Reading Frame of *map30* gene composed of 789 nucleotides. The result of the nucleotide and deduced amino acid sequences is shown in Figure 3.5, the gene encoded for 263 amino acids. The DNA sequence was aligned with known *map30* gene from NCBI database by using program Clustal X. The alignment showed 7 different nucleotides between *map*30 gene from cloned pUCmap and that of Lee-Haung (1995) and 1-4 nucleotides were difference from other sources of *map30* gene. (Figure3.6). However, comparison of deduced amino acid with MAP30 amino acid sequence from protein data bank showed that 2 amino acid residues (His47 and Ile166) were different from other sources (Arg47 and Tyr166) (Figure 3.7), but it did not interfere with residues of active site (Tyr70, Tyr109 and Ile153) which show in Figure 3.8. The molecular mass of deduced amino acid that was expected by program compute pI/MW on website http://kr.expasy.org/tools/pi_tool.html was 29.62 kDa and pI was 8.57.

Figure 3.1 Extraction of genomic DNA from Mara Khee Nok. Genomic DNA was extracted and separated in 0.7 % agarose gel electrophoresis (Section 2.20.1)

Lane M = λ/*Hin*dIII DNA marker

Lane 1-6 = genomic DNA extracts of Mara Khee Nok

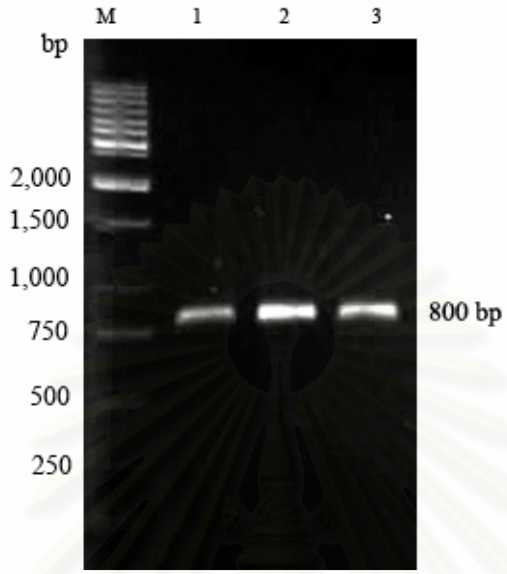


Figure 3.2 PCR product from full length *map30* amplification. PCR amplification of map30 started with predenaturing at 94 ^{o}C for 5 minutes and amplification was 30 cycles of denaturation at 94 72 ^{o}C for 45 seconds

Lane M = λ/*Hin*d III DNA marker

Lane 1-3 = PCR product

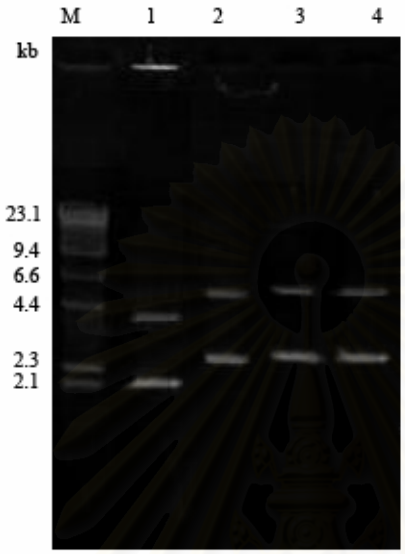


Figure 3.3 Recombinant plasmid pUCmap in 0.7% agarose gel.

Lane M = λ/*Hin*dIII DNA marker

Lane 1 = pUC18

Lane 2-4 = pUCmap

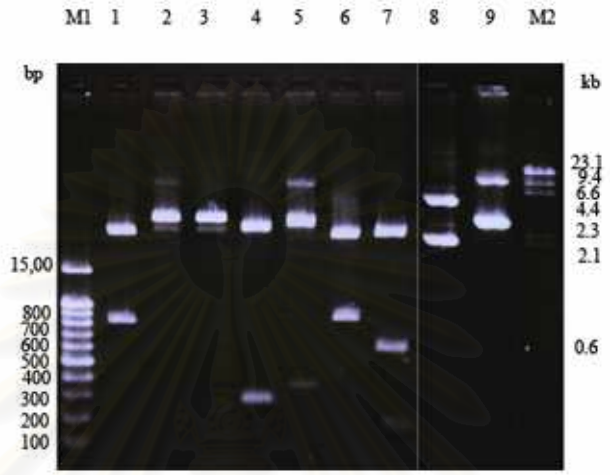


Figure 3.4A Restriction mapping of pUCmap. (A) The recombinant plasmid was digested by restriction enzyme as described in section 2.11.5.1

Lane M1 = 100 bp DNA ladder

Lane 1 = pUCmap digested with *Eco*R I and *Bam*H I

Lane 2 = pUCmap digested with *Eco*R I

Lane 3 = pUCmap digested with *Bam*H I

Lane 4 = pUCmap digested with *Pst* I

Lane 5 = pUCmap digested with *Hin*d III

Lane 6 = pUCmap digested with *Nde* I

Lane 7 = pUCmap3 digested with *Sal* I

Lane 8 = pUC18undigested

Lane 9 = pUCmap undigested

Lane M2 = λ / *Hin*d III DNA marker

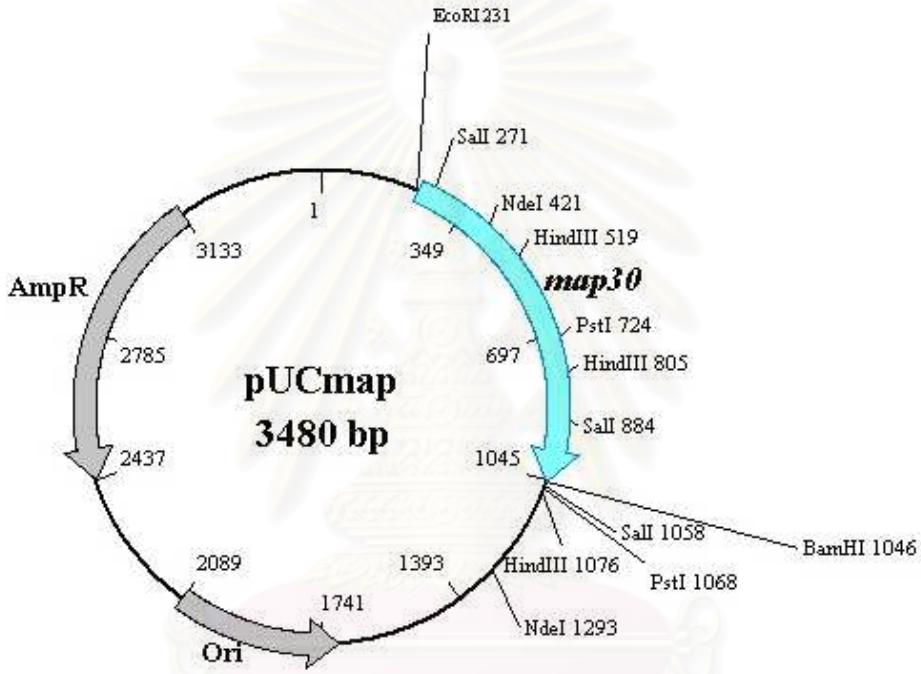


Figure 3.4B Restriction mapping of pUCmap. Restriction map of pUCmap as applied from a Bioedit Program.

```
                                                                CG GAATTC CG AGGAAAGAGACC

ATG GAT GTT AAC TTC GAT TTG TCG ACT GCC ACT GCA AAA ACC TAC ACA AAA TTT ATC
  M   D   V   N   F   D   L   S   T   A   T   A   K   T   Y   T   K   F   I
GAA GAT TTC AGG GCG ACT TTT CCA TTT AGC CAT AAA GTG TAT GAT ATA CCT CTA CTG
 E   D   F   R   A   T   F   P   F   S   H   K   V   Y   D   I   P   L   L
TAT TCC ACT ATT TCC GAC TCC AGA CAT TTC ATA CTC CTC AAT CTC ACA AGT TAT GCA
 Y   S   T   I   S   D   S   R   H   F   I   L   L   N   L   T   S   Y   A
TAT GAA ACC ATC TCG GTG GCC ATA GAT GTG ACG AAC GTT TAT GTT GTG GCC TAT CGC
 Y   E   T   I   S   V   A   I   D   V   T   N   V   Y   V   V   A   Y   R
ACC CGC GAT GTA TCC TAC TTT TTT AAA GAA TCT CCT CCT GAA GCT TAT AAC ATC CTA
 T   R   D   V   S   Y   F   F   K   E   S   P   P   E   A   Y   N   I   L
TTC AAA GGT ACG CGG AAA ATT ACA CTG CCA TAT ACC GGT AAT TAT GAA AAT CTT CAA
 F   K   G   T   R   K   I   T   L   P   Y   T   G   N   Y   E   N   L   Q
ACT GCT GCA CAC AAA ATA AGA GAG AAT ATT GAT CTT GGA CTC CCT GCC TTG AGT AGT
 T   A   A   H   K   I   R   E   N   I   D   L   G   L   P   A   L   S   S
GCC ATT ACC ACA TTG TTT TAT TAC AAT GCC CAA TCT GCT CCT TCT GCA TTG CTT GTA
 A   I   T   T   L   F   Y   Y   N   A   Q   S   A   P   S   A   L   L   V
CTA ATC CAG ACG ACT GCA GAA GCT GCA AGA TTT AAG TAT ATC GAG CGA CAC GTT GCT
 L   I   Q   T   T   A   E   A   A   R   F   K   Y   I   E   R   H   V   A
AAG TAT GTT GCC ACT AAC TTT AAG CCA AAT CTA GCC ATC ATA AGC TTG GAA AAT CAA
 K   Y   V   A   T   N   F   K   P   N   L   A   I   I   S   L   E   N   Q
TGG TCT GCT CTC TCC AAA CAA ATA TTT TTG GCG CAG AAT CAA GGA GGA AAA TTT AGA
 W   S   A   L   S   K   Q   I   F   L   A   Q   N   Q   G   G   K   F   R
AAT CCT GTC GAC CTT ATA AAA CCT ACC GGG GAA CGG TTT CAA GTA ACC AAT GTT GAT
 N   P   V   D   L   I   K   P   T   G   E   R   F   Q   V   T   N   V   D
TCA GAT GTT GTA AAA GGT AAT ATC AAA CTC CTG CTG AAC TCC AGA GCT AGC ACT GCT
 S   D   V   V   K   G   N   I   K   L   L   L   N   S   R   A   S   T   A
GAT GAA AAC TTT ATC ACA ACC ATG ACT CTA CTT GGG GAA TCT GTT GTG AAT TGA GGA
 D   E   N   F   I   T   T   M   T   L   L   G   E   S   V   V   N  *** TCC
```


Figure 3.5 The nucleotide and deduced amino acid sequences of *map30* gene clone from Mara khee Nok. The amino acids are single letter code in blue color alphabet.

```
clUSTAL W (1.82) multiple sequence alignment

Alignment Score Sequence Number Sequence Name Sequence Length

SeqA Name        Len(nt)  SeqB Name       Len(nt)  Score
=========================================================
1    myMAP       798      2    S79450     839      98
1    myMAP       798      3    AF284811   792      99
1    myMAP       798      4    AJ294541   792      99
2    S79450      839      3    AF284811   792      99
2    S79450      839      4    AJ294541   792      98
3    AF284811    792      4    AJ294541   792      99
=========================================================


myMAP            GATGTTAACTTCGATTTGTCGACTGCCACTGCAAAAACCTACACAAAATTTATCGAAGAT 60
AJ294541         GATGTTAACTTCGATTTGTCGACTGCCACTGCAAAAACCTACACAAAATTTATCGAAGAT 60
AF284811         GATGTTAACTTCGATTTGTCGACTGCCACTGCAAAAACCTACACAAAATTTATCGAAGAT 60
S79450           GATGTTAACTTCGATTTGTCGACTGCCACTGCAAAAACCTACACAAAATTTATCGAAGAT 60
                 ************************************************************

myMAP            TTCAGGGCGACTCTTCCATTTAGCCATAAAGTGTATGATATACCTCTACTGTATTCCACT 120
AJ294541         TTCAGGGCGACTCTTCCATTTAGCCATAAAGTGTATGATATACCTCTACTGTATTCCACT 120
AF284811         TTCAGGGCGACTCTTCCATTTAGCCATAAAGTGTATGATATACCTCTACTGTATTCCACT 120
S79450           TTCAGGGCGACTCTTCCATTTAGCCATAAAGTGTATGATATACCTCTGCTGTATTCCACT 120
                 ********************************************** ************

myMAP            ATTTCCGACTCCAGACATTTCATACTCCTCAATCTCACAAGTTATGCATATGAAACCATC 180
AJ294541         ATTTCCGACTCCAGACGTTTCATACTCCTCAATCTCACAAGTTATGCATATGAAACCATC 180
AF284811         ATTTCGGACTCCAGACGTTTCATACTCCTCAATCTCACAAGTTATGCATATGAAACCATC 180
S79450           ATTTCCGACTCCAGACGTTTCATACTCCTCAATCTCACAAGTTATGCATATGAAACCATC 180
                 ***** ********** ******************************************

myMAP            TCGGTGGCCATAGATGTGACGAACGTTTATGTTGTGGCCTATCGCACCCGCGATGTATCC 240
AJ294541         TCGGTGGCCATAGATGTGACGAACGTTTATGTTGTGGCCTATCGCACCCGCGATGTATCC 240
AF284811         TCGGTGGCCATAGATGTGACGAACGTTTATGTTGTGGCCTATCGCACCCGCGATGTATCC 240
S79450           TCGGTGGCCATAGATGTGACGAACGTTTATGTTGTGGCCTATCGCACCCGCGATGTATCC 240
                 ************************************************************

myMAP            TACTTTTTTAAAGAATCTCCTCCTGAAGCTTATAACATCCTATTCAAAGGTACGCGGAAA 300
AJ294541         TACTTTTTTAAAGAATCTCCTCCTGAAGCTTATAACATCCTATTCAAAGGTACGCGGAAA 300
AF284811         TACTTTTTTAAAGAATCTCCTCCTGAAGCTTATAACATCCTATTCAAAGGTACGCGGAAA 300
S79450           TACTTTTTTAAAGAATCTCCTCCTGAAGCTTATAACATCCTATTCAAAGGTACGCGGAAA 300
                 ************************************************************

myMAP            ATTACACTGCCATATACCGGTAATTATGAAAATCTTCAAACTGCTGCACACAAAATAAGA 360
AJ294541         ATTACACTGCCATATACCGGTAATTATGAAAATCTTCAAACTGCTGCACACAAAATAAGA 360
AF284811         ATTACACTGCCATATACCGGTAATTATGAAAATCTTCAAACTGCTGCACACAAAATAAGA 360
S79450           ATTACACTGCCATATACCGGTAATTATGAAAATCTCCAAACTGCTGCACACAAAATAAGA 360
                 ********************************** ***********************

myMAP            GAGAATATTGATCTTGGACTCCCTGCCTTGAGTAGTGCCATTACCACATTGTTTTATTAC 420
AJ294541         GAGAATATTGAGCTTGGACTCCCTGCCTTGAGTAGTGCCATTACCACATTGTTTTATTAC 420
AF284811         GAGAATATTGATCTTGGACTCCCTGCCTTGAGTAGTGCCATTACCACATTGTTTTATTAC 420
S79450           GAGAATATTGATCTTGGACTCCCTGCCTTGAGTAGTGCCATTACCACATTGTTTTATTAC 420
                 *********** ************************************************

myMAP            AATGCCCAATCTGCTCCTTCTGCATTGCTTGTACTAATCCAGACGACTGCAGAAGCTGCA 480
AJ294541         AATGCCCAATCTGCGCCTTCTGCATTGCTTGTACTAATCCAGACGACTGCAGAAGCTGCA 480
AF284811         AATGCCCAATCTGCTCCTTCTGCATTGCTTGTACTAATCCAGACGACTGCAGAAGCTGCA 480
S79450           AATGCCCAATCTGCTCCTTCTGCATTGCTTGTACTAATCCAGACGACTGCAGAAGCTGCA 480
                 ************** ***********************************************
```

Figure 3. 6 Nucleotide sequence alignment of *map30* gene. Nucleotide sequence of *map30* gene from Mara Khee Nok (myMAP) was aligned with *map30* gene of *Momordica charantia* from NCBI database using clustalW, The sequence which reported by Lee-Haung (1995) is S79450

```
myMAP          AGATTTAAGTATATCGAGCGACACGTTGCTAAGTATGTTGCCACTAACTTTAAGCCAAAT 540
AJ294541       AGATTTAAGTATATCGAGCGACACGTTGCTAAGTATGTTGCCACTAACTTTAAGCCAAAT 540
AF284811       AGATTTAAGTATATCGAGCGACACGTTGCTAAGTATGTTGCCACTAACTTTAAGCCAAAT 540
S79450         AGATTTAAGTATACTGAGCGACACGTTGCTAAGTATGTTGCCACTAACTTTAAGCCAAAT 540
               ************  ********************************************

myMAP          CTAGCCATCATAAGCTTGGAAAATCAATGGTCTGCTCTCTCCAAACAAATATTTTTGGCG 600
AJ294541       CTAGCCATCATAAGCTTGGAAAATCAATGGTCTGCTCTCTCCAAACAAATATTTTTGGCG 600
AF284811       CTAGCCATCATAAGCTTGGAAAATCAATGGTCTGCTCTCTCCAAACAAATATTTTTGGCG 600
S79450         CTAGCCATCATAAGCCTGGAAAATCAATGGTCTGCTCTCTCCAAACAAATCTTTTTGGCG 600
               ************** ********************************** *********

myMAP          CAGAATCAAGGAGGAAAATTTAGAAATCCTGTCGACCTTATAAAACCTACCGGGGAACGG 660
AJ294541       CAGAATCAAGGAGCAAAATTTAGAAATCCTGTCGACCTTATAAAACCTACCGGGGAACGG 660
AF284811       CAGAATCAAGGAGGAAAATTTAGAAATCCTGTCGACCTTATAAAACCTACCGGGGAACGG 660
S79450         CAGAATCAAGGAGGAAAATTTAGAAATCCTGTCGACCTTATAAAACCTACCGGGGAACGG 660
               ************* ********************************************

myMAP          TTTCAAGTAACCAATGTTGATTCAGATGTTGTAAAAGGTAATATCAAACTCCTGCTGAAC 720
AJ294541       TTTCAAGTAACCAATGTTGATTCAGATGTTGTAAAAGGTAATATCAAACTCCTGCTGAAC 720
AF284811       TTTCAAGTAACCAATGTTGATTCAGATGTTGTAAAAGGTAATATCAAACTCCTGCTGAAC 720
S79450         TTTCAAGTAACCAATGTTGATTCAGATGTTGTAAAAGGTAATATCAAACTCCTGCTGAAC 720
               ************************************************************

myMAP          TCCAGAGCTAGCACTGCTGATGAAAACTTTATCACAACCATGACTCTACTTGGGGAATCT 780
AJ294541       TCCAGAGCTAGCACTGCTGATGAAAACTTTATCACAACCATGACTCTACTTGGGGAATCT 780
AF284811       TCCAGAGCTAGCACTGCTGATGAAAACTTTATCACAACCATGACTCTACTTGGGGAATCT 780
S79450         TCCAGAGCTAGCACTGCTGATGAAAACTTTATCACAACCATGACTCTACTTGGGGAATCT 780
               ************************************************************

myMAP          GTTGTGAATTGAGGATCC---------------------------------------- 798
AJ294541       GTTGTGAATTGA-------------------------------------------- 792
AF284811       GTTGTGAATTGA-------------------------------------------- 792
S79450         GTTGTGAATTGAAAGTTTAATAATCCACCCATATCGAAATAAGGCATGTTCATGACAT
```

