การโคลนและการแสดงออกของยีนแคทาเลสจากข้าว *Oryza sativa* L. ใน *Escherichia coli* Rosetta-gami

นางสาวสุนทรียา บุญคมรัตน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2553

CLONING AND EXPRESSION OF CATALASE GENES FROM RICE *Oryza sativa* L. IN *Escherichia coli* Rosetta-gami

Miss Suntareeya Boonkomrat

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
Faculty of Science
Chulalongkorn University
Academic Year 2010

Thesis Title	CLONING AND EXPRESSION OF CATALASE GENES FROM RICE *Oryza sativa* L. IN *Escherichia coli* Rosetta-gami
By	Miss Suntareeya Boonkomrat
Field of Study	Biotechnology
Thesis Advisor	Nuchanat Wutipraditkul, Ph.D.
Thesis Co-advisor	Associate Professor Teerapong Buaboocha, 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 Supot Hannongbua, Dr.rer.nat.)

THESIS COMMITTEE

…… Chairman
(Associate Professor Piamsook Pongsawasdi**,** Ph.D.)

…… Thesis Advisor
(Nuchanat Wutipraditkul, Ph.D.)

…… ……... Thesis Co-advisor
(Associate Professor Teerapong Buaboocha, Ph.D.)

…… Examiner
(Assistant Professor Kanoktip Packdibamrung, Ph.D.)

…… External Examiner
(Kittisak Yokthongwattana, Ph.D.)

สุนทรียา บุญคมรัตน์: การโคลนและการแสดงออกของยีนแคทาเลสจากข้าว *Oryza sativa* L. ใน *Escherichia coli* Rosetta-gami (CLONING AND EXPRESSION OF CATALASE GENES FROM RICE *Oryza sativa* L. IN *Escherichia coli* Rosetta-gami) อ . ที่ปรึกษาวิทยานิพนธ์หลัก: ดร.นุชนาถ วุฒิประดิษฐกุล , อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร.ธีรพงษ์ บัวบูชา, 125 หน้า.

แคทาเลสเป็น หนึ่งในเอนไซม์ที่ สำคัญในระบบการกำจัดออกซิเจนที่ไวต่อปฏิกิริยาของสิ่งมีชีวิตที่หายใจแบบใช้ออกซิเจน ในพืชพบแคทาเลสในส่วนเพอรอกซิโซมทำหน้าที่กำจัดไฮโดรเจนเปอร์ออกไซด์ให้อยู่ในรูปของออกซิเจนและน้ำ ในข้าว *Oryza sativa* L. พบแคทาเลสอย่างน้อย 3 ไอโซฟอร์ม ในงานวิจัยนี้ ได้ศึกษาลักษณะของ ยีนทั้งสาม คือ *OsCatA, OsCatB* และ *OsCatC* จากข้าว *Oryza sativa* L. โดยการสร้างพลาสมิดรีคอมบิแนนต์ของยีน ดังกล่าวในเวกเตอร์ pET-21a (+) และส่งถ่ายเข้าสู่เซลล์ *E. coli* สายพันธุ์ Rosetta-gami หลังจากเหนี่ยวนำให้เกิดการสร้างโปรตีนด้วยการเติม IPTG พบโปรตีนรีคอมบิแนนท์ของ OsCAT ทั้งสามอยู่ในส่วนของอินคลูชันบอดี ที่สามารถละลายด้วย ฟอสเฟตบัพเฟอร์ pH 12.0 โปรตีนที่ได้มีขนาด 58.8, 58.7 และ 58.9 kDa ตามลำดับ หลังผ่านการตรวจสอบด้วย SDS-PAGE และ Western blot และเมื่อนำโปรตีนมาผ่านการทำให้บริสุทธิ์บางส่วนด้วย คอลัมน์นิกเกิล พบแอกทิวิตีของ OsCATA, OsCATB และ OsCATC เท่ากับ 54.9, 130.8 and 5.4 μmol/min/mg ตามลำดับ โดยมีความบริสุทธิ์เพิ่ม 4, 2.2 และ 2.2 เท่าตามลำดับ ค่า K_m เท่ากับ 80.0, 66.67 และ 40 mM ตามลำดับ และมีค่า k_{cat} ของ OsCATA, OsCATB และ OsCATC เท่ากับ 6.60×10^{-3}, 20.0×10^{-3} และ 0.60×10^{-3} min^{-1} ตามลำดับ ส่วนpH และอุณหภูมิ ที่เหมาะสมในการเร่งปฏิกิริยา ของ OsCATA คือ 8.0 และ 30 องศาเซลเซียส ตามลำดับ, OsCATB คือ 7.5 และ 25 องศาเซลเซียส ตามลำดับ และ OsCATC คือ 7.0 และ 30 องศาเซลเซียส ตามลำดับ และผลของเกลือโซเดียมคลอไรด์ต่อแอก ทิวิตีของโปรตีนรีคอมบิแนนท์ OsCATs พบว่าโปรตีน OsCAT ทุกชนิดถูกยับยั้งเมื่อเพิ่มความเข้มข้นของเกลือโซเดียมคลอไรด์ ลักษณะเดียวกับแอกทิวิตีของเอนไซม์ที่ได้จากต้นข้าว

ภาควิชา…....เทคโนโลยีชีวภาพ.... ลายมือชื่อนิสิต …….....

ปีการศึกษา…..2553…….. ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก……

ลายมือชื่อ อ .ที่ปรึกษาวิทยานิพนธ์ร่วม…….

5072521923 : MAJOR BIOTECHNOLOGY
KEYWORDS: Catalase / rice *Oryza sativa* L. / *Escherichia coli* (Rosetta gami)

SUNTAREEYA BOONKOMRAT: CLONING AND EXPRESSION OF CATALASE GENES FROM RICE *Oryza sativa* L. IN *Escherichia coli* Rosetta-gami. THESIS ADVISOR: NUCHANAT WUTIPRADITKUL, Ph.D., THESIS CO-ADVISOR: ASSOC. PROF. TEERAPONG BUABOOCHA, Ph.D., 125 pp.

Catalase is an important enzyme in cellular active-oxygen scavenging system of aerobic organisms. In plants, catalase is located in peroxisomes where it is responsible for removing H_2O_2 by converting it to oxygen and water. Rice *Oryza sativa* L. contains at least three isoforms of catalase. In this study, *OsCatA, OsCatB* and *OsCatC* from the rice *Oryza sativa* L. genome were identified and constructed into the expression vector pET21a(+). The resulting recombinant plasmids were introduced into *Escherichia coli* strain Rosetta-gami. After protein production was induced by IPTG, the recombinant proteins OsCATA, OsCATB and OsCATC of approximately 58.8, 58.7 and 58.9 kDa were detected in the inclusion bodies by SDS-PAGE and western blot analysis. After, the inclusion bodies were successfully solubilized with phosphate buffer pH 12.0, the active enzyme was then partially purified using Ni-Sepharose column and characterized. The specific activities of OsCATA, OsCATB and OsCATC determined were 54.9, 130.8 and 5.4 μmol/min/mg, respectively, with 4, 2.2 and 2.2 -purification fold, respectively. The calculated K_m were 80.0, 66.67 and 40.0 mM, respectively and k_{cat} of OsCATA, OsCATB and OsCATC were 6.60 x 10^{-3}, 20.0 x 10^{-3} and 0.60 x 10^{-3} min^{-1}, respectively. The optimum temperatures and pH values for activity of the enzymes were 30 ^oC and 8.0 for OsCATA, 25 ^oC and 7.5 for OsCATB and 30 ^oC and 7.0 for OsCATC. In addition, OsCAT activities were shown to be deactivated with high concentrations of NaCl similar to the determined catalase activity which was extracted from rice.

Field of Study: …..Biotechnology…. Student's Signature…….
Academic Year: …2010…….. Advisor's Signature…….....
Co-Advisor's Signature.……..…….....

ACKNOWLEDGMENTS

I would sincerely like to acknowledge the efforts of many people who contribute to this work. This thesis could not be achieved without their following assistance, my thesis advisor Dr. Nuchanat Wutipraditkul and my co-advisor Associate Professor Dr. Teerapong Buaboocha for his generous advice, skillful assistance, technical helps, guidance, encouragement, support, fruitful and stimulating discussion through the period of my study.

Sincere thanks and appreciation are due to Associate Professor Piamsook Pongsawasdi**,** Assistant Professor Kanoktip Packdibamrung**,** and Dr. Kittisak Yokthongwattana for serving as the member of the master thesis committee, for their useful comments and suggestions.

I would like to thank the graduate school, Chulalongkorn University for financially supporting the research through the thesis fund. Special thanks are also extended to Srivilai Phean-o-pas, Aumnart Chinpongpanich, Thunchanok Dumrisuk, all members of 707, 708, 709 and other friends of the Biochemistry and Biotechnology programs for lending a helping hand whenever needed, sharing the great time in laboratory, for their friendships.