Figure 3.6 Nucleotide sequence alignment of *map30* gene (continued).

```
CLUSTAL W (1.82) multiple sequence alignment


SeqA Name        Len(aa)  SeqB Name        Len(aa)  Score
=========================================================
1    AJ294541    263      2    myMAP       263      98
1    AJ294541    263      3    AF284811    263      99
1    AJ294541    263      4    S79450      263      98
2    myMAP       263      3    AF284811    263      99
2    myMAP       263      4    S79450      263      98
3    AF284811    263      4    S79450      263      99
=========================================================


AF284811         DVNFDLSTATAKTYTKFIEDFRATLPFSHKVYDIPLLYSTISDSRRFILLNLTSYAYETI 60
S79450           DVNFDLSTATAKTYTKFIEDFRATLPFSHKVYDIPLLYSTISDSRRFILLNLTSYAYETI 60
myMAP            DVNFDLSTATAKTYTKFIEDFRATLPFSHKVYDIPLLYSTISDSRRFILLNLTSYAYETI 60
AJ294541         DVNFDLSTATAKTYTKFIEDFRATLPFSHKVYDIPLLYSTISDSRRFILLNLTSYAYETI 60
                 ************************************************************

AF284811         SVAIDVTNVYVVAYRTRDVSYFFKESPPEAYNILFKGTRKITLPYTGNYENLQTAAHKIR 120
S79450           SVAIDVTNVYVVAYRTRDVSYFFKESPPEAYNILFKGTRKITLPYTGNYENLQTAAHKIR 120
myMAP            SVAIDVTNVYVVAYRTRDVSYFFKESPPEAYNILFKGTRKITLPYTGNYENLQTAAHKIR 120
AJ294541         SVAIDVTNVYVVAYRTRDVSYFFKESPPEAYNILFKGTRKITLPYTGNYENLQTAAHKIR 120
                 ************************************************************

AF284811         ENIDLGLPALSSAITTLFYYNAQSAPSALLVLIQTTAEAARFKYIERHVAKYVATNFKPN 180
S79450           ENIDLGLPALSSAITTLFYYNAQSAPSALLVLIQTTAEAARFKYTERHVAKYVATNFKPN 180
myMAP            ENIDLGLPALSSAITTLFYYNAQSAPSALLVLIQTTAEAARFKYIERHVAKYVATNFKPN 180
AJ294541         ENIELGLPALSSAITTLFYYNAQSAPSALLVLIQTTAEAARFKYIERHVAKYVATNFKPN 180
                 ***:**************************************** ***************

AF284811         LAIISLENQWSALSKQIFLAQNQGGKFRNPVDLIKPTGERFQVTNVDSDVVKGNIKLLLN 240
S79450           LAIISLENQWSALSKQIFLAQNQGGKFRNPVDLIKPTGERFQVTNVDSDVVKGNIKLLLN 240
myMAP            LAIISLENQWSALSKQIFLAQNQGGKFRNPVDLIKPTGERFQVTNVDSDVVKGNIKLLLN 240
AJ294541         LAIISLENQWSALSKQIFLAQNQGAKFRNPVDLIKPTGERFQVTNVDSDVVKGNIKLLLN 240
                 ***********************.**********************************

AF284811         SRASTADENFITTMTLLGESVVN 263
S79450           SRASTADENFITTMTLLGESVVN 263
myMAP            SRASTADENFITTMTLLGESVVN 263
AJ294541         SRASTADENFITTMTLLGESVVN 263
                 ***********************
```

Figure 3.7 Amino acid alignment of MAP30 protein. Deduced amino acid of MAP30 from Mara Khee Nok (myMAP) was aligned with known MAP30 sequence of *Momordica charantia* from NCBI database. The sequence which reported by Lee-Haung (1995) is S79450

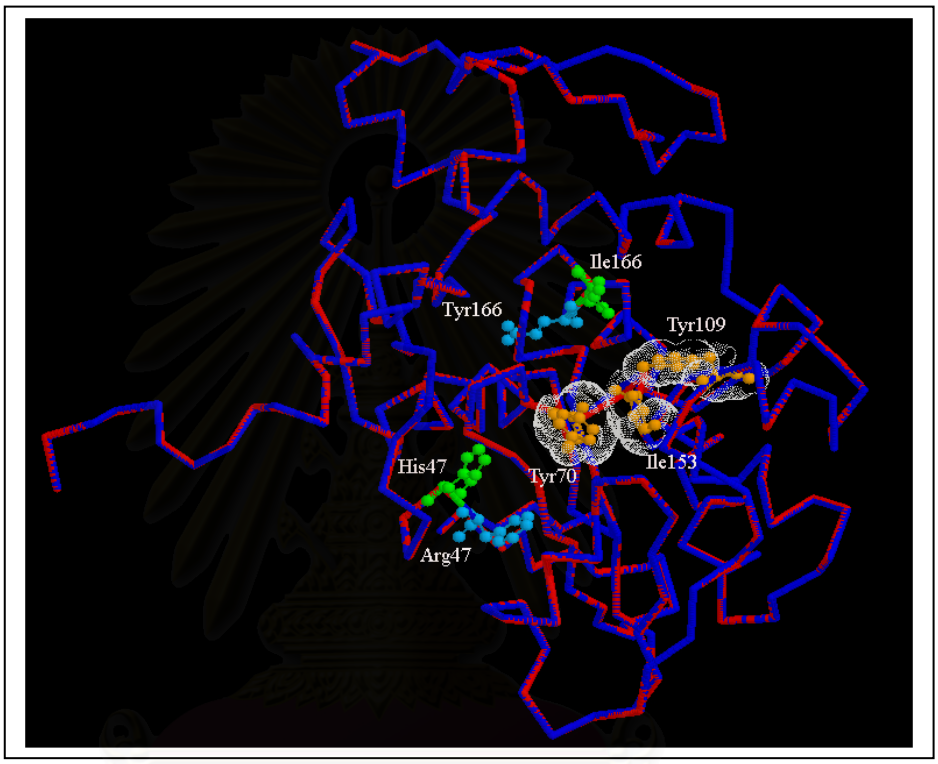


Figure 3.8 Superimpose of MAP30 structure from Lee-Haung and Mara Khee Nok. The His47 and Ile166 are residues from MAP30 of Mara Khee Nok, the Phe47 and Glu166 are residues from MAP30 of Lee-Huang and Tyr70, Tyr109 and Ile153 are active site residues.

3.2 Expression of pUCmap in *E. coli* JM109

The expression of *map30* gene inrecombinant clone by induction with 1mM IPTG for 0.5, 1, 2, 3, 4 and 6 hours were observed. The SDS PAGE patterns of total cell protein were visualized by Coomassie Brilliant Blue staining. It appeared that the protein patterns of wild type cell, non induced-recombinant cell (control) and induced cell were similar without marked 30 kDa protein band (Figure 3.9). It was possible that MAP30 protein was not expressed or expressed at vary small amount in this system. To improve the expression, *map30* gene from pUCmap was further subcloned to other potent expression vector such as pET19b

3.3 Cloning and expression level of *map30* gene using pET vector

3.3.1 Subcloning of *map*30 gene

The *map*30 gene from recombinant pUCmap was subcloned to pET19b. Briefly, the pUCmap was amplified with two specific primers, mapFN and mapRB (Table 2.1). The PCR-product of *map*30 gene fragment (~800 bp) was double digested with restriction enzyme *Nco*I and *Bam*HI as described in 2.13.1 and prior to ligation containing the recombinant plasmid (designated pETmap) with linear pET19b that was previously cutted with the same restriction enzymes. The ligation mixture was electroporated into competent cell *E. coli* Rosetta DE3 (pLysS) and 50 µl of the culture was spreaded for selection in LB plate supplemented with chloramphenicol and amplicilin (section 2.13.1). Ten white colonies were randomly picked for plasmid extraction and digested with *Nco*I and *Bam*HI. The size of the pET19b was about 6 kb. After digestion, two DNA fragments of 5.8 kb and and ~800 bp were detected which corresponded with linear pET19b and *map30* gene, respectively (Figure 3.10, lane 4). These pETmap were used for large scale production of MAP30.

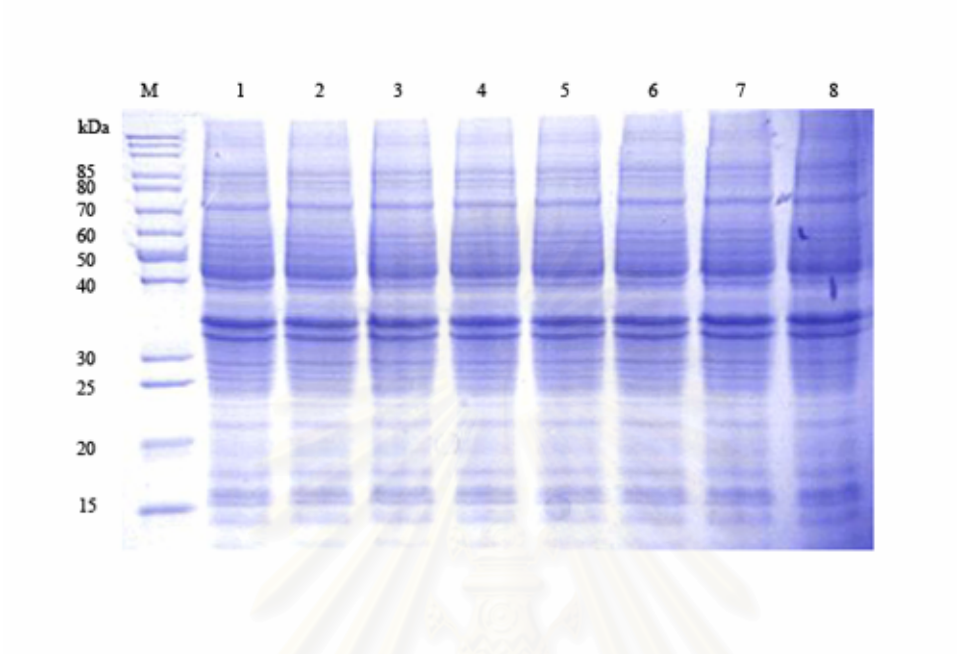


Figure 3.9 Expression of pUCmap in *E. coli* JM109.

The protein from whole cell lysate was detected by SDS-PAGE as described in section 2.12

Lane M = Low molecular weight protein marker

Lane 1 = Wild type *E. coli* JM109 induced with 1 mM IPTG for 3 hours

Lane 2-7 = recombinant *E. coli* JM109 induced with 1 mM IPTG for 0.5, 1, 2, 3, 4 and 6 hours, respectively

Lane 8 = recombinant cell at t_o

.

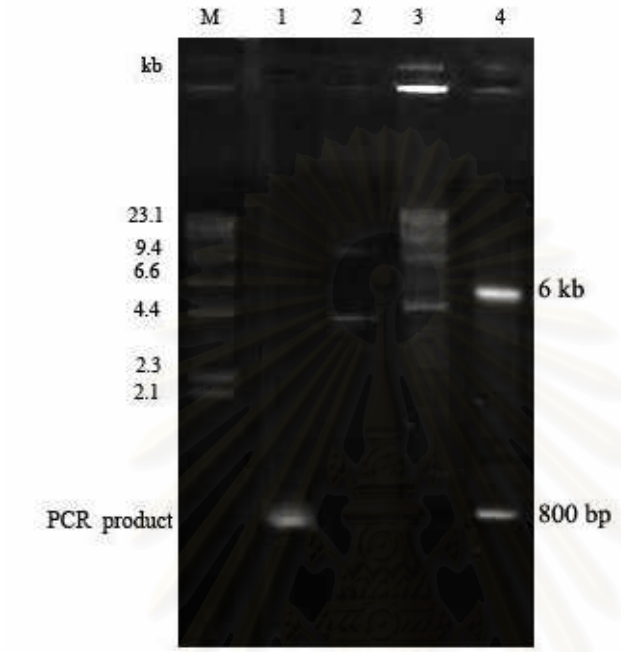


Figure 3.10 Restriction analysis of pETmap.

Lane M = λ / *Hin*dIII DNA marker

Lane 1 = PCR product from amplification with primers mapFN and mapRB.

Lane 2 = undigested pET19b

Lane 3 = undigested pETmap

Lane 4 = pETmap digested by *Nco*I and *Bam*HI

3.3.2 Induction of pETmap in *E. coli* Rosetta DE3 (pLysS)

3.3.3 IPTG concentration for induction

The vector pET19b contains a strong phage T_7 promoter and could be induced by addition of 1 mM IPTG following the method by Novagen (2002). The SDS-PAGE pattern of total cell protein, visualized by Coomassie Brilliant Blue with various IPTG induction time indicated that a band of about 30 kDa was present in all induced recombinant cells but not in the wildtype or non induced cell (Figure 3.11). The maximal level of the protein was achieved about 3 hours after IPTG induction. The soluble and insolubled fractions of these *E. coli* cell lysate were later separated by centrifugation and each resolved by SDS-PAGE. The protein pattern show that the expressed protein MAP30 was located largely in the insoluble fraction, suggesting that recombinant MAP30 is aggregated as insoluble inclusion bodies in the cytoplasm (Figure 3.12). In order to avoid the expression of MAP30 as inclusion bodies, the induction condition was varied either by lowering the IPTG concentration or incubation temperature

E.coli Rosetta DE3 (pLysS) containing the pETmap was cultured at 37^{o}C with shaking and induction was done by addition of 0.1 or 0.5 mM of IPTG for 3 hours. The protein pattern was analyzed by 12% SDS-PAGE for insoluble or soluble localization of induced MAP30 protein. Figure 3.12 showed it was still located in both soluble and insoluble fraction even at 0.1 mM of IPTG induction. The expression signal of all IPTG concentration was similarity and about 30kDa protein band was observed in both soluble and insoluble fraction. Therefore, 0.1 mM of IPTG would be used for induction in future experiments.

3.3.4 Temperature for induction

E.coli Rosetta DE3 (pLysS) containing pETmap were grown at 25 ^{o}C, 30 ^{o}C and 37 ^{o}C in the presence of 0.1 mM IPTG for 3 hours. The protein pattern of total cell lysate, soluble and insoluble fractions of each cell cultures was analyzed by SDS-PAGE. Figure 3.14 showed that the putative MAP30 protein mainly appeared in the insoluble fraction of 30 ^{o}C cell culture similar to that of 37 ^{o}C cells culture (data not shown for 37 ^{o}C). However, n cells grown at 25^{o}C show that the corresponding band was weaker.

For protein purification, the recombinant cells would be induced with 0.1 mM IPTG and cells were grown at 30^{o}C. Both the soluble and insoluble fractions would be subjected to purification and analysis of MAP30.

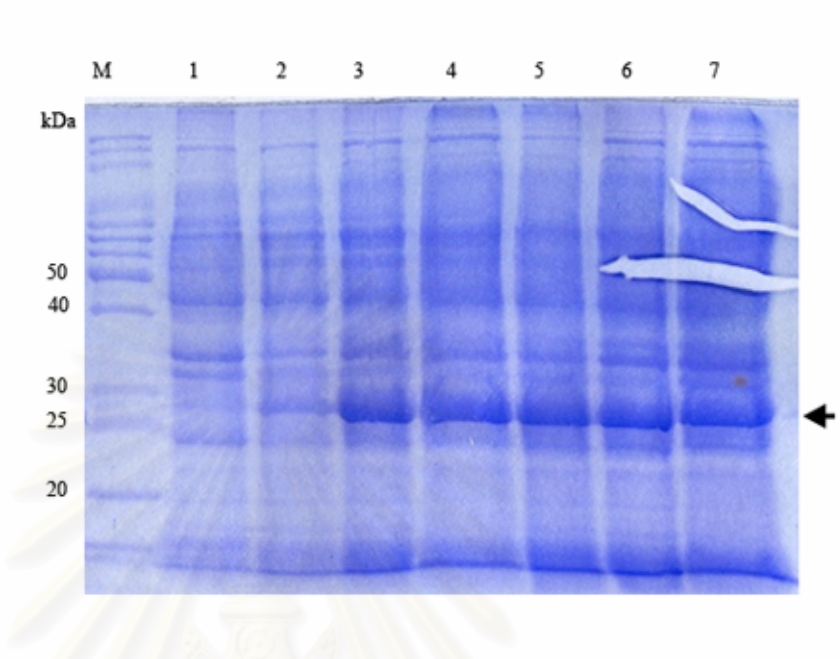


Figure 3.11 Expression of MAP30 in recombinant *E. coli* Rosetta (pLysE) containing pETmap. The protein from whole cell lysate was detected by SDS-PAGE as described in 2.12.3

Lane M = protein marker

Lane 1 = wild type *E coli* Rosetta (pLysE) induced with 1 mM IPTG

Lane 2-7 = recombinant *E coli* induced with 1 mM IPTG and harvested at 0.5,1,2,3,4 and 6 hours, respectively