Finally, I wish to thank my deepest gratitude to my parents and my family for their love, care, understanding and encouragement extended throughout my study.

CONTENTS

Page

THAI ABSTRACT…… iv

ENGLISH ABSTRACT…… v

ACKNOWLEDGMENT……..... vi

CONTENTS……..... vii

LIST OF TABLES……. xii

LIST OF FIGURES…… xiii

LIST OF ABBREVIATIONS…… xvi

CHAPTER I INTRODUCTIONS……. 1

Salt stress……..... 4

Oxidative stress……..... 4

Sites of activated oxygen production……. 6

Antioxidant defense systems……..... 8

Role of antioxidant system……..... 9

Effect of salt stress on enzymatic…… 10

Catalase……... 11

CHAPTER II MATERIALS AND METHODS……..... 16

2.1 Materials……. 16

2.1.1 Rice seeds……... 16

2.1.2 cDNA clones…….. 16

2.1.3 Instruments……. 16

2.1.4 Inventory supplies ……..... 17

2.1.5 Chemicals and reagents…….. 18

Page

2.1.6 Enzymes……. 21

2.1.7 Microorganisms…….. 21

2.1.8 Kits and plasmids…….. 22

2.1.9 Antibiotics…….. 22

2.1.10 Oligonucleotide primers……. 22

2.1.11 Softwares……. 22

2.2 Bacterial growth medium……... 23

2.3 Methods…….. 23

2.3.1 Quantitative method for determination of DNA concentration…. 23

2.3.2 Sequence analysis…… 23

2.3.3 Cloning of the *Oscat* genes into cloning vector (pTZ57R/T)..... 24

2.3.3.1 Primer design……... 24

2.3.3.2 PCR amplification…… 25

2.3.3.3 Agarose gel electrophoresis…….. 25

2.3.3.4 Elution of DNA fragments from agarose gel…… 26

2.3.3.5 Ligation of *OsCat* genes products to pTZ57R/T…….. 27

2.3.3.6 Transformation of ligated products to *E. coli* host cells by $CaCl_2$ Method……. 27

a) Preparation of *E. coli* competent cells……. 27

b) Transformation by $CaCl_2$ method…… 28

2.3.3.7 Analysis of recombinant plasmids…… 29

a) Plasmid DNA isolation by alkaline lysis method…… 29

b) Restriction enzyme analysis……. 30

Page

2.3.4 Cloning of *OsCat* genes into expression vector pET21a(+)…… 30

2.3.4.1 Vector DNA preparation…….. 30

2.3.4.2 Preparation of *OsCat* gene fragments……..... 31

2.3.4.3 Ligation of *OsCat* gene fragments to pET21a(+)…… 31

2.3.4.4 Transformation of *E. coli* XL-1Blue cell by $CaCl_2$ method..... 31

2.3.4.5 Analysis of recombinant plasmids…… 32

2.3.5 Nucleotide and amino acid sequences analysis…… 32

2.4 Expression of the *OsCat* genes……. 33

2.4.1 Preparation of cell expression…… 33

2.4.2 Optimization for *OsCat* genes expression……..... 33

2.4.3 Determination of protein concentration…… 34

2.4.4 SDS-polyacrylamide gel electrophoresis…….. 34

2.4.5 Protein staining……. 35

2.5 Purification of the OsCAT proteins……. 35

2.5.1 Crude extract preparation……. 36

2.5.2 Preparation of inclusion bodies…… 36

2.5.3 Solubilization and refolding of inclusion bodies……. 37

2.5.4 OsCAT proteins purification by Ni-Sepharose column…… 37

2.6 Western blot detection of the His-tagged protein……... 38

2.7 Determination of catalase activity…… 40

2.7.1 Effect of NaCl on OsCAT activity…… 40

2.7.2 Optimal pH ……...……40

2.7.3 Optimal temperature.……...……...41

Page

2.8 Determination of catalase from rice *Oryza sativa* L. cultivar Khao Dok Ma Li 105 (KDML105)…… 41

2.8.1 Preparation of rice seedlings……..... 41

2.8.2 Preparation of crude enzyme extract…… 42

2.8.3 Effect of NaCl on CAT activity…… 42

CHAPTER III RESULTS…… 43

3.1 Catalase sequence analysis……43

3.2 Molecular cloning of the *OsCat* genes……..43

3.3 Expression of the recombinant *OsCat* genes…….51

3.4 Purification and characterization of the recombinant OsCAT protein…..60

3.4.1 Preparation of OsCAT proteins from inclusion bodies……..60

3.4.2 Solubilization and refolding of OsCAT from inclusion bodies……. 67

3.4.3 Purification of the refolded OsCAT proteins by Ni sepharose column..... 67

3.4.4 Western blot detection of the His-tagged protein…… 68

3.5 Biochemical characterization of purified catalases..... 76

3.6 Effect of NaCl on OsCAT activity…… 86

3.7 CAT activity of rice *Oryza sativa* L. cultivar Khao Dok Ma Li 105 (KDML105)…….. 86

3.7.1 Measurement of CAT activity in rice which grown under salt stress condition…….. 86

3.7.2 Effect of NaCl on CAT activity in rice……. 89

Page

CHAPTER IV DISCUSSION……. 92

4.1 Cloning and expression of *OsCat* genes…….. 92

4.2 Solubilization and refolding of OsCAT from inclusion bodies…….. 93

4.3 Purification and characterization of recombinant OsCAT proteins…… 94

4.4 Biochemical characterization of catalase purified…….. 94

4.5 Effect of NaCl on activity of recombinant OsCAT proteins…….. 96

4.6 CAT activity of rice *Oryza sativa* L. cultivar Khao Dok Ma Li 105 (KDML105)…… 97

4.6.1 Measurement of CAT activity in rice which grown under salt stress condition…… 97

4.6.2 Effects of NaCl on CAT activity of rice seedlings leaves…….. 97

CHAPTER V CONCLUSION……... 99

REFERENCES…… 101

APPENDICES……. 111

APPENDIX A…….. 112

APPENDIX B……..... 115

APPENDIX C……..... 117

BIOGRAPHY…….. 125

LIST OF TABLES

Table		Page
Table 2.1	The sequences and the length of oligonucleotide primers used for PCR amplification……	24
Table 3.1	Purification table of the recombinant OsCATA protein from *E.coli* Rosetta-gami..…….	73
Table 3.2	Purification table of the recombinant OsCATB protein from *E.coli* Rosetta-gami..…….	74
Table 3.3	Purification table of the recombinant OsCATC protein from *E.coli* Rosetta-gami..…….	75
Table 3.4	Kinetic parameters of recombinant OsCAT proteins from *E.coli* Rosetta-gami..…..……..	87

LIST OF FIGURES

Figure		Page
1.1	A generic pathway of signal perception and signal transduction in plants…….	3
1.2	Generation and scavenging of reactive oxygen species……	5
1.3	Model proposed for the function or the ascorbate-glutathione cycle in leaf peroxisomes……	13
2.1	Exploded view of the Trans-Blot® SD (Bio-Rad)……	39
3.1	Alignment of the deduced amino acid sequence of rice catalase (*OsCATA*, *OsCATB* and *OsCATC*) with Arabidopsis catalase (*AtCAT1, AtCAT2* and *AtCAT3*)……...	45
3.2	Agarose gel electrophoresis of the amplified fragment of the *OsCatA* gene at various annealing temperatures……..	47
3.3	Agarose gel electrophoresis of the amplified fragment of the *OsCatB* gene at various annealing temperatures…….	48
3.4	Agarose gel electrophoresis of the amplified fragment of the *OsCatC* gene at various annealing temperatures…….....	49
3.5	Analysis of the *OsCat* gene inserts in pTZ57R/T by digestion with *Nde*I and *Eco*RI restriction enzymes and separation in 1% agarose gel electrophoresis…….	50
3.6	Analysis of the *OsCat* genes inserts in pET21a(+) by digestion with *Nde*I and *Eco*RI restriction enzymes and separation in 1% agarose gel electrophoresis…….	52
3.7	Nucleotide and deduced amino acid sequences of *OsCatA* gene……	54

Figure **Page**

3.8 Nucleotide and deduced amino acid sequences of *OsCatB* gene…… 56

3.9 Nucleotide and deduced amino acid sequences of *OsCatC* gene…… 58

3.10 Direction of the *OsCat* gene inserted into pET21a(+)……. 59

3.11 The expression pattern of OsCATA in soluble protein fraction of *E. coli* Rosetta-gami under various final concentrations of IPTG…….. 61

3.12 The expression pattern of OsCATA in insoluble protein fraction of *E. coli* Rosetta-gami under various final concentrations of IPTG……... 62

3.13 The expression pattern of OsCATB in soluble protein fraction of *E. coli* Rosetta-gami under various final concentrations of IPTG…….. 63

3.14 The expression pattern of OsCATB in insoluble protein fraction of *E. coli* Rosetta-gami under various final concentrations of IPTG……... 64