Lane 8 = recombinant cell at t_o

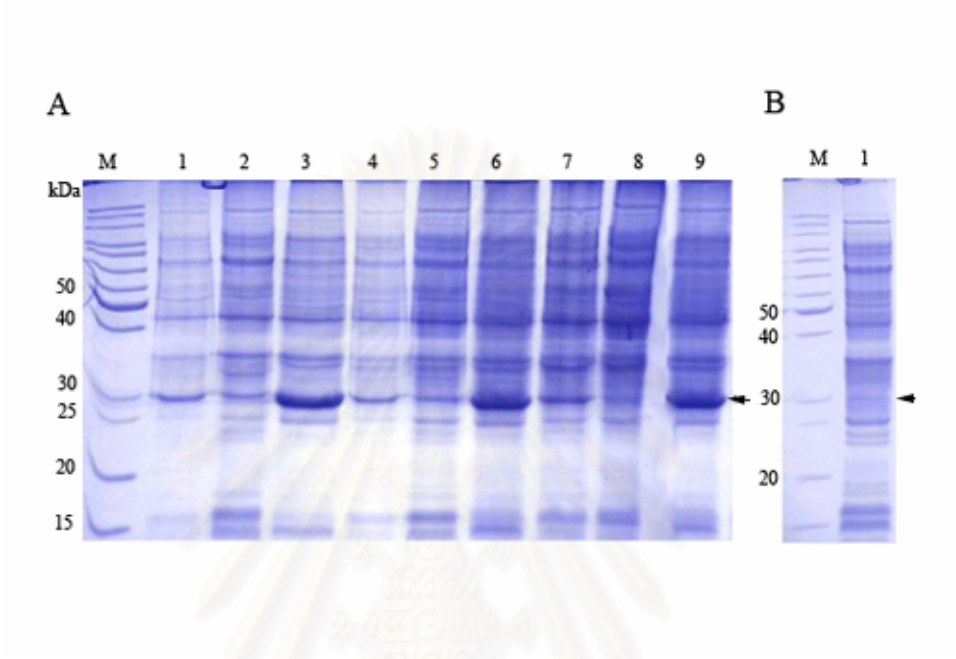


Figure 3.12 Localization of MAP30 from pETmap in *E. coli* Rosetta (pLysS). (**A**) 1 mM Induction of expression in recombinant *E. coli* Rosetta DE3 (pLysS) and (**B**) in wild type cell. Fractionation and SDS-PAGE analysis was performed as described in 2.12.3

Lane M = Protein marker

Lane 1, 4, 7 = Cell lysate from IPTG treated cells induced for 2, 3 and 4 hours induction, respectively

Lane 2, 5, 8 = Soluble fraction at 2, 3 and 4 hours induction, respectively

Lane 3, 6, 9 = Insoluble fraction at 2,3 and 4 hours induction, respectively

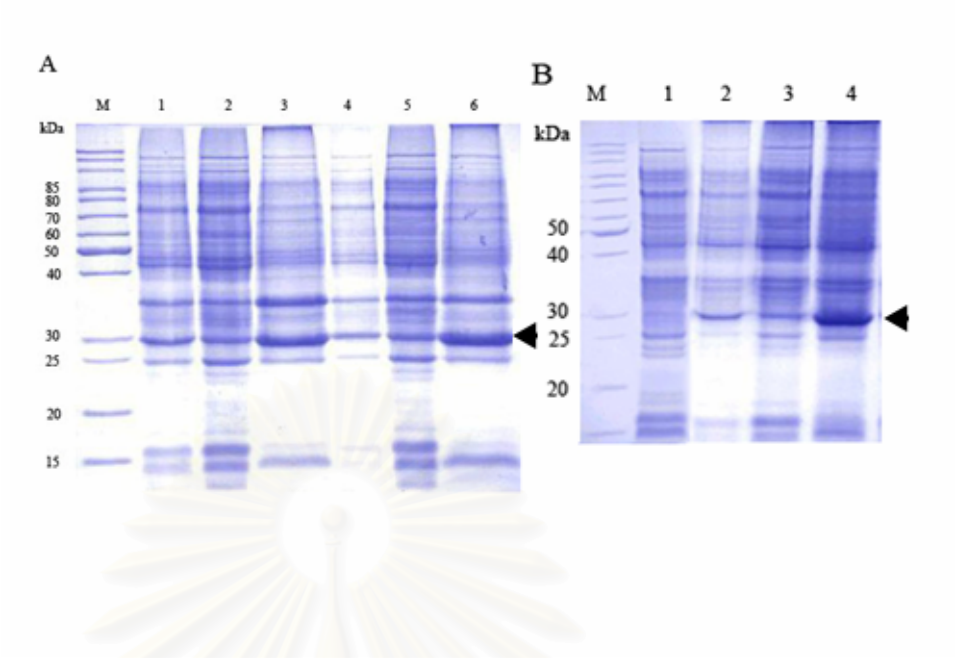


Figure 3.13 Induction of MAP30 expression by various IPTG concentration. Cells were induced at 30°C for 3 hours before and analysis by SDS-PAGE. Except for lane 1 and 4 in figure A, the protein sample were from recombinant cells

A. M = Low molecular weight protein marker

Lanes 1,4 = Crude cell lysate from 0.1 and 0.5 mM IPTG treated cells,

Lanes 2,5, = Soluble fractions of induced cells with 0.1 and 0.5 mM IPTG, respectively

Lanes 3,6, = Insoluble fraction of induced cell with 0.1 and 0.5 mM IPTG, respectively

B. M = Low molecular weight protein marker

Lane 1 = Wild type cell induced with 1 mM IPTG

Lane 2 = Cell lysate from IPTG cells induced with 1 mM IPTG

Lane 3 = Soluble fractions of cells with 1 mM IPTG

Lane 4 = Insoluble fraction of cell with 1 mM IPTG

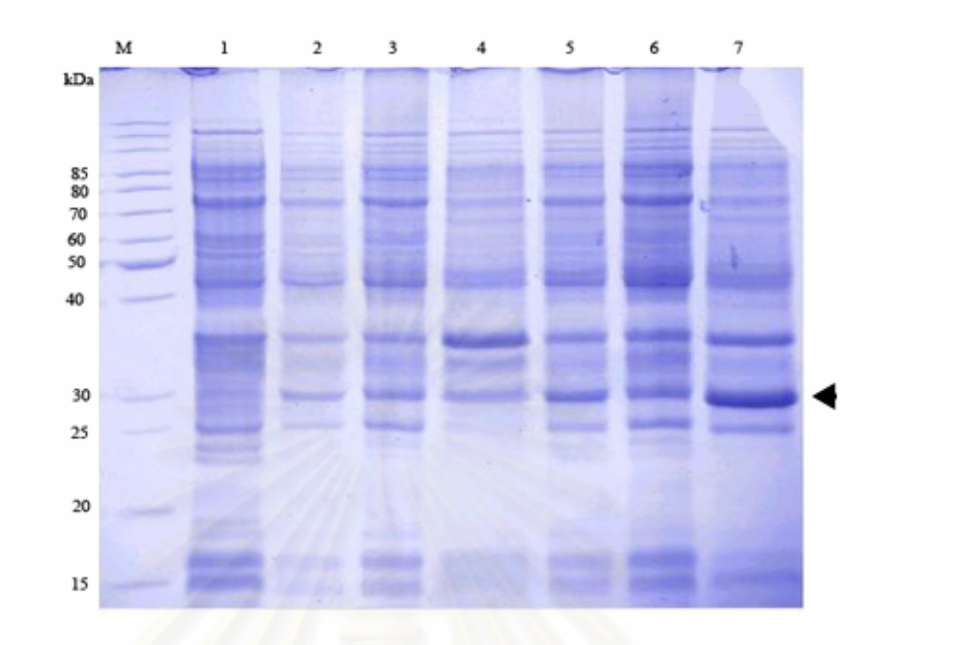


Figure 3.14 SDS-PAGE analysis of MAP30 localization in cells grown at difference Temperature.

Lane M = Low molecular weight protein marker

Lane 1 = Total cell protein of non- induced recombinant cell

Lane 2, 5 = Crude cell lysate from IPTG induced cells culture at 25 ^{o}C and 30 ^{o}C, respectively

Lane 3, 6 = Soluble fractions of induced cells culture at 25 ^{o}C and 30 ^{o}C, respectively

Lane 4, 7 = Insoluble fraction of induced cells culture at 25 ^{o}C and 30 ^{o}C, respectively

3.4 Purification and characterization of recombinant MAP30

3.4.1 Preparation of MAP30 from inclusion bodies fraction

As shown in Figure 3.12 that MAP30 from pETmap recombinant cells was mostly expressed in the insoluble fraction most likely as inclusion bodies, this fraction was subjected to protein purification.

3.4.1.1 Solubilization and refolding of MAP30 from inclusion bodies

The inclusion bodies from crude protein extract was solubilized and refolded to activate protein activity. Solubilization of inclusion bodies was achieved using 50 mM Tris-HCl buffer, pH 8.0, containing 8 M urea. The remaining precipitates were removed by centrifugation at 12,000 rpm for 30 minutes. About 10.2 mg of protein was obtained (about 20 % of total cell protein) and was proceeded for protein refolding.

Refolding was achieved by slowly diluting the sample with glycerol to 20% final concentration and subsequently exhaustive dialysis in 50 mM phosphate buffer, pH 6.0. The cloudy, unrefolded protein was removed by centrifugation. SDS–PAGE analysis of proteins pattern in the crude precipitates, solubilized and refolded preparations revealed the presence of a major protein band with estimated molecular mass of about 30 kDa (Figure 3.15). The final yield of the refolded MAP30 was estimated as 20.20 mg, which corresponded to approximately 19.5 % of total cellular protein.

3.4.1.2 Purification of refolded MAP30 by column chromatography

The refolded recombinant MAP30 was purified using ion exchange chromatography. Five milliters (10.10 mg) of the solubilized, refolded protein from

3.5.1.1 was applied to the CM Sephadex C50 column that was equilibrated with 50 mM phosphate buffer, pH 6.0, washed with the same buffer and eluted with elution gradient of the same buffer containing 0-200 mM NaCl. The elution profile was show in Figure 3.17. Two unbound were obtained, namely iCmI and iCmII (Figure 3.17). Pooled protein from each peak was screened for MAP30 protein by topoisomerase activity assay and SDS-PAGE. Figure 3.18 show that the iCmII peak contained the approximate 30 kDa band with high purity whereas the iCmI peak contained most other proteins. Further purification with phosphocellulose P11 column which is an affinity column for MAP30 using five milliters (9.88 mg) of the protein from iCmII pool was performed. The column was washed with 50 mM phosphate buffer, pH 6.0, and eluted with the same 0-200 mM NaCl gradient. The elution profile showed that most protein was again washed from the column and scarcely any bound protein (Figure 3.17). Observation from SDS PAGE showed that only a single 30 kDa band protein was obtained in the unbound fraction indicating that the encoded MAP30 was purified to homogeneity in this step (Figure 3.18). Since, too little protein bound to the column, it was not analysed by SDS-PAGE by the method described in 2.12.3. Figure 3.20 also showed the protein from each step. Analysis of topoisomerase activity of all sample derived from MAP30 in the inclusion bodies revealed no retaining activity at all (Figure 3.16 and 3.19) implying that perhaps refolding of the protein was not achieved.

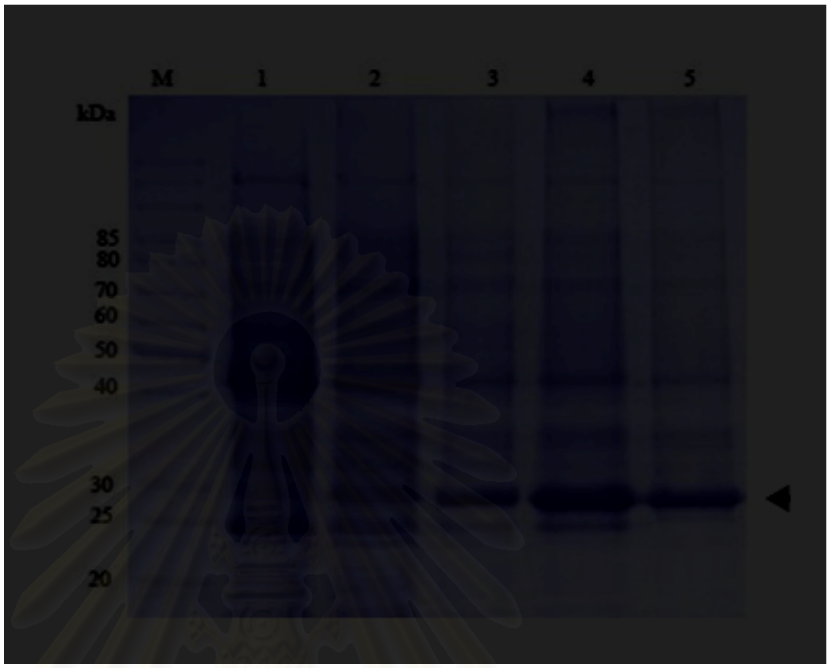


Figure 3.15 SDS-PAGE analysis of inclusion bodies from various treatment

Lane M = Low molecular weight protein marker

Lane 1 = Total cell protein of non – induced cell

Lane 2 = Total cell protein of induced cell

Lane 3 = Crude inclusion bodies

Lane 4 = Solubilized inclusion bodies

Lane 5 = Refolded inclusion bodies

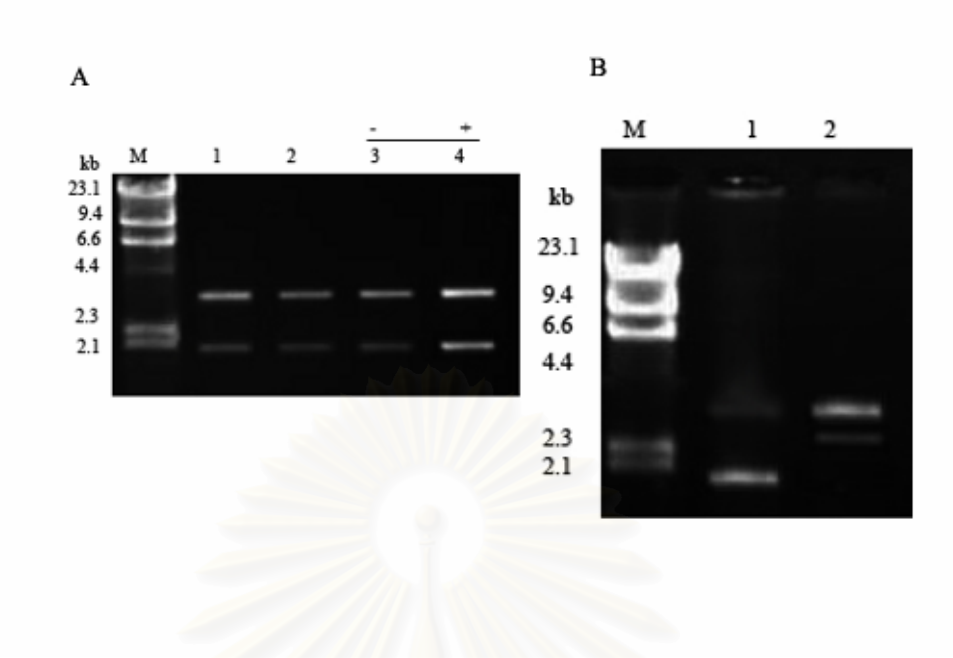


Figure 3.16 Topoisomerase activity of MAP30 from inclusion bodies preparation.

The plasmid pGEM was incubated with the protein sample in 20 µl reaction at 30°C minute for 30 minutes

A

Lane M = λ/*Hin*d III DNA marker

Lane 1 = plasmid pGEM

Lane 2 = plasmid incubated with buffer

Lane 3 = plasmid incubated with boiled-protein form refolded inclusion body

Lane 4 = plasmid was incubated with protein from refolded inclusion body

\- = The reaction incubate with boiled-sample as control

\+ = The reaction incubate with sample as test

B

Lane M= λ/*Hin*d III DNA marker

Lane 1 = plasmid pGEM

Lane 2 = plasmid incubated with Human topoisomerase I

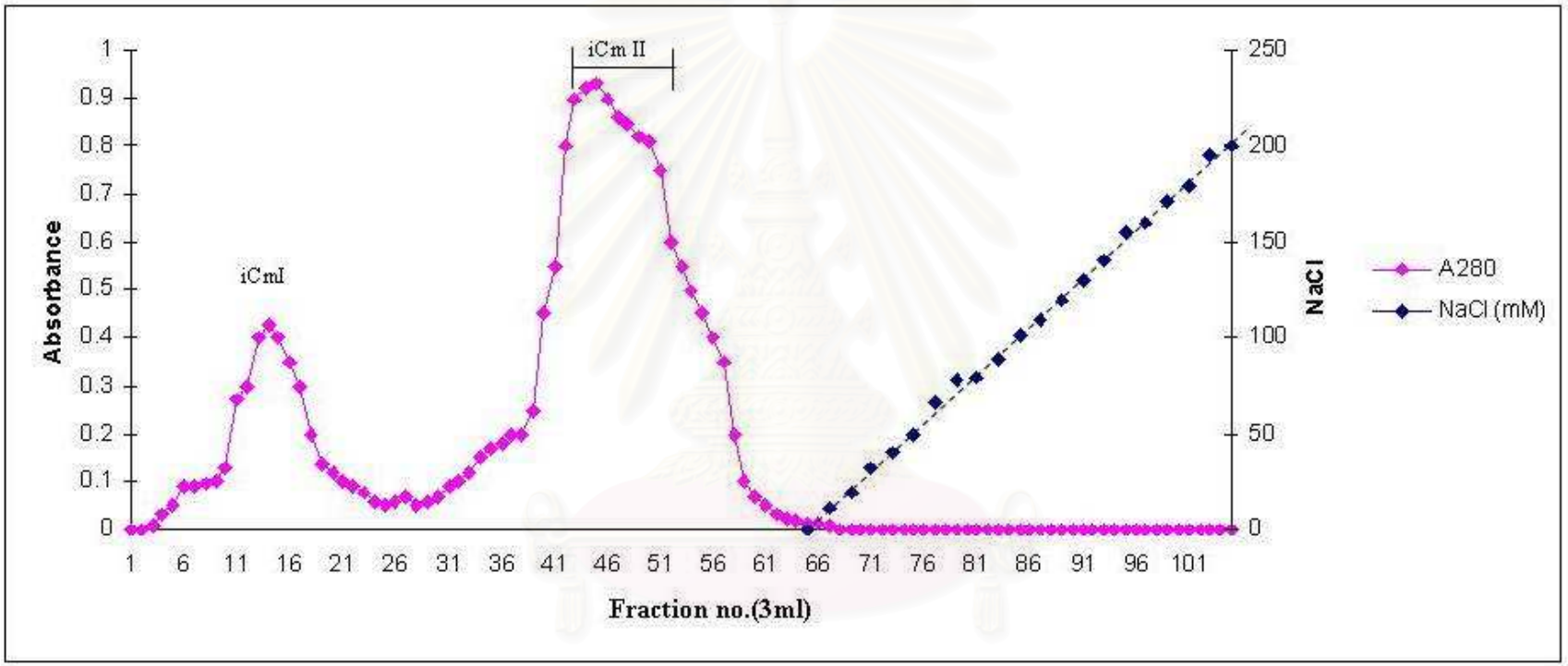


Figure 3.17 Purification profiles of refolded recombinant MAP30 by CM Sephadex C 50. Elution profile of protein purification by CM Sephadex C50 column chromatography, The protein was applied to the column and wash by 50 mM Phosphate buffer, pH 6.0, ⊢⊣ fraction 40-60 were pooled.