3.15 The expression pattern of OsCATC in soluble protein fraction of *E. coli* Rosetta-gami under various final concentrations of IPTG…..… 65

3.16 The expression pattern of OsCATC in insoluble protein fraction of *E. coli* Rosetta-gami under various final concentrations of IPTG…..…. 66

3.17 Purification of his-tagged recombinant *OsCatA* expressed in *E. coli* Rosetta-gami using Nickel-Sepharose……..…… 69

3.18 Purification of his-tagged recombinant *OsCatB* expressed in *E. coli* Rosetta-gami using Nickel-Sepharose……..…… 70

3.19 Purification of his-tagged recombinant *OsCatC* expressed in *E. coli* Rosetta-gami using Nickel-Sepharose……..…… 71

Figure **Page**

3.20 Western blot analysis of recombinant proteins from *E. coli* Rosetta-gami harboring *OsCat* genes. Proteins were run on 12% SDS-polyacrylamide gel and visualized by Coomassie blue staining…… 72

3.21 The effect of pH on OsCATA activity…… 77

3.22 The effect of temperature on OsCATA activity…….....……..... 78

3.23 The effect of pH on OsCATB activity…….… 79

3.24 The effect of temperature on OsCATB activity…….…….…..... 80

3.25 The effect of pH on OsCATC activity…….…..... 81

3.26 The effect of temperature on OsCATC activity……..…..... 82

3.27 The apparent Lineweaver-Burk plot of OsCATA with H_2O_2 of different concentration (0 to 100 mM) as substrate……... 83

3.28 The apparent Lineweaver-Burk plot of OsCATB with H_2O_2 of different concentration (0 to 20 mM) as substrate…….…… 84

3.29 The apparent Lineweaver-Burk plot of OsCATC with H_2O_2 of different concentration (0 to 20 mM) as substrate…….…… 85

3.30 Effects of NaCl on OsCAT activity……...….. 88

3.31 Effect of salt stress on CAT activities of KDML105 rice seedling leaves at 0, 1, 2 and 3 days after salt stress treatments……... 90

3.32 Effects of NaCl on CAT activity of KDML105 rice seedlings leaves… 91

LIST OF ABBREVIATIONS

A	absorbance, 2′-deoxyadenosine (in a DNA sequence)
bp	base pairs
C	2′-deoxycytidine (in a DNA sequence)
^{o}C	degree Celsius
C-terminus	carboxyl terminus
Ca^{2+}	Calcium ion
cDNA	complementary deoxyribonucleic acid
Da	Dalton
DNA	deoxyribonucleic acid
dNTP	2′-deoxynucleoside 5′-triphosphate
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
EtBr	ethidium bromide
G	2′-deoxyguanosine (in a DNA sequence)
g	gram
HCl	hydrochloric acid
IPTG	isopropyl-thiogalactoside
kb	kilobase pairs in duplex nucleic acid, kilobases in single-standed nucleic acid
KCl	potassium chloride
kDa	kiloDalton
l	liter
LB	Luria-Bertani
Mg^{2+}	magnesium ion
µg	microgram
µl	microliter
µM	micromolar
M	mole per liter (molar)
mg	milligram
min	minute

ml	milliliter
mM	millimolar
MW	molecular weight
N-terminus	amino terminus
OD	optical density
PCR	polymerase chain reaction
μmol	micromole
SDS	sodium dodecyl sulfate
T	2′-deoxythymidine (in a DNA sequence)
Tris	tris (hydroxyl methyl) aminomethane
UV	ultraviolet
V	voltage
v/v	volume by volume
w/w	weight by weight

CHAPTER I

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important stable food crops in the world. In Asia, more than two billion people are getting 60-70% of their energy requirement from rice and its derived products. In India, rice occupies an area of 44 million hectare with an average production of 90 million tones with productivity of 2.0 tones/ha. Demand for rice is growing every year and it is estimated that in 2010 and 2025 AD the requirement would be 100 and 140 million tones respectively. To sustain present food self-sufficiency and to meet future food requirements which is the principal source of food for more than one third of the world's population.

Salinity is the most serious threats to agriculture and for more important globally (Sahi, 2006) water consisted minerals and salt materials are major harmful factors in arid and semi-arid region of worldwide. Salt stress causes the reduction of rice yield, and sometimes-severe salt stress may even threaten survival. Although rice is considered as a sensitive crop to salinity, it is one of the most widely grown crops in coastal areas frequently inundated with saline seawater during high tidal period (Akbar *et al*., 1972; Mori and Kinoshita, 1987). In saline soil, the low water potential directly affects efficiency of water use, because plants need to develop more negative water potential to maintain a downhill gradient of water potential between the soil and the plant. Moreover, salts in saline soil directly damage the plant cells, tissues and organelles. Salt sensitive species are susceptible to injury by salt stress, resulting in loss of leaf expansion and chlorophyll synthesis prior to plant death (Cha-um *et al.*, 2006). Plants have developed multiple strategies to achieve salt tolerance. During the

process of salt adaptation, the expression of many salt-stress related genes was induced, and the synthesis of a variety of proteins was increased (Xujun *et al.*, 2005). Salt stress tolerance requires a complex interplay of signaling cascades (Xiong *et al.*, 2002), and is determined by multiple factors consisting of effectors and regulators (Hasegawa *et al.*, 2000).

Figure 1.1 shows the generic signal transduction pathway that starts with signal perception, followed by the generation of second messengers. Signal transduction requires the proper spatial and temporal coordination of all signaling molecules. Thus, there are certain molecules that participate on modification, delivery, or assembly of, signaling components, but do not directly relay the signal (Xiong *et al.*, 2002). Because different signals often elicit distinct and specific cellular responses it is important to determine how plants sense various signals and produce an appropriate response. Salt stress signal transduction is made up of multiple pathways that consist of ionic and osmotic homeostasis signaling pathways, detoxification response pathways, pathways for growth regulation and calcium signaling pathway (Zhu, 2001). Components of signaling pathways operating in salt stress-affected cells and interactions between these pathways are main research subjects in many laboratories.

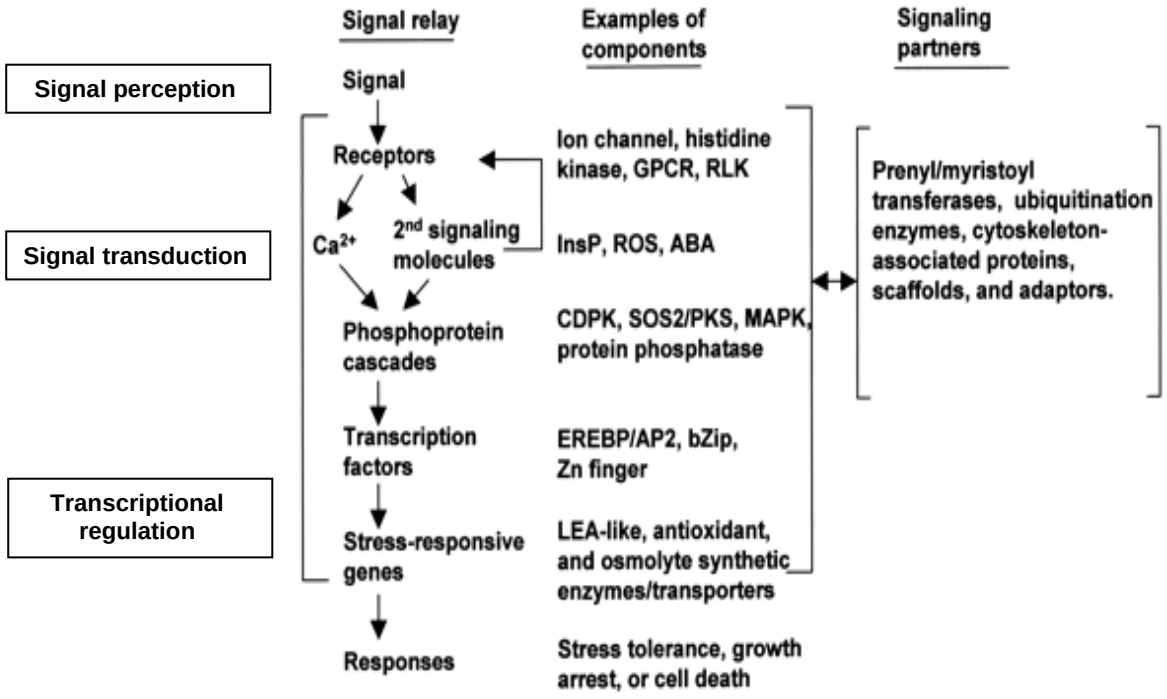


Figure 1.1 A generic pathway of signal perception and signal transduction in plants. Examples of signaling components in each of the steps are shown. Secondary signaling molecules can cause receptor-mediated Ca^{2+} release (indicated with a feedback arrow). Examples of signaling partners that modulate the main pathway are also shown. These partners can be regulated by the main pathway. Signaling can also bypass Ca^{2+} or secondary signaling molecules in early signaling steps. GPCR, G-protein coupled receptor; InsP, inositol polyphosphates; RLK, receptor-like kinase; ROS, reactive oxygen species; ABA, abscisic acid; CDPK, Ca^{2+}-dependent protein kinases; SOS2/PKS, Salt Overly Sensitive 2/Protein kinases; MAPK, mitogenactivated protein kinase; EREBP/AP2, ethylene-responsive element binding proteins/APETALA2; bZIP, basic leucine zipper; Zn finger, Zinc finger; and LEA-like, late embryogenesis–abundant. (Xiong *et al.*, 2002).