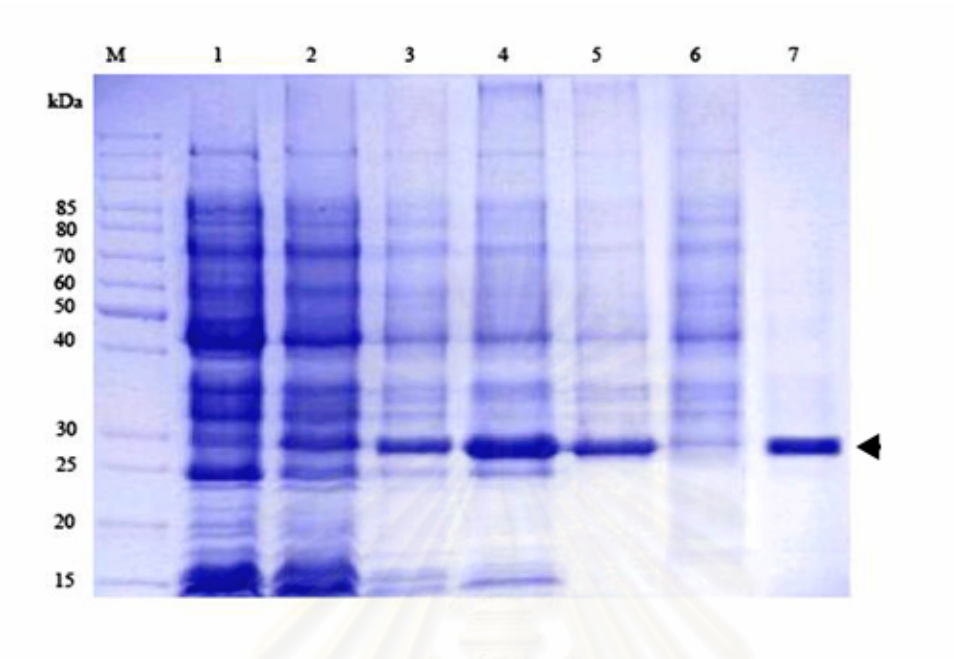


Figure 3.18 The SDS-PAGE of refolded inclusion bodies from CM-Sephadex C50 column.

Lane M = Low molecular weight protein marker

Lane1 = Total cell protein of non -induced recombinant cell

Lane2 = Total cell protein of induced recombinant cell

Lane3 = Inclusion body

Lane4 = Crude solubilized inclusion body

Lane5 = Refolded inclusion body

Lane6 = Protein solution of iCm I fraction

lane7 = Protein solution of iCm II fraction

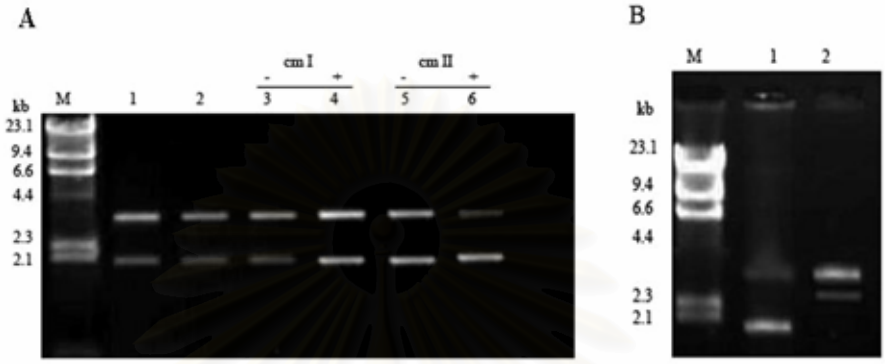


Figure 3.19 Topoisomerase activity of reMAP30 from CM Sephadex C50 column.

A. LaneM = λ/*Hin*d III DNA marker

Lane1 = pGEM

Lane2 = plasmid inclubated with buffer

Lane3 = plasmid inclubated with boiled-protein from iCm I

Lane4 = plasmid inclubated with protein form iCmI

Lane5 = plasmid inclubated with boiled-protein from iCm II

Lane6 = plasmid inclubated with protein from iCm II

\- = control as described in Figure 3.15

\+ = test as described in Figure 3.15

B Lane M= λ/*Hin*d III DNA marker

Lane 1 = plasmid pGEM

Lane 2 = plasmid incubated with human topoisomerase I

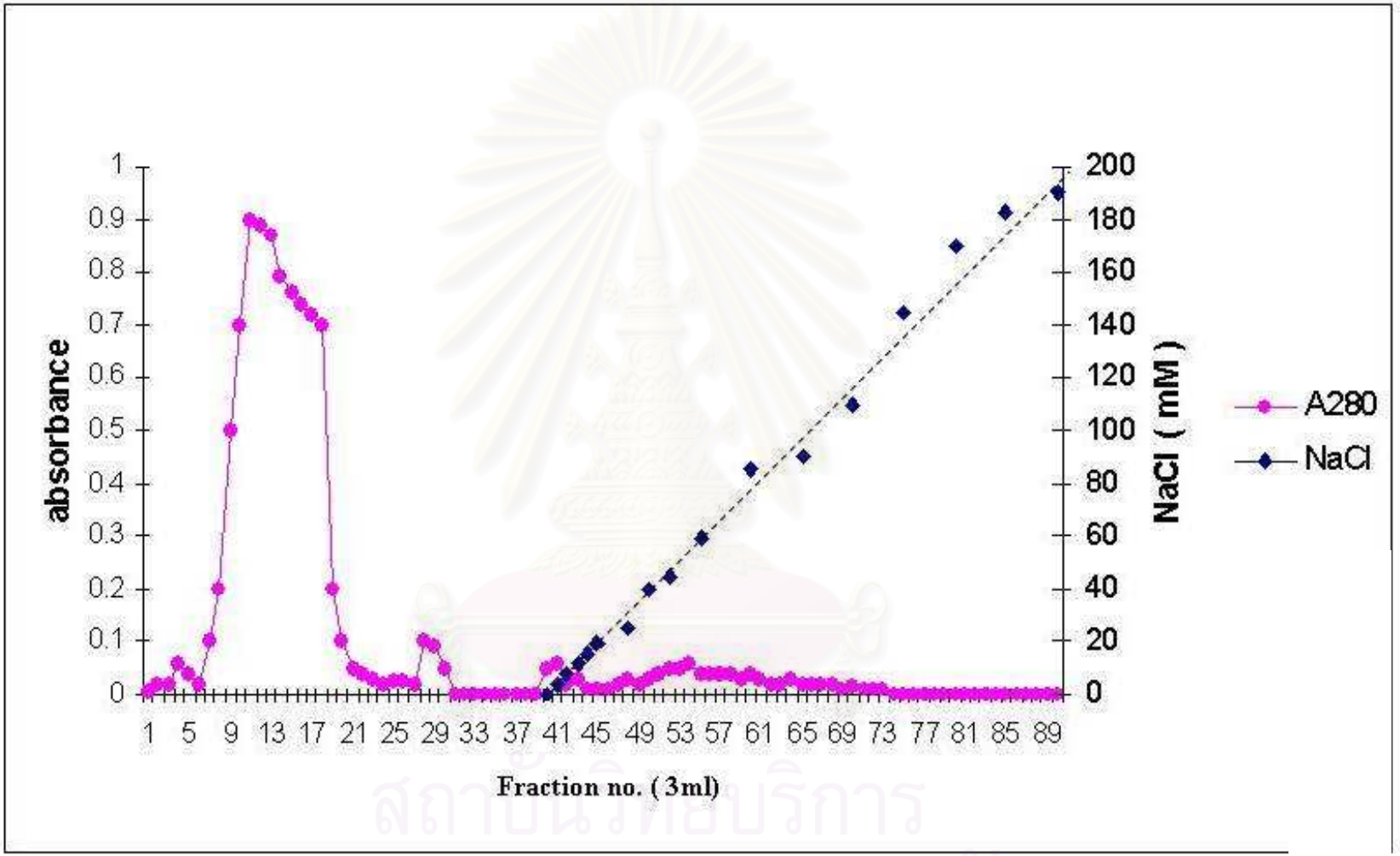


Figure 3.20 Purification profiles of recombinant MAP30 by affinity column Phosphocellulose P11. .The prot
applied to the column and washed with 50 mM Phosphate buffer pH 6.0, the bound protein was eluted by a gradier 0
mM NaCl

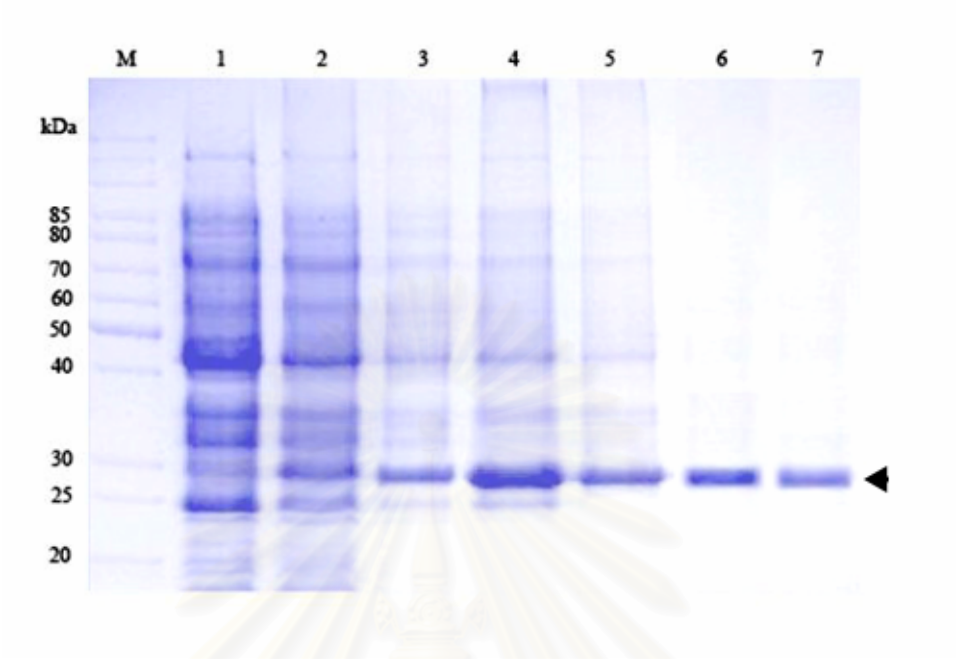


Figure 3.21 The SDS-PAGE pattern of proteins from different purification step.

Lane M = low molecular weight protein marker

Lane 1 = Total cell protein of non – induced cell

Lane 2 = Total cell protein of induced cell

Lane 3 = crude inclusion bodies

Lane 4 = solubilized inclusion bodies

Lane 5 = refolded inclusion bodies

Lane 6 = purified refolded inclusion bodies form CM-Sephadex C50, CmII

Lane 7 = purified refolded inclusion bodies form Phosphocellulose P11, unbound

3.4.2 Preparation of crude extract

Attempt to obtain active MAP30 from the highly expressed insoluble fraction (inclusion bodies) had failed, the soluble protein from cell supernatant was used instead. MAP30 was expressed in *E. coli* Rosetta DE3 (pLysS) harboring the pETmap expression vector. Crude protein solution was prepared from 180 mg wet weight cell which was cultivated in 500 ml of LB medium supplemented with 100 µg/ml ampicilin, 36 µg/ml chrolamphenicol and 0.1 mM IPTG. As described in section 2.10, cells were lysed by pulsed sonication and the soluble protein and inclusion body were fractionated by centrifugation at 12,000 rpm for 30 minutes. The supernatant contained 74 mg protein or about 75 % of total cell protein.

3.4.3 Purification of soluble protein

3.4.3.1 Ammonium sulfate precipitation

Supernatant containing the soluble recombinant MAP30 protein was precipitated with 0-30%, 30-60% and 60-90 % saturated ammonium sulfate, respectively. The precipitate was dissolved in 50 mM phosphate buffer, pH 6.0, dialyzed and concentrated with aquasorb. Each protein preparation was screened for MAP30 by observing its mobility in SDS-PAGE and measured the topological inactivation activity represented by topoisomerase I activity using pGEM as substrate and human topoisomerase I as the control (Section 2.15.3). Figure 3.23 demonstrated that both precipitates from both 0-30% and 30-60% fractions exhibited topoisomerase activity as observed from the alteration of supercoiled plasmid topology to relaxed form (lane 5 and 3). On the other hand, the 60- 90% fraction gave smear DNA pattern without distinct plasmid band. Analysis by SDS-PAGE (Figure 3.22) showed that the protein band of approximately 30 kDa was resided mainly in the 30-60% ammonium sulfate precipitate. This precipitation was used for further purification by column chromatography.

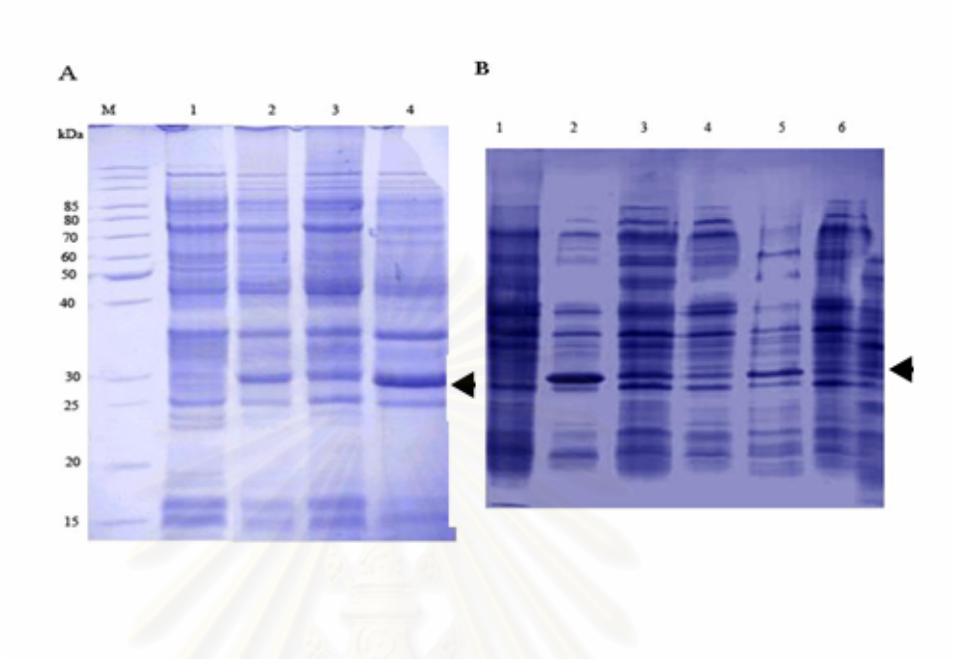


Figure 3.22 Screening of MAP30 from ammonium sulfate precipitates. The proteins were separated by 12% SDSPAGE and stained with Coomassie Brilliant Blue R250 staining

Lane 1 = Total cell protein of non- induced recombinant cell

Lane2 = Inclusion bodies

Lane3 = crude soluble protein

Lane4 = 0-30 % ammonium sulfate precipitates of supernatant from induced cell

Lane5 = 30 -60 % ammonium sulfate precipitates of supernatant from induced cell

Lane6 = 60-90% ammonium sulfate precipitates of supernatant from induced cell

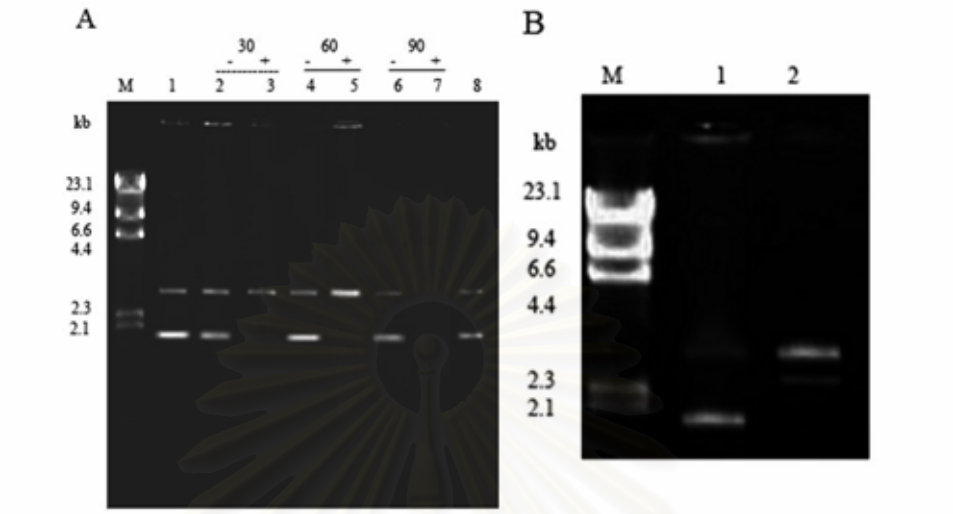


Figure 3.23 Topoisomerase activity of MAP30 from ammonium sulfate precipitations.

A.

Lane1 = Plasmid pGEM

Lane (-) = Control sample in which the precipitate was boiled before assay

Lane (+) = Test sample from each precipitate

The percentage of ammonium sulfate precipitate was indicated in the top row and of the figure .

B

Lane M = λ/Hind III DNA marker

Lane 1 = Plasmid pGEM

Lane 2 = Plasmid incubated with human topoisomerase I

3.4.3.2 CM Sephadex C-50 columns

Five milliliters (61.65mg) of the concentrated 30- 60% ammonium sulfate preparation was further fractionated by CM-Sephadex C-50 column which was equilibrated in 50 mM phosphate buffer pH 6.0 and eluted with 0-200mM MaCl gradient. The elution profile gave 2 unbound protein peaks (CmI and CmII) (Figure 3.24). Each peak was pooled for investigation of topoisomerased activity and molecular mass estimation by SDSPAGE. The observation show that both pooled fractions contained topoisomerase activity by alteration of supercoiled to linear and relax plasmids, but protein of approximately 30kDa was obtained from CmII fraction (Figure 3.25 and 3.24, respectively). The result also indicated topoisomerase activity in the CmI peak but with less MAP30 and more impurities. Thus CmII was chose for purification by affinity chromatography.

Since MAP30 binds to DNA, further attempts to purify the protein were done by affinity column chromatography using pPhosphocellulose P11.

3.4.3.3 Cellulose phosphate p11 column

Ten milliliters (49.33 mg) of the pooled CmII fraction containing the recombinant MAP30 protein were applied onto cellulosephosphate P11 column equilibrated with the 50 mM phosphate buffer pH 6.0. The bound protein was eluted with a gradient of NaCl (0-0.2M). Three-militer fractions were collected. The elution profile showed one major peak of the unbound and bound fraction (Figure 3.27). Both peaks were individually pooled and analyzed for their topoisomerase activities and molecular weights by SDS PAGE

The topoisomerase activity obtained from the bound protein fraction showed alteration of plasmid supercoiled topology to relaxed and linear forms, while the same activity was not observed in the unbound protein (Figure

3.28). The protein purity was investigated by 12% SDS-PAGE. A single protein band of 30kDa expected of recombinant MAP30 was obtained from the phosphocellulose P11 bound protein fraction (Figure 3.29). The result also indicated that advantage of using phosphocellulose P11 for the purification process. The recombinant protein was released from the column in the range of about 70-150 mM NaCl. The recovery of the recombinant protein was 30.5 mg which represents about 15% of total *E. coli* protein extract from 500 ml culture (1.8 g cell wet weight).The recovery yield was summarized in Table3.2.