Salt stress

Salinity is the presence of the excessive concentrations of soluble salts in the soil that suppress plant growth. The major cations contributing to salinity are Na^{+}, Ca^{+2}, Mg^{+2}, K^{+} and anions are Cl^{-}, SO_4^{-2}, HCO_3^{-}, CO_3^{-2} and NO_3^{-2}. There exist also trace elements including B, Sr, Li, Rb, F, Mo, Ba, and Al. High concentrations of the Na^{+} cation gives damage to the plant by two means; osmotic stress and toxic effect (Tanji, 1990). The low osmotic potential of the soil salt solution makes it necessary for plants exposed to these media to maintain a lower intracellular osmotic potential, otherwise they would experience osmotic desiccation because water would move osmotically from the cells to the soil. Salts may affect plants in two ways: (1) osmotically, i.e., low osmotic potential may limit the water available to the plant; and (2) by the toxicity of different ions in the media. Several researchers have found that the total salt in the media seems to be a more important factor than ionic composition, or kinds of salt (Magistad, 1943; Hayward and Bernstein, 1958; Nieman, 1962).

Oxidative stress

Responses by plants to extreme temperature, drought and salt stress correlate with responses typically observed from increased oxidative stress. Oxidative stress may be also be toxic due to the activities of derivatives such as superoxide (O_2^{-}), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^{-}). Toxic superoxide radicals (O_2^{-}) are generated in many biological oxidations, such as the univalent reduction of dioxygen during photosynthetic electron transport in chloroplasts (Figure 1.2), especially under conditions of limiting carbon dioxide and high light intensity