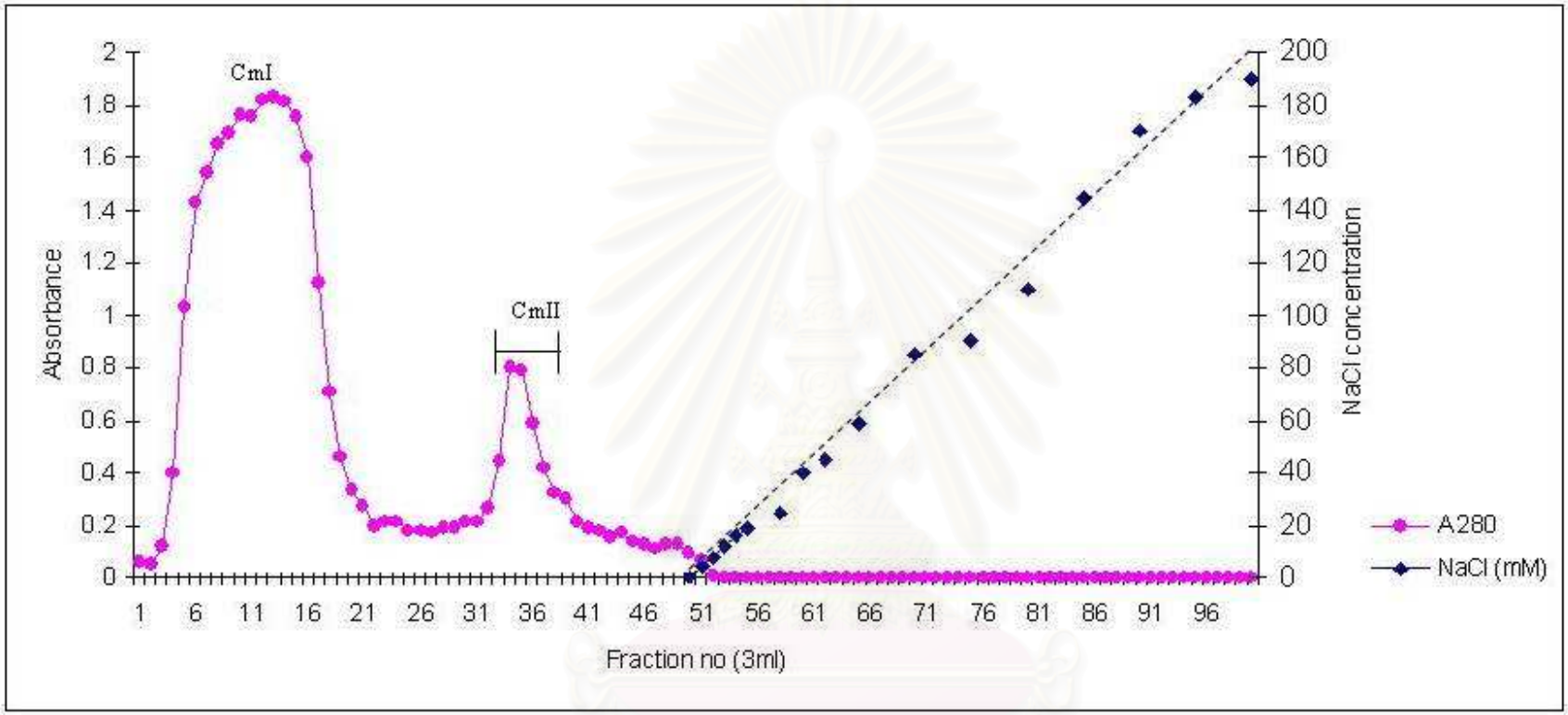


Figure 3. 24 Purification profiles of recombinant MAP30 by CM Sephadex C 50 The protein was applied to the column and wash by 50 mM Phosphate buffer pH 6.0 at flow rate of 3ml and eluted with 0-0.2 M NaCl gradient, ⊢⊣ fraction 30-40 were pooled.

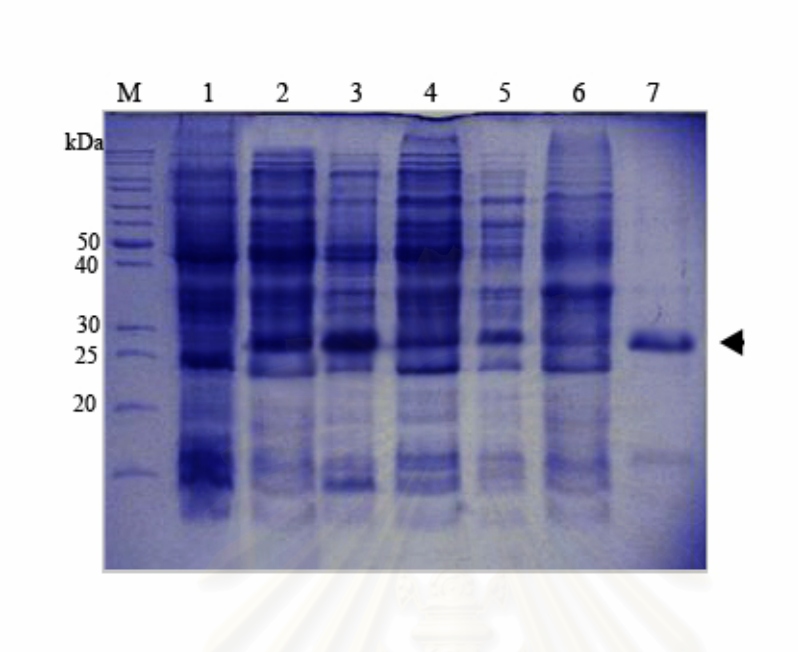


Figure 3.25 The SDSPAGE pattern showing profiles of protein purification.

Lane5-7= are samples prepared from the supernatant.

Lane M = Low molecular weight protein marker

Lane1 = Total cell protein of non – induced cell

Lane2 = Total cell protein of induced cell

Lane3 = Inclusion body

Lane4 = Crude solubilized inclusion body

Lane5 = Precipitaes from 30-60% amomonium sulfate

Lane6 = Protein solution of Cm I fraction

lane7 = Protein solution of Cm II fraction

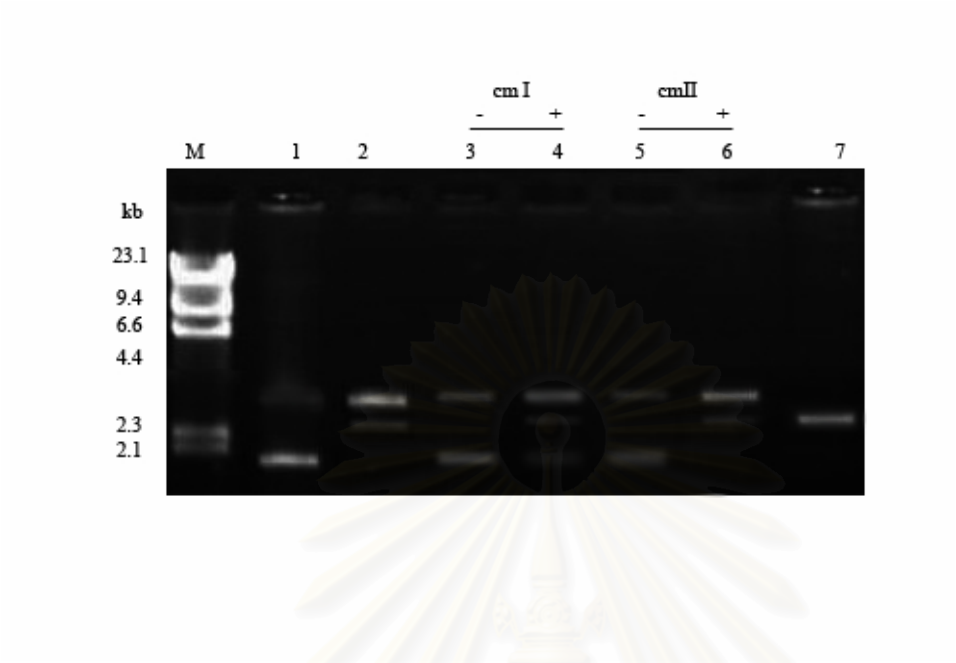


Figure 3.26 Topoisomerase I activity of protein from CM Sephadex C50 column.

Lane M= λ/Hind III DNA marker

Lane 1 = plasmid treated with buffer

Lane 2 = plasmid treated human topoisomerase I

Lane 3 = plasmid treated with boiled- protein from CmI

Lane 4 = plasmid treated with protein from CmI

Lane 5 = plasmid treated with boiled- protein from CmII

Lane 6 = plasmid treated with protein from CmII

Lane 7 = lineared plasmid by

- = control sample. The protein was boiled before assayed

+ = test sample

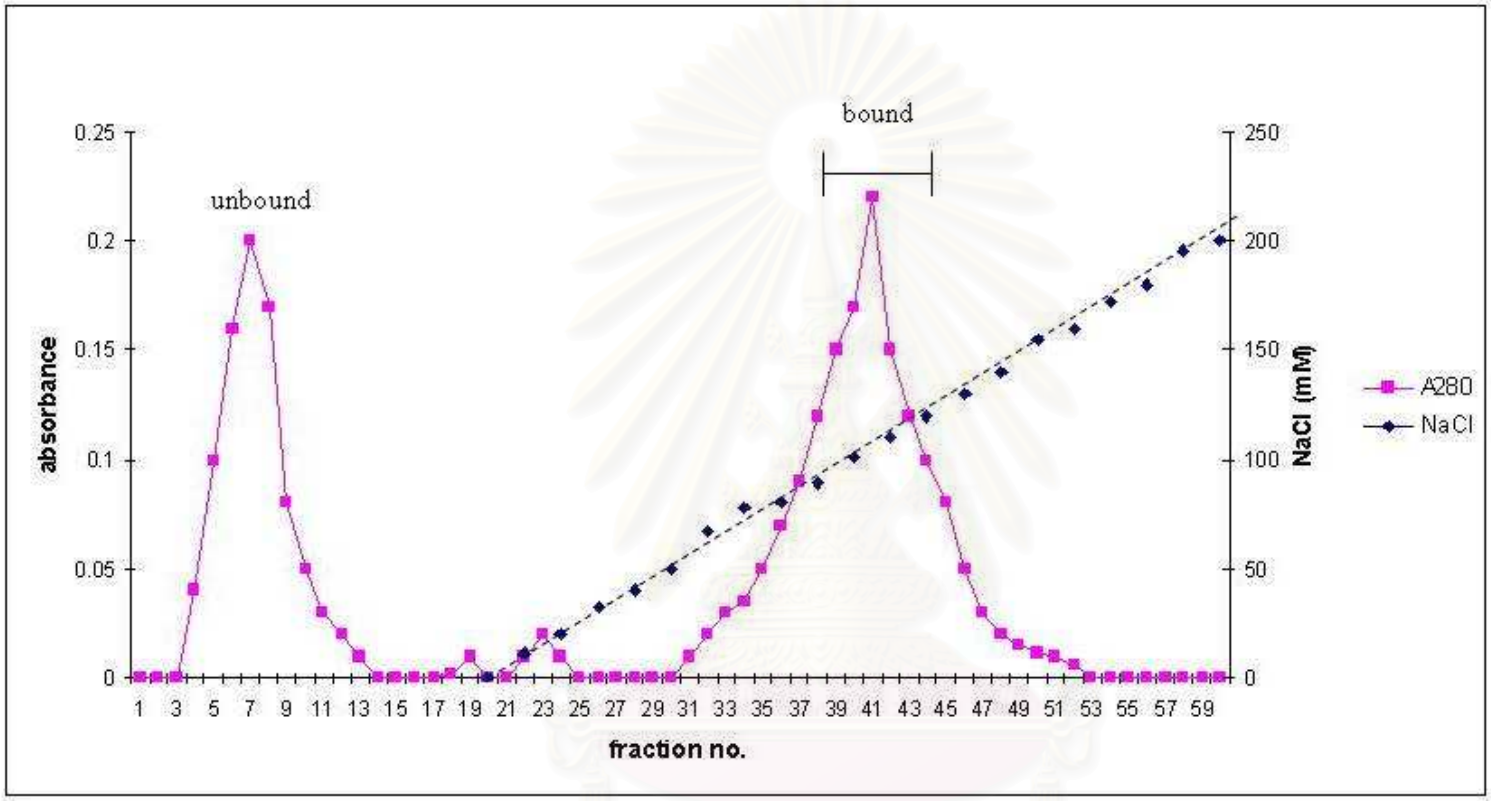


Figure 3.27 Purification profiles of recombinant MAP30 by Phosphocellulose P11. Protein was applied to the column and washed with 50 mM Phosphate buffer pH 6.0, and eluted by 0-200 mM NaCl gradient. ⊢⊣ fraction 35-45 were pooled.

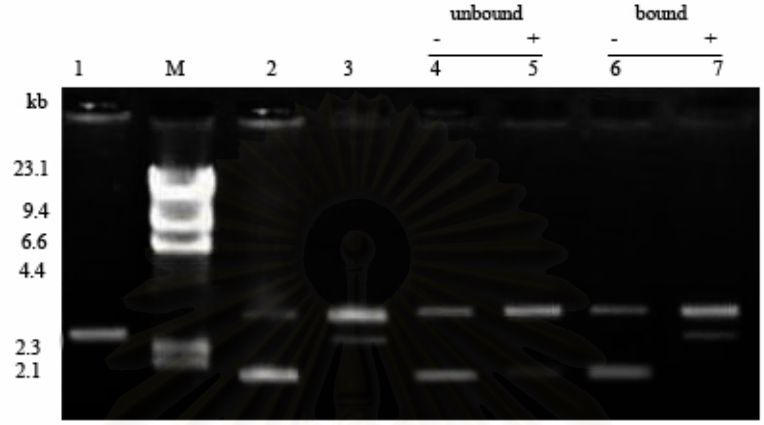


Figure 3. 28 Topoisomerase activity of MAP30 from phosphocellulose P11 column.

Lane M = λ/*Hin*d III DNA marker

Lane 1 = lineared plasmid pGEM

Lane 2 = plasmid pGEM

Lane 3 = plasmid treated with topoisomerase I

Lane 4 = plasmid treated with boiled-unbound protein

Lane 5 = plasmid treated with unbound protein

Lane 6 = plasmid treated with boiled-bound protein

Lane 7 = plasmid treated with bounded protein

Lane (-) = control

Lane (+) = test

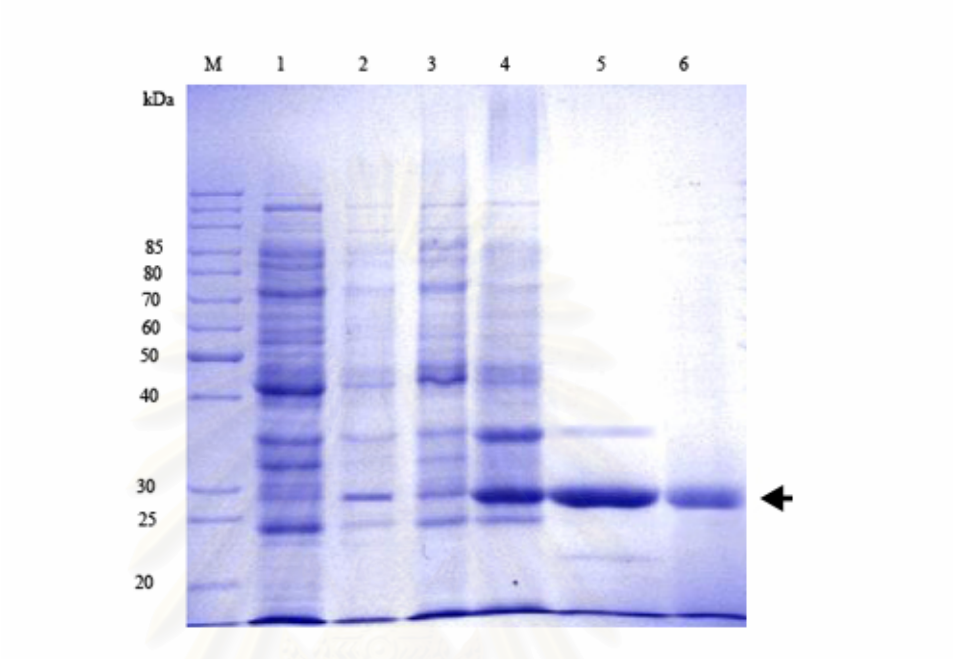


Figure 3.29 The SDS-PAGE protein pattern from different step of purification.

Lane M = low molecular weight protein marker

Lane 1 = cellular protein of non – induced cell

Lane 2 = inclusion bodies

Lane 3 = crude soluble protein

Lane 4 = 30-60 % ammonium sulfate fraction

Lane 5 = CmII from CM Sephadex C50

Lane 6 = Bound protein from Phosphocellulose P11

Table 3.1 Protein recovery from each purification step

Sample	Purification step	Protein (mg)	% yield
Crude protein (180 mg wet weight cell)		**210.0**	**100**
Soluble protein	**Crude soluble extract** 60%Ammonium sulfate precipitate After CM sephadex After Phospho cellulose P11	**148.0** 122.7 68.3 30.5	**75.0** 58.7 47.0 15.0
Inclusion body	**precipitate** Solubilized fraction Refolded fraction iCm II fraction from CM –Sephadex	**52.0** 20.4 20.1 18.9	**25.0** 20.0 19.5 19.0

3.5 Characterization of MAP30

3.5.1 Molecular weight analysis

Estimation of the molecular mass of encoded MAP30 from pETmap by SDS-PAGE analysis as compared to low molecular weight marker is 26.33 kDa (figure 3.30), while calculation by computing program is 29.62 kDa.

3.5.2 N-terminal sequence

The putative protein which was purified to homogeneity from Phosphocellulose column was confirmed to be MAP30 by N-terminal sequencing (section 2.15.2). The first 20 amino acid N-terminal sequence were Met Asp Val Asn Phe Asp Lle Ser Thr Ala Thr Ala Lys Thr Tyr Thr Lys Phe Ile and Glu. This is identical to the reported sequence of the mature MAP30 by Lee-Haung (Lee Haung *et al*., 1990).

3.5.3 Dose reponse topoisomerase activity of purified MAP30

MAP30 has topoisomerase-like activity but the DNA topological inactivation was irreversible (Lee-Haung *et at*., 1995). It is capable of introducing irreversible single and double-stranded breakage of supercoiled DNA to form topologically inactive products. Figure 3.40 show the results of incubating supercoiled plasmid DNA with 0.01-10 μg purified re-MAP30 in 20 μl of total reaction volume. It was shown that the lowest concentration of MAP30 which could significantly convert supercoiled DNA to relax forms was 0.05 μg or 0.85 μM.

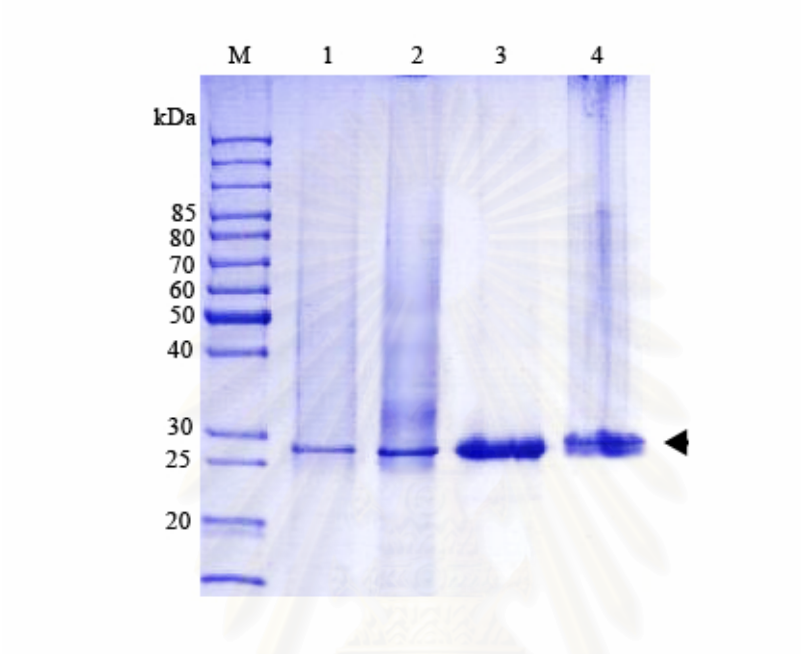


Figure 3.30 Molecular weight determination by 12% SDS PAGE

Lane M= low molecular weight protein marker

Lane 1= Purified MAP30 from inclusion body

Lane 2= Purified MAP30 from inclusion body was treat with β-marcaptoethanol

Lane 3= Purified soluble MAP30

Lane 4= Purified soluble MAP30 was treat with β-marcaptoethanol

Figure 3.31 Doseresponse activity of MAP30

Lane M = λ/*Hin*d III DNA marker

Lane 1 = plasmid pGEM

Lane 2 = plasmid was incubated with buffer

Lane 3-15 = plasmid was incubated with 0.01, 0.05, 0.1, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 µg of MAP30 in 20 µl reaction mixture.

CHARPTER IV

DISCUSSION

MAP30 from *M. charantia* is an anti-HIV plant protein that is also effective against several viruses and a vast group of human tumor cells, in vitro and in vivo. It contains multiple activities and capable of inhibiting various stages of viral life cycle. Such anti-viral and anti-tumor activities are not unique to MAP30 many RIPs and plant-derived proteins also exhibited these interesting property. Few example of these proteins are TAP29 (Tricosanthin) from the root tuber of *Trichosanthes kirilowii*, GAP31 (Gelonin) from *Gelonium multiflorum* seeds, DAP30 and 32 (Dianthus of 30 and 32 kDa) from leaves of carnation, ricin from *Ricinus communis* and PAP (Pokeweed antiviral protein) from pokeweed plant (Lee-Huang *et al*., 1990, Rajamohan *et al*., 1999). However, when assayed under identical condition, MAP30 showed higher potency and less cytotoxic effect than others (Lee-Huang *et al*., 1990). Structurally, MAP30 shared similar three-dimensional fold with ricin A with 25% sequence homology and 57% sequence homology to trichosanthin. Lee Huang *et al*. had studied MAP30 quite extensively and postulated that although MAP30 is a type I ribosome inactivating protein (RIP), the anti-viral activity lies mainly to its inhibition of viral integration at 3 steps in a dose-dependent fashion: (a) 3'-processing of viral DNA, (b) strand transfer of viral DNA into host chromosome and (c) the excision of the viral DNA from host genome (Lee-Huang *et al*., 1995)

Although, bitter melon is widely used as an alternative therapy due to its many pharmaceutical active ingredients, including MAP30, but some undesirable side-effect could occur from excessive/uncontrolled ingestion of bitter melon juice. It

may create diarrhea and abdominal pain, for instance. Consuming excessive amount of the seeds is linked with fever, headache and coma in extreme case. Abortive effect was also reported in pregnant animals. Without careful monitoring, hypoglycemia may be a negative effect by bitter melon, trigger or warning the condition of diabetes patients. With this bear in mind, MAP30 should be isolated and purified before it could be used to avoid toxic contaminants.

The present study reported the production of MAP30 in *E. coli*. The source of the *map30* gene is Mara Khee Nok, a local *M. charantia* variety in Thailand. *E. coli* was used because its cultivation is not costly and is fast growing. Firstly, genomic DNA of Mara Khee Nok was extracted and amplified with primers designed from the published sequence of Lee-Huang (1995). A DNA fragment of approximately ~800 bp was obtained (Figure 3.2) which correlated well with the size of mature MAP30 devoid of leader peptide (263 amino acids). This fragment was cloned into the *Eco*RI-*Bam*HI site of pUC18. The pUC based vector is generally used for cloning in *E. coli* basing on its high copy number properly and contain the moderately strong *lac* promoter.

In addition, its expression could be controlled by ITPG and recombination cells could be easily identified by the inactivation of *lac* Z' gene at the polylinker site. The recombinant plasmid was used for nucleotide sequencing and the corresponding amino acid sequence was deduced. From the result, it was proved that map 30 gene from Mara Khee Nok had been cloned into the pUC18 (Figure 1.8). The resulting recombinanat plasmid was named pUC map.

Since There are many varieties of *M. charantia* with diverse morphology and intraspecific variation (Para-or, 2001) and that the origin of *map* 30 from Lee-Huang studies was not specified, it would be interesting to compare MAP30 from Mara Khee

Nok with other sources, especially that of Lee–Huang *et al* who is the main research group for this protein and had patented the gene. It was hoped that this result could lead to comparison of their biological activities and finally strain development. Alignment of Mara Khee Nok MAP30 with DNA database revealed that MAP30 is a highly conserve protein. Only 1-4 nucleotides and 2 amino acids variations was observed. Using Program RASMOL to superimpose 3-D structure of this study with that of Lee-Huang showed that the overall structure are similar and the amino acids His^{47} and Ile^{166} (Arg^{47} and Tyr^{166} for reported sequence) was not in the vicinity of N-glycosidase active site (Tyr^{70},Tyr^{109} and Ile^{158})(Figure 3.8)

As MAP30 was not expressed by pUC map *E. coli*. JM109 when induced with 1 mM IPTG (Figures 3.9), It is possible that MAP30 is a plant protein in nature. *M. charantia* has many codon usages that are different from the *E. coli* system. The *map30* gene contains a lot of codons rarely used in *E. coli* such as AGA, AGG, CGG, CGA for Arg, ATA for Ile, CTA for Leu and CCC for Pro (Novy *et al*., 2001). These rare codons can stall out stop polypeptide synthesis in cell contain a little, or without, the cognate tRNAs. Otherwise, the DNA supercoiled structure is required in prokaryote for initiation of DNA replication, site-specific recombination and transcription of some gene (Kerbs *et al*., 1996). Therefore, irreversible change of DNA relaxed form by acting of topoisomerase-liked activity of MAP30 cause cell are not growth. However, the recombinant cell that is not produce MAP30 can be survived by natural adaptation. May be it is another reason to show that MAP30 is not expressed in pUCmap harboring *E. coli* JM 109. While, MAP30 can be expressed by pET system because the system contains strong T7 promoter, it produces many RNA molecule that increase an opportunity of MAP30 production more than Lac promoter in pUC plasmid. However, inclusion bodies were observed after induction 1-2 hours.

Since, inclusion bodies are non-function polypeptide. It is possible that the cells produced MAP30 as inclusion bodies to decrease toxicity of this protein.

However, it should also be noted that the present expression system was not optimized and could be another reason for the result. The putative MAP30 gene was further subcloned into pET196 to be expressed in *E. coli* Roseta DE3 (pLysS). The pET expression system contains a high selectivity T7 RNA polymerase for its cognated promoter, the high level of the polymerase and the high translation efficiency mediated by the T7 gene translation signals (Novagen, 2002). In the pET system, the protein coding sequence of interest is cloned to downstream of the T7 promoter and then transformed into *E. coli* strains Rosetta DE3 (pLysS). Protein expression was achieved by IPTG induction of a chromosomally integrated cassette in which the T7 RNA polymerase was expressed from the lacUV5 promoter and then polymerase would be specific binding to the T7 promoter in expression vector. Next, host RNA polymerase would be transcribed by the highly activity, together with high efficiency translation and then target protein may contaminate the majority of the cellular protein after only a few hours. However, host system would supply the eukaryotic codon usage. For this reason, *E. coli* Rosetta DE3 (pLysS) was selected. The Rosetta strain contains host plasmid which contain gene of rare codon.

Induction of *map30* with 1 mM IPTG at 37 °C resulted in the appearance of MAP30 as cellular soluble protein and inclusion bodies (Figure 3.12). The majority of MAP30 was in the inclusion bodies fraction. Since inclusion bodies are aggregates of inactive protein, generally produced when that protein is expressed in high level at fast rate that proper folding of the molecule. Intermolecular hydrophobic and ionic interactions of these partially folded proteins with exposed hydrophobic moieties occurred causing aggregate. Although, there are advantages aspects of inclusion

bodies for making such as high purity concentrated protein of interest with few contaminants (mostly from lipoprotein envelope of the cell debris), the process from renaturation of this protein often require a lot of trials for suitable renaturation. The active yield are often low to avoid the problem from inclusion body formation, optimization experiments were performed using lower concentration of IPTG (0.1-0.5 mM) and incubated at 25-37 °C. Unfortunately, all tested conditions still provide MAP30 mainly in the inclusion bodies fraction (25% of total cell protein). The induction was, at last, chose to use 0.1 mM IPTG at 30 °C. At this condition, MAP30 was produced in *E. coli* Rosetta DE3 (pLysS) in large amount.

Purification method for target protein is based on several molecular weight, or specific interaction between the protein and separation matrix. For MAP30 purification, recombinant cells were broken by sonication and the soluble and insoluble fractions were separated by centrifugation. Ammonium sulfate precipitation of crude extract was chosen because it combines many useful features such as solution and low price (Bollage *et al*., 1991). The result showed that similar to the report by Lee-Huang (1990). The SDS-PAGE result indicated that inclusion bodies contained major 30 kDa protein band. For the inclusion body fraction, this step was omitted because the preparation was quite pure.

The soluble MAP30 from 30-60% ammonium sulfate precipitation is a weak cation-exchanger. The result showed that MAP30 fraction (indicated by approximately 30 kDa band in SDS-PAGE, Figure 3.22) was not bound to the column and was washed out in the latter peak (CmII). Since the MAP30 is a basic protein (composed of about 20% basic amino acid residues) with a calculated pI of 8.57, the protein should bind to the column at pH 6.0. Either the CM matrix was not properly activated or the ionic strength of the washing buffer was too strong (50 mM) may

contribute to the observations. Although, the protein samples failed to attach to the column, the quality of protein from CmII fraction was almost pure, so further attempt on this ion exchanger was disregarded. MAP30 binds to both DNA and RNA as observed from its RIP activities and topological inactivation of double stranded DNA. Therefore, Phosphocellulose P11 was used as affinity matrix for MAP30 purification. The result in Figure 3.28 confirmed this assumption. Proteins passing through phosphocellulose P11 column were separated into 2 peaks: bound and unbound (Figure 3.27). The bound fraction showed a single band protein in SDS-PAGE. The result demonstrated that the putative MAP30 was pure at this step. Amino acid sequencing of the first 20 residues at N-terminals confirmed that this protein is generally derived from *map30* gene of Mara Khee Nok.

For the solubilized and refolded protein from the inclusion body fraction, passing through CM-Sephadex C50 and phosphocellculose P11 also rendered pure protein of approximately 30 kDa. Regretfully, the corresponding molecular mass does not reflect the folding of correct conformation as could be observed from the unbinding of the protein sample to Phosphocellulose P11 column. Activity assay by topological inactivation also confirmed that somehow the renaturation attempt by direct dilution of the solubilized sample with 20% glycerol failed to render the correct folding of the protein. In general, addition of detergents such as Triton X-100 (non-ionic detergent) chotropic compound prior to cell breakage or for washing the inclusion body preparation will allow the removal of membrane proteins or other contaminants in microbial cell (Vallejo and Rinas, 2004) and the inclusion bodies was dissolved in guanidium hydrochloride or urea. In this study, urea was chosen because it is less expensive than guanidium hydrochloride. It should also be noted that there are evidences that urea solutions may contain and spontaneously produce

cyanate which can carbamylate the amino group of the protein .Other types of solubilized agents include detergents low concentration of various kinds of determinants, aggregation suppressor arginine. The solubilization of inclusion body results in a soluble protein that is devoid of its native conformation. To archive the biological activity, this protein must be refolded in an appropriate condition. Protein refolding requires intramolecular interactions and can require a few seconds to several days. During this period, more often than not, misfolding occurs more. Refolding yield also decreases with increase initial concentration of the protei (Vallejo and Rinas, 2004). The simplest refolding method is direct dilution of the solubilized sample in buffer or buffer in the presence of chemicals (such as glycerol L-arginine) that aids refolding. Proper method should be concluded from several trail experiments of varying technique. Beside its size, MAP30 could be detects and quantitative by its biological properties. Lee-Huang *et al* accessed MAP30 by measuring (a) *in vitro* translation inhibitory activity using reticulocyte system or viral core protein p24 expression, (b) inhibition of HIV-1 integrase and (c) inhibition of syncytial formation for antiviral and anti-tumor activity. MAP30 could cleave supercoiled or nicked plasmid by introduce one strand breakage at a time. The break is irreversible so MAP30 is likened to topoisomerase in the presence of inhibitor. It was postulated that this reaction renders the viral DNA an in appropriate substrate for integrase. Studies also suggested that binding of MAP30 to DNA alone could result in the inhibition of integrase. In this study, the topoisomerase activity was chosen for reasons of simplicity and cost effectiveness. The result in this study showed that MAP30 could introduce strand break of supercoiled plasmid to become relaxed and/or linear forms. Since the purified MAP30 was used in the assay, contamination by *E. coli* topoisomerase I (96 kDa) was excluded. The reaction was also controlled for

nuclease nicle by addition of EDTA. From this study, it was shown that MAP30 could be easily screen for by using topoisomerase activity. With the multiple activities of this small protein, particularly the anti viral activities, it would be interesting to test MAP30 against RNA viruses which cause severe epidemic in Thailand. The efficacy of the protein from various varieties should also be tested to see if strain conservation and/or improvement would be benefited.

CHARPTER V

CONCLUSIONS

1. The *map30* gene from Mara Khee Nok is composed of 789 nucleotides (excluding the leader peptide) and encodes for 263 amino acids.
2. The comparison of *map30* gene from Mara Khee Nok with known *map30* from DNA database showed that MAP30 is a highly conserve protein, even though their morphology of *M. charantia* is vary diverse.
3. The molecular mass of the purified MAP30 is 29.62 kDa by calculation and the first 20-amino acid sequence at the N-terminus is identical to the reported MAP30 gene by Lee Huang *et* al (Lee-Haung *et al*., 1995).
4. The recombinant MAP30 was expressed both as soluble and inclusion bodies in *E. coli* Rosetta DE3 (pLysS) by using pET19b at the condition used.
5. Purified MAP30 was obtained from precipitation with 30-60% saturated ammonium sulfate, CM-Sephadex C50 and Phosphocellulose P11 column chromatography as observed by SDS-PAGE analysis.
6. Minimal concentration of MAP30 exhibiting topoisomerase activity was 0.85 μM.
7. Topoisomerase activity is an easy and fast method for detection of MAP30.

REFERENCES

Arazi, T., Lee Huang, P., Lin Huang, P., Zhang, L., Shiboleth, Y. M., On, A. G. and Lee-Huang, S. 2002. Production of antiviral and antitumor proteins MAP30 and GAP31 in Cucurbits using the plant virus vector ZYMV-AGII. *Biochemical and Biophysical Research Communications*. 292: 441-448.

Barbieri, L., Brigotti, M., Perocco, P., Carcncelli, D., Ciani, M., Mercatali, L. and Stirpe, F. 2003. Ribosome-inactivating proteins depurinate poly (ADPribosyl) ated poly (ADP-ribose) polymerase and have transforming activity for 3T3 fibroblasts. *FEBS Lett.* 538: 178–182.

Barbieri, L., Balteli, M.G. and Stirpe, F.1993. Ribosome inactivating proteins from plants. *Biohim Biochem Biophys Acta.* 1154: 237–284.

Barbieri, L., Valbonesi, P., Bonora, E., Gorini, P., Bolognesi, A. and Stirpe, F.1997. Polynucleotide:adenosine glycosidase activity of ribosome-inactivating proteins: effect on DNA, RNA and poly(A). *Nucleic Acids Res.* 25: 518–522.

Barbieri, L., Valbonesi, P., Righi, F., Zuccheri, G., Monti, F., Gorini, P., Samorı, B. and Stirpe, F. 2000. Polynucleotide:adenosine glycosidase is the sole activity of ribosome-inactivating proteins on DNA. *J Biochem.* 128: 883–889.

Basu, I., Ferens W. A., Stone, D. M. and Hovde, C. J. 2003. Antiviral activity of shiga toxin requires enzymatic activity and is associated with increased permeability of the target cells. *Infection and imunity.* pp. 327–334.

Basch, E., Gabardi, S., and Ubricht, C. 2003. CLINICAL REVEIWS: Bitter melon Bitter melon (*Momordica charantia*): A review of efficacy and safety. *Am J Health-Syst Pharm*. V. 60

Bax, A., and Torchia, D. A. 1999. Solution structure of anti-HIV-1 and anti-tumor protein MAP30: structural insights into its multiple functions. *Cell.* 99:433–442.

Benjamin,D., Sharma, V., Kubin, M., Klein, J. L., Sartori, A., Holliday, J., and Trinchieri, G.1996. IL-12 expression in AIDS related lymphoma B cell lines. *J. Immunol.* 156:1626–1637.

Bollag D. M. and Edelstein S. J.1991. Concentrating protein solutions: Preparative methods*, Protein methods*. pp. 79-82.

Bolognesi, A., Polito, L., Tazzari, P. L., Lemoli, R. M., Lubelli, C., Fogli, M., Boon, L., Boer, M. D. and Stirpe, F., 2000. In vitro anti-tumour activity of anti-CD80 and anti-CD86 immunotoxins containing type 1 ribosome-inactivating proteins. *British Journal of Haematology.* 110:351-361

Bourinbaiar A. S., and Lee-Huang S.1996. The activity of plant-derived antiretroviral proteins MAP30 and GAP31 against herpes simplex virus in vitro. *Biochem. Biophys. Res. Commun.* 219: 923–929.

Chulabhorn Mahidol, Prawa, H.V.and Ruchirawat, S. 2002. Investigation of some bioactive Thai medicinal plants. *Photochemistry Reviews.* 1: 287–297

Christopher, D. Putnam and John, A. Tainer. 2000. The food of sweet and bitter fancy. *nature structural biology.* Vol.7 No.1:17 -18

Cowan, M. M. 1999. Plant Products as Antimicrobial Agents. *Clinical microbiology reviews*. pp. 564–582

David, C. Chan and Peter, S. Kim 1998. HIV Entry and Its Inhibition. *Minireview Cell*. 93:681–684

DeRisi, J., Penland, L., Brown, P. O., Bittner, M. L., Meltzer, P. S., Ray, M., Chen, Y., Su, Y. A., and Trent, J. M. 1996. Use of a cDNA microarray to analyse

gene expression patterns in human cancer. *Nat. Genet.* 14: 457–460

Emmanuelle, N., Goodyer, I. D. and Theodore, F. T. 1997. An additional mechanism of ribosome-inactivating protein cytotoxicity : degradation of extrachromosomal DNA. *Biochem. J.* 327: 413-417

Huang, P. L., Sun, Y., Chen, H. C., Kung, H. F., and Lee-Huang, S. 1999. Proteolytic fragments of anti-HIV and anti-tumor proteinsMAP30 and GAP31 are biologically active. *Biochem. Biophys.Res. Commun*. 262: 615–623.

Krebs, J. E. and Dunaway, M.1996. DNA Length Is a Critical Parameter for Eukaryotic Transcription In Vivo. *Molecular and cellular biology*. pp. 5821-5829

Lee-Huang, S., Huang, P. L., Sun, Y., Chen, H. C., Kung, H. F. and Murphy, W. J. 2000. Inhibition of MDA-MB-231 human breast tumor xenografts and HER2 expression by anti-tumor agents GAP31 and MAP30. *Anticancer Res.* 20:653–659.

Lee-Huang, S., Huang, P. L., Nara, P. L., Chen, H. C., Kung, H. F., Huang, P., and Huang, H. I. 1990. MAP 30: A new inhibitor of HIV-1 infection and replication. *FEBS Lett.* 272:12–18.

Lee-Huang, S., Kung, H. F., Huang, P. L., Li, B. Q., Huang, P., Huang, H. I., and Chen, H. C. 1991. A new class of anti-HIV agents: GAP31, DAPs 30 and 32. *FEBS Lett.* 291:139–144.

Lee-Huang, S., Kung, H. F., Huang, P. L., Bourinbaiar, A. S., Morell, J. L., Brown, J. H., Tsai, W. P., Chen, A. Y. and Huang, H. I. 1994. Human immunodeficiency virus type 1 (HIV-1) inhibition, DNA-binding,RNA-binding, and ribosome inactivation activities in the N-terminal segments of the plant anti-HIV protein GAP31. *Proc. Natl. Acad. Sci.* USA. 91:12208–12212.

Lee-Huang, S., Huang, P. L., Bourinbaiar, A. S., Chen, H. C., and Kung, H. F. 1995. Inhibition of the integrase of human immunodeficiency virus (HIV) type 1 by anti-HIV plant proteins MAP30 and GAP31. Proc. *Natl. Acad. Sci*. USA. 92: 8818–8822.

Lee-Huang, S., Huang, P. L., Chen, H. C., Bourinbaiar, A., Huang, H. I., and Kung, H. F. 1995. Anti-HIV and anti-tumor activities of recombinant MAP30 from bitter melon. *Gene.* 161: 151–156.

Lin Huang, P., Sun, Y., Chen, H. C., Kung, H.F., Lee Huang, P. and Lee-Huang, S. 1999. Proteolytic Fragments of Anti-HIV and Anti-tumor Proteins MAP30 and GAP31 Are Biologically Active. *Biochemical and Biophysical Research Communications.* 262: 615–623

Lodge, J.K, Kaniewski, W.K, Tumer, N.E. 1993. Broad-spectrum virus resistance in transgenic plants expressing pokeweed antiviral protein. *Proc Natl Acad Sci. USA*. 90: 7089–7093

Luban, J., *HIV Molecular biology & pathogenesis*.

Middelberg, P. J., 2000. Preparative protein refolding, *Trends in biotechnology*, Vol. 20, No. 2 : 437-443

Martoglio, A. M., Tom, B. D., Starkey, M., Corps, A. N., Charnock-Jones, D. S., and Smith, S. K. 2000. Changes in tumorigenesis- and angiogenesis-related gene transcript abundance profiles in ovarian cancer detected by tailored high density cDNA arrays. *Mol. Med.* 6: 750–765

Narayanan, S., Surolla, A. and Karande, A. 2004. A Ribosome-inactivating protein and apoptosis: abrin causes cell death via mitochondrial pathway in Jurkat cells. *Biochem. J*. 377: 233–240

Nicholson, D. W., and Thornberry, N. A.1997. Caspases: Killer proteases. *Trends*

Biochem. Sci. 22: 299–306.

Novagen. 2000. pET System Vectors and Hosts. *Instruction manual.*

Novagen. 2000. *pET System Manual*.

Novagen. 2000. Prokaryote system. *Protein Expression*.

Novy, R., Drott D. , Yaeger, K. and Mierendorf, R. Novagen, Inc. overcoming the codon bias of E. coli for enhanced protein expression. *New sletter of Novagen* , I N C .No. 12.

Park, S. W., Stevens, N. M., Vivanco, J. M. 2002., Enzymatic specificity of three ribosome-inactivating proteins against fungal ribosomes, and correlation with antifungal activity. *Planta.* 216: 227–234

Parikh, B. A., Coetzer, C. and Tumer, N. E. 2002. Pokeweed Antiviral protein regulates the stability of its own mRNA by a mechanism that requires depurination but can be separated from depurination of the alpha-Sarcin/Ricin Loop of rRNA. *The journal of biological chemistry.* Vol. 277, No. 44, pp. 41428–41437

Phan, G. Q., Attia, P., Steinberg, S. M., White, D. E., and Rosenberg, S. A. 2001. Factors associated with response to high-dose interleukin-2 in patients with metastatic melanoma. *J. Clin. Oncol.* 19: 3477–3482

Peumans, W. J., Hao Q. and Vandmme, Els J. M. 2001. Ribosome-inactivating proteins from plants: more than RNA N-glycosidases? *The FASEB Journal.* 15 :1493-1506

Rajamohan, F., Engstrom, C. R., Denton, T. J., Engen, L. A., Kourinov, I. and Uckun, F. M. 1999. High-level expression and purification of biologically active recombinant pokeweed antiviral protein. *Protein expression and purification.* 16:356-368.

Rebecca, S., Hugh, M., Nagarajan, S., Wang, Y. C., Sell, K. W. and Selvaraj, P. 1999. Protein transfer of glycosyl-phosphatidylinositol-B7-1 into tumor cell membranes: a novel approach to tumor immunotherapy1. *cancer research.* 59: 2433–2437

Ready, M. P, Brown, D. T, Robertus, J. D. 1986. Extracellular localization of pokeweed protein. *Proc Natl Acad Sci. USA.* 84:5053–5056

Robert, E., Hanclock, W. AND Chapple, D. S. 1999. MINIREVIEW:Peptide Antibiotics. *Antimicrobial agents and chemotherapy*. pp. 1317–1323

Rudolph, R. and Lilie, H. 1996, In vito folding of inclusion body proteins, *FASEB J.* 10:49-56.

Rybak, S., Lin, J., Newton, D., Kung, H., Monks, A., Chen, H., Huang, P. and Lee-Huang, S. 1994. In vitro anti-tumor activity of the plant ribosome inactivating proteins MAP30 and GAP31. *Int. J. Oncol.* 5:1171–1176

Saluta, M. and Bel, P. A.1998. Life Science News: Troubleshooting GST fusion protein expression in *E. coli*. *Amersham Pharmacia Biotech.*

Scherf, U., Ross, D. T., Waltham, M., Smith, L. H., Lee, J. K., Tanabe, L., Kohn, K. W., Reinhold, W. C., Myers, T. G., Andrews, D. T., Scudiero, D. A., Eisen, M. B., Sausville, E. A., Pommier, Y., Botstein, D., Brown, P. O., and Weinstein, J. N. 2000. A gene expression database for the molecular pharmacology of cancer. *Nat. Genet*. 24: 236–52.

Sharma, N., Park, S.W., Vepachedu, R., Barbieri, L., Ciani, M., Stirpe, F., Savary, B. J., and Vivanco, J. M. 2004. Isolation and Characterization of an RIP (Ribosome-Inactivating Protein)-Like Protein from Tobacco with Dual Enzymatic Activity. *Plant Physiology.* 134: 171–181

Stirpe, F.2004. Review: Ribosome-inactivating proteins. *Toxicon* . 44:371–383

Sun, Y., Huang, P. L., Li, J. J., Huang, Y. Q., Zhang, L. and Lee-Huang, S. 2001. Anti-HIV agent MAP30 modulates the expression profile of viral and cellular genes for proliferation and apoptosis in AIDS-related lymphoma cells infected with Kaposi's sarcoma-associated virus. *Biochem. Biophys. Res. Commun.* 287: 983–994.

Taylor, L. 2002. Technical Data Report for Bitter melon (Momordica charantia). *Herbal Secrets of the Rainforest*. 2nd edition.

Wang, Y. X., Neamati, N., Jacob, J., Palmer, I., Stahl, S. J., Kaufman, J. D., Huang, P. L., Winslow,H. E., Pommier, Y., Wingfield, P. T., Lee-Huang, S., Bax, A., and Torchia, D. A. 2000. AntiHIV and anti tumor protein MAP30, a 30 kDa single type-I RIP, shares similar secondary structure and bete-sheet topology with the A chain of ricin, a type II RIP, *Protein Science.* 9:138-144

Wasternack, C., Miersch, O., Kramel, R., Hause, B.,Ward, J., Beale, M., Boland, W., Parthier, B. and Ivo Feussner L.1998. Jasmonic acid: biosynthesis, signal transduction, *gene expression Fett/Lipid.* 100, Nr. 4-5, S. 139–146

Wang, P. and Tumer, N. E. 1999. Pokeweed antiviral protein cleaves double-stranded supercoiled DNA using the same active site required to depurinat rRNA. *Nucleic Acid Research* V. 27 No. 8 .pp. 1900-1905.

You, Z., Ouyang, H., Lopatin, D., Polver, P. J., and Wang, C. Y. 2001. Nuclear factor-kappa B-inducible death effector domaincontaining protein suppresses tumor necrosis factor-mediated apoptosis by inhibiting caspase-8 activity. *J. Biol. Chem*. 276:26398–2640

APPENDIX A

Bradford Method and reagents

Solutions

1.Bradford stock solution

100 ml 95% ethanol

200 ml 88% phosphoric acid

350 ml Serva Blue G

2. Bradford working buffer

450 ml distrilled water

15 ml 95% ethanol

30 ml 88% phosphoric acid

30 ml Bradford stock solution

Filter through whatman No.1 paper, store at room temperature in brown glass bottle. Usable for several weeks, but may need to be refiltered.

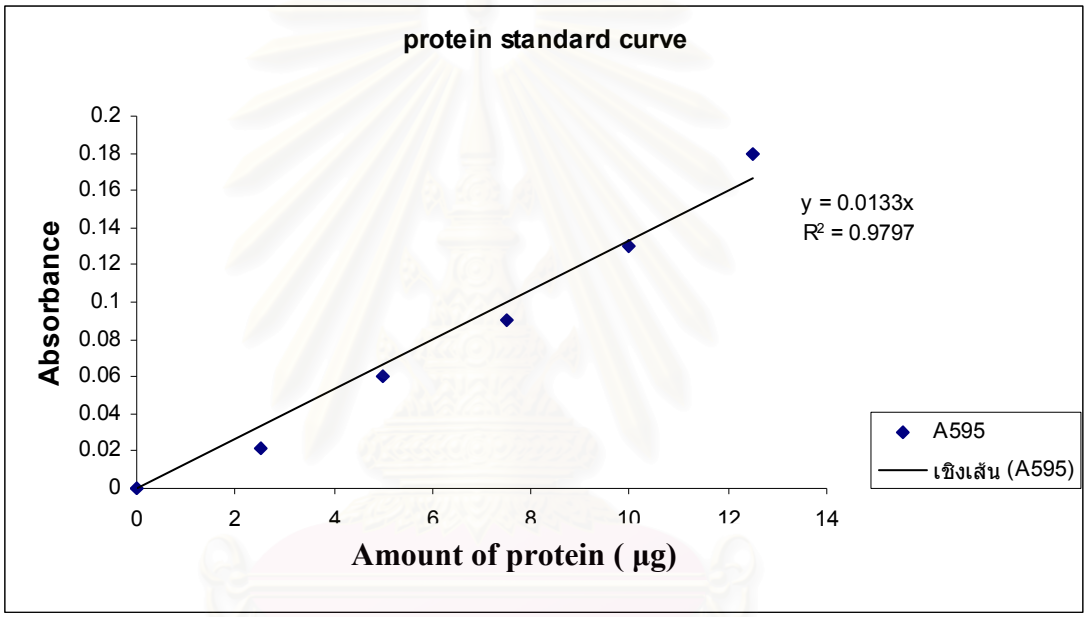
Assay

1. Pipet protein solution into tube
2. Add experiment buffer to make a total volume of 100 µl
3. Add 1 ml Bradford working buffer and vortex.
4. Read A_{595} after 2 minutes but before 1 hour

APPENDIX B

Standard curve for determine protein concentration by Bradford's method..

Standard curve for protein was made by mornitoring the absorbance at 595 nm of standard concentration BSA according to the Bradford's method.



APPENDIX C

Preparation of denaturing polyacrylamide gel electrophoresis

Solution A (Acrylamide Stock Solution)

a. 29.2 g acrylamide

b. 0.8 g *N, N'*-dimethylene-bis-acrylamide

Add ultrapure water to make 100 mL and stir until completely dissolved.

Solution B (4X Separating Gel Buffer)

a. 75 mL of 2 M Tris-HC, pH 8.8 (final concentration is 1.5 M)

b. 4 mL of 10%SDS (final concentration is 0.4%)

c. 21 mL of ultrapure water (final volume is 100 mL)

Solution C (4X Stacking Gel Buffer)

a. 50 mL of Tris-HCl, pH6.8 (final concentration is 0.5 M)

b. 4 mL of 10%SDS (final concentration is 0.4%)

c. 46 mL of ultrapure water (final volume is 100 mL)

10% Ammonium persulfate, 1 mL

a. 0.1 g of ammonium persulfate

b. 1 mL of ultrapure water

Stable for months in a capped tube in the refrigerator.

10X Electrophoresis Buffer, 1 liter

a. 30 g of Tris (final concentration is 0.25 M)

b. 144 g of glycine (final concentration is 1.92 M)

c. 10 g of SDS (final concentration is 1%)

d. Ultrapure water to make 1 liter

5X Sample Buffer, 10 mL

a. 0.6 mL of a M Tris-HCl, pH 6.8 (final concentration is 60 mM)

b. 5 mL of 50% (v/v)glycerol (final concentration is 25 %)

c. 2 mL of 10%SDS (final concentration is 2%)

d. 0.5 mL of β-mercaptoethanol (final concentration is 14.4 mM)

e. 1 mL of 1% (w/v) bromophenol blue (final concentration is 0.1%)

f. 0.9 mL of ultrapure water

Chemical	10%Seperating Gel (μL)	2.5%Stacking Gel (μL)
Solution A	1875	670
Solution B	1250	-
Solution C	-	1000
2.5%Substrate	100	-
10%ammonium persulfate	60	40
TEMED	20	10
Ultrapure water	1695	2300
Total	5000	4000

APPENDIX D

Staining a gel with coomassie blue

Reagents

1. Coomassie blue R-250
2. Methanol-CH_3OH
3. Glacial acetic acid-CH_3COOH

Stock solutions

1. Coomassie Gel stain, 1 liter
   1.0g Coomassie Blue R 250
   450 ml methanol
   450 ml H2O
   100 ml glacial acetic acid
2. Coomassie Gel Destain
   100 ml methanol
   100 ml glacial acetic acid
   800 ml H2O

Staining Procedure

1. Wearing gloves to prevent transfer of fingerprints to the gel, pick up the gel and transfer it to a small container containing a small amount of coomassie stain, or gently agretate the glass plate in stain solution until gel separate from plate
2. Agitate for 5-10 minutes for 0.75 mm, 10-20 minutes for 1.5 mm gel on slow rotary or rocking shaker. Cover the container with the lid or plastic wrab during staining and destain
3. Pour out stain and rinse the gel with a few changes of water. Use gloves to avoid saining hand
4. Add coomassie Destain. Strong bands are visible immediately on the light box, and the gel is largely destained within an hour. . Used destain can be washed down the sink with ample water.
5. To destain completely, change deatain solution and agitate overnight. 1-2 cm of yarn or a piece of Styrofoam can be added to absob coomassie stain which diffuses from the gel

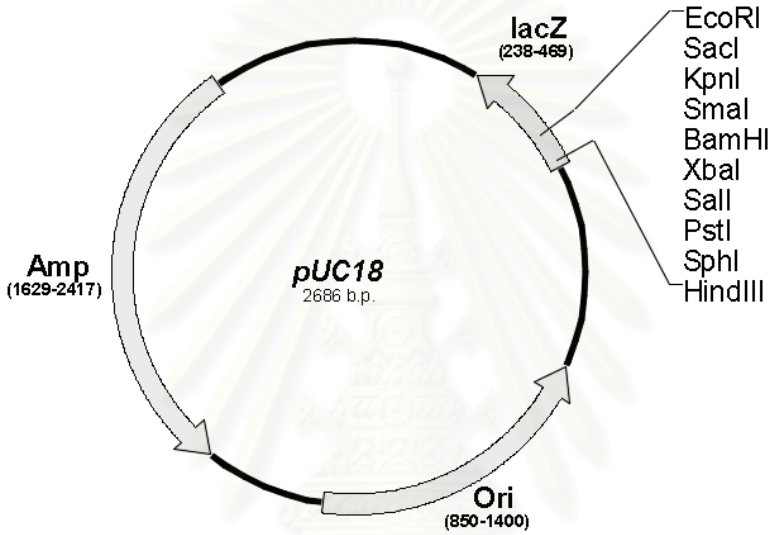
APPENDIX E

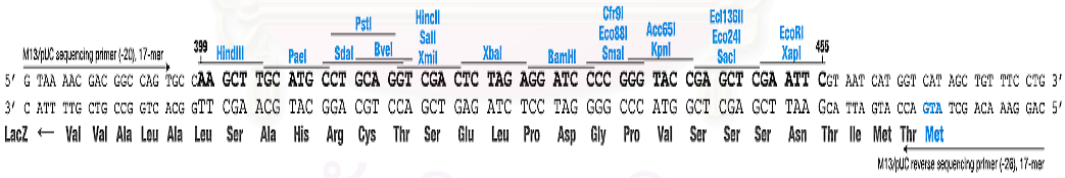
Abbrevation for amino acid residues

Amino acid	3 Letter-Abbreviation	1 Letter-Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cystein	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Iso	I
Lysine	Lys	K
Luecine	Lue	L
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Try	W
Tyrosine	Tyr	Y
Valine	Val	V

APPENDIX F

Restriction map of pU18

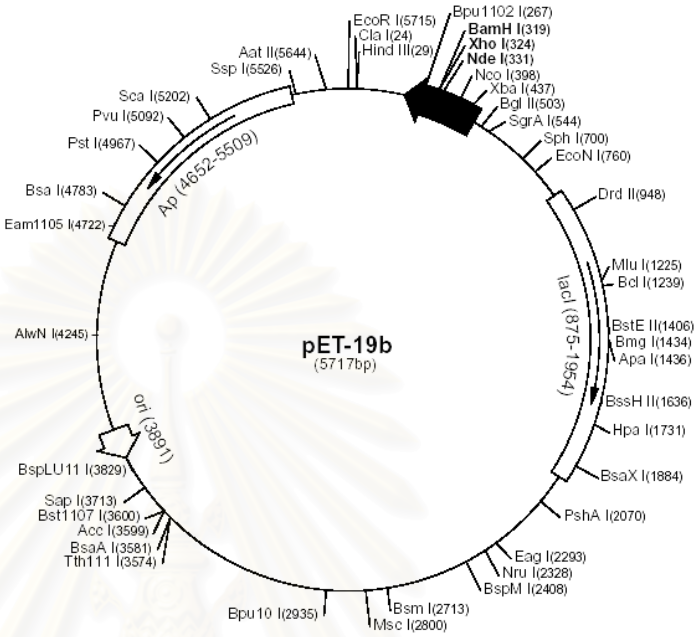


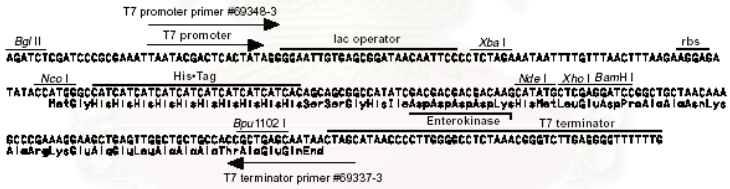


APPENDIX G

Restriction map of pET19b

pET-19b sequence landmarks	
T7 promoter	472-488
T7 transcription start	471
His•Tag coding sequence	366-395
Multiple cloning sites	
(*Nde* I -*Bam*H I)	319-335
T7 terminator	213-259
lacI coding sequence	875-1954
pBR322 origin	3891
bla coding sequence	4652-5509





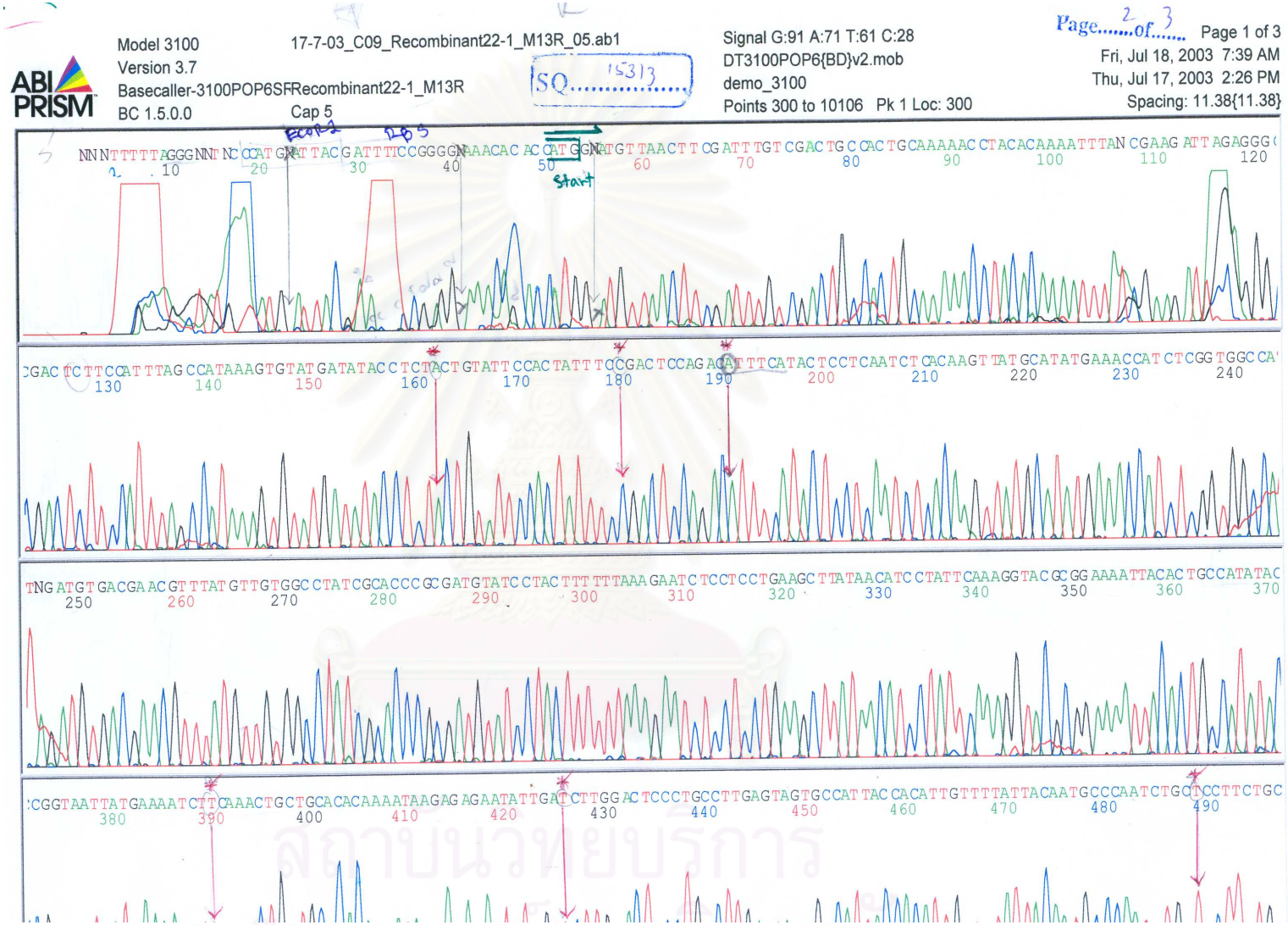
pET-19b cloning/expression region

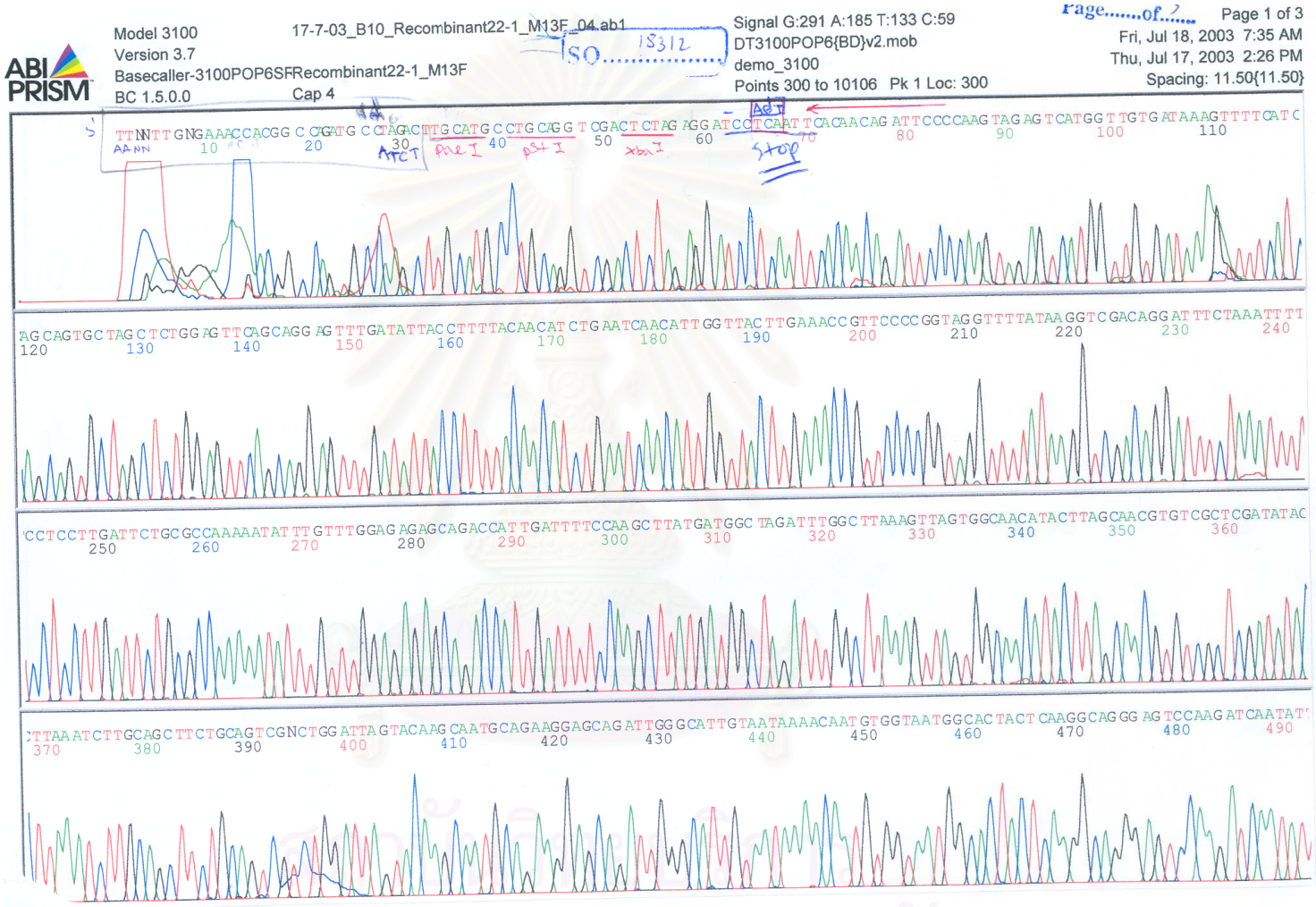
APPENDIX H

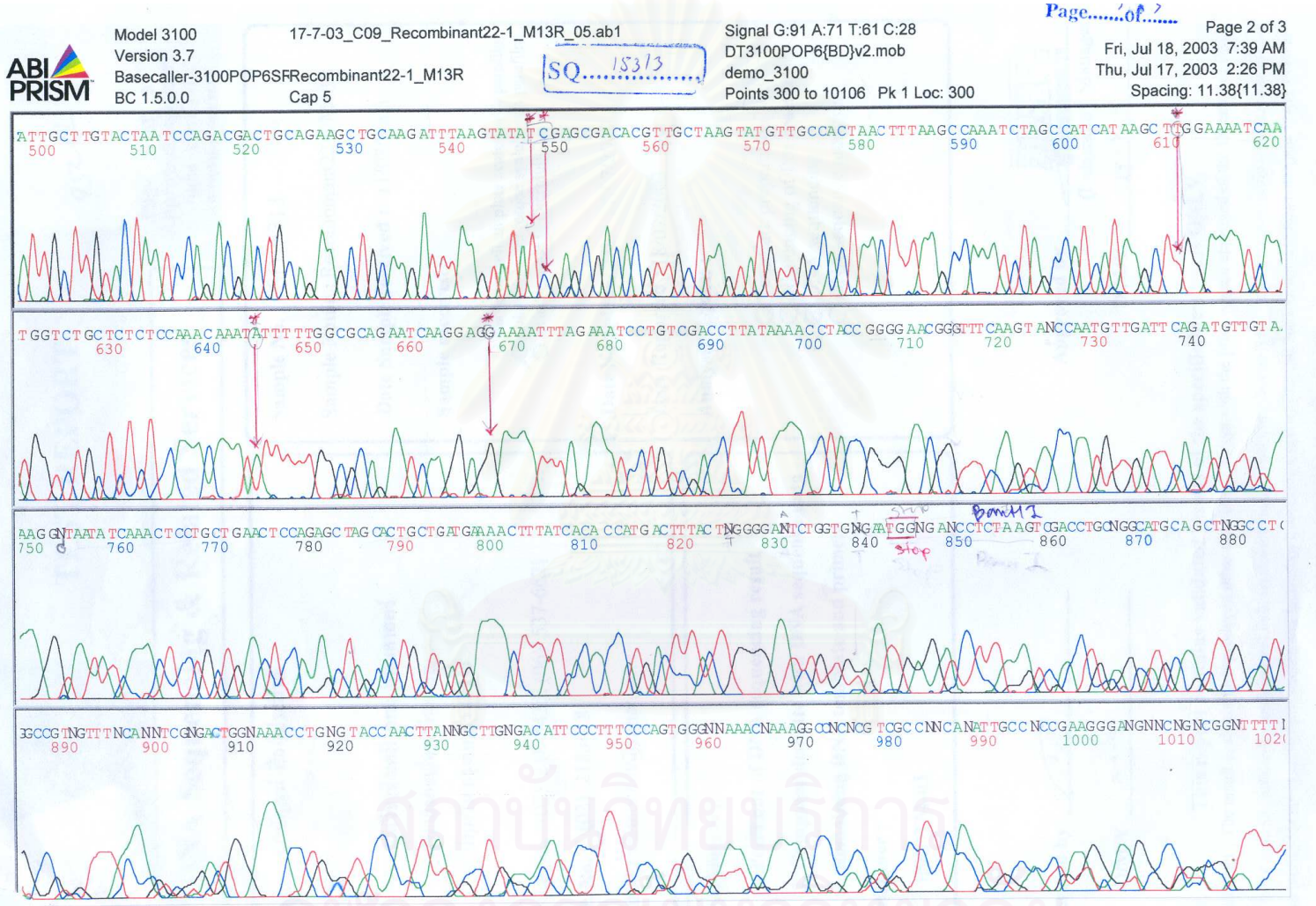
The DNA sequencing profile of the MAP30 gene from recombinant clone

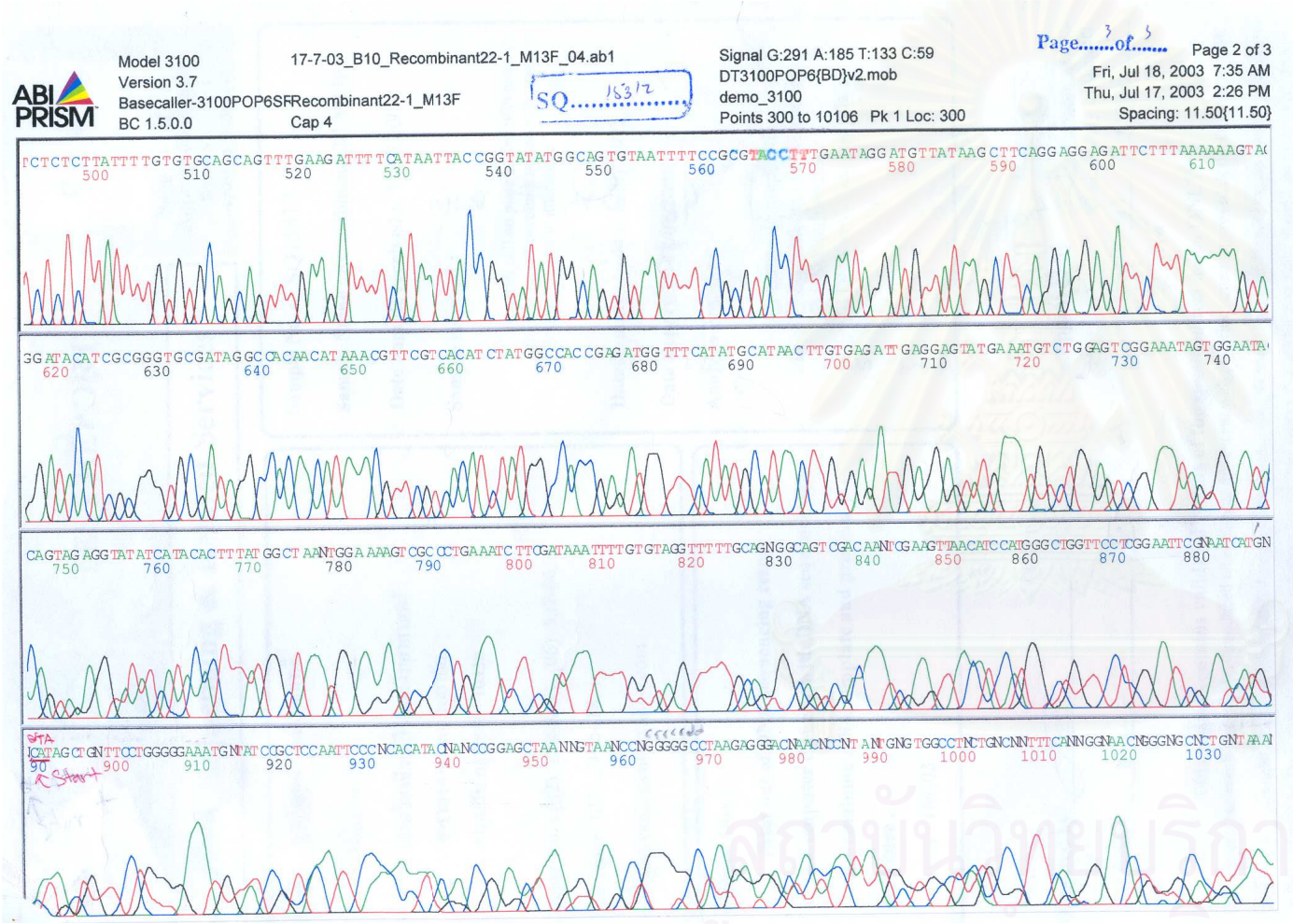
(a) = The DNA sequencing profile of the MAP30 gene from recombinant clone using primer Fn

(b) = The DNA sequencing profile of the MAP30 gene from recombinant clone using primer Rb









BIOGRAPHY

Mr. Kun Silprasit was born on December 30, 1978. He graduated with the degree of Bachelor of Science from department of Biochemistry at Khon Kaen University in 2000. He has studied for the degree of Master of Science at the Department of Biochemistry, Chulalongkorn University since 2000.