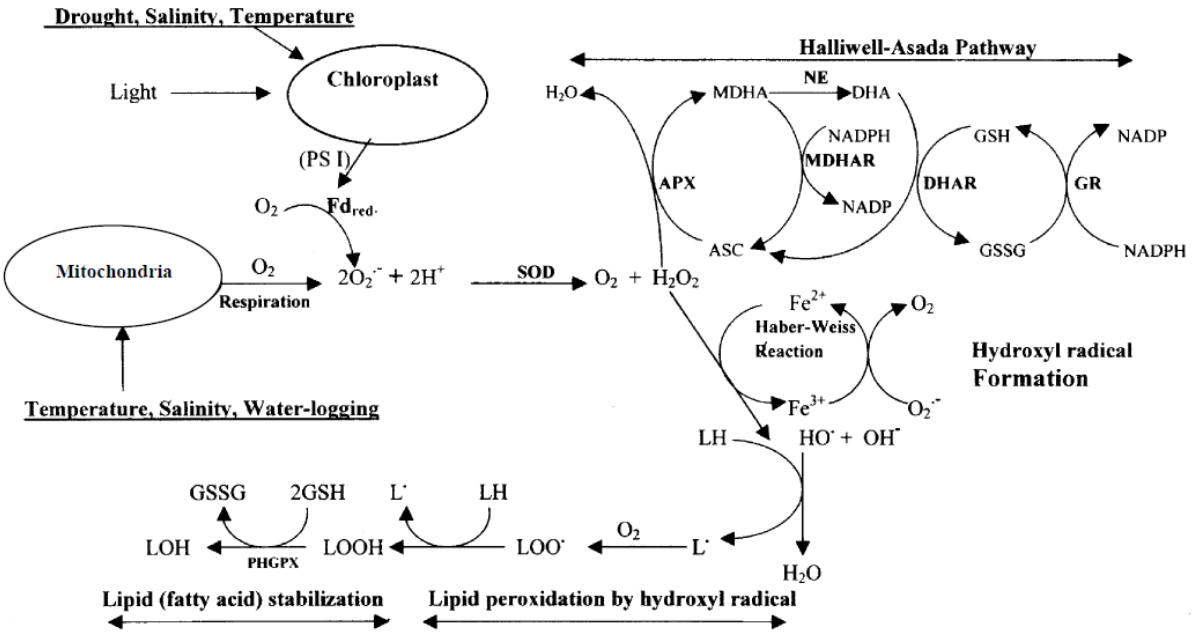


Figure 1.2 Generation and scavenging of superoxide radical, hydrogen peroxide, hydroxyl radical-induced lipid peroxidation and glutathione peroxidase-mediated lipid (fatty acid) stabilization. APX, Ascorbate peroxidase; ASC, Ascorbate; DHA, Dehydroascorbate; DHAR, Dehydroascorbate reductase; Fd, Ferredoxin; GR, Glutathione reductase; GSH, Red glutathione; GSSG, Oxi-glutathione; $HO^{\cdot}$, Hydroxyl radical; LH, Lipid; $L^{\cdot}$, $LOO^{\cdot}$; LOOH, Unstable lipid radicals and hydroperoxides; LOH, Stable lipid (fatty acid); MDHA, Monodehydro-ascorbate; MDHAR, Mono dehydro-ascorbate reductase; NE, Non-enzymatic reaction; PHGPX, Phospholipid-hydroperoxide glutathione peroxidase; SOD, Superoxide dismutase. (Sairam and Aruna, 2004)

(Asada and Takahashi, 1987). In chloroplasts, xanthine oxidase can generate superoxide anions from molecular oxygen. In this reaction, xanthine, hypoxanthine or acetaldehyde can act as electron donors (Elstner, 1987). Mitochondrial respiration also produces superoxide radicals. The superoxide anions can convert into hydrogen peroxide either spontaneously or by the action of superoxide dismutase. Superoxide radicals and H_2O_2 can readily interact in the presence of Fe (III) to produce hydroxyl radicals which are the most reactive oxygen species. These radicals can react with nucleic acids, proteins, lipids and almost any other organic cellular component causing considerable damage (Rabinowitch and Fridovich, 1983).

Sites of activated oxygen production

The reduction of oxygen to form superoxide, hydrogen peroxide, and hydroxyl radicals is the principle mechanism of oxygen activation in most biological systems. However, in most photosynthetic plants, the formation of singlet oxygen by the photosystems has importance. Activated oxygen is often formed as a component of metabolism to enable complex chemical reactions or by the disfunctioning of enzymes or electron transport systems as a result of perturbations in metabolism caused by chemical or environmental stress. Chloroplasts, mitochondria, endoplasmic reticulum, microbodies, plasma membranes, and cell walls are the major sites of activated oxygen production in a plant cell (McKersie, 1996).

As described by Elstner (1991), there are at least four sites within the chloroplast that can activate oxygen. Firstly, the reducing side of PSI is thought to contribute significantly to the monovalent reduction of oxygen under conditions where NADP is limiting. Secondly, under conditions that prevent the captured light

energy from being utilized in the electron transport systems, the excitation energy of photoactivated chlorophyll can excite oxygen from the triplet to singlet form. Thirdly, leaks of electrons from the oxidizing side of PSH to molecular oxygen, or release of partially reduced oxygen products contribute to activated oxygen production. Lastly, due to photorespiration glycolate is formed, whose subsequent metabolism in the peroxisomes leads to the generation of activated oxygen.

The main superoxide generators in the mitochondria are the ubiquinone radical and NADH dehydrogenases. Superoxide is formed by the autooxidation of the reduced components of the respiratory chain (Dat *et al.,* 2000). The various Fe-S proteins have also been implicated as possible sites of superoxide and hydrogen peroxide formation in mitochondria (Turrens *et al.,* 1982).

Various oxidative processes occuring on the smooth endoplasmic reticulum involves the transfer of oxygen into an organic substrate using NAD(P)H as the electron donor. Superoxide is produced by microsomal NAD(P)H dependent electron transport involving cytochrome P_{450} (Winston and Cederbaum, 1983). Cytochrome $_{P450}$ reacts with its organic substrate (RH) and forms a radical intermediate ($cytP_{450}$-$R^{\cdot}$) that can readily react with triplet oxygen (forming $cytP_{450}$-$ROO^{\cdot}$) since each has one unpaired electron. This oxygenated complex may be reduced by cytochrome b or occasionally the complex may decompose releasing superoxide (McKersie, 1996).

Peroxisomes and glyoxysomes contain enzymes involved in β-oxidation of fatty acids and the glyoxylic acid cycle including glycolate oxidase, catalase, and various peroxidases. Glycolate oxidase produces H_2O_2 in a reaction involving two electron transfer from glycolate to oxygen (Lindqvist *et al.*, 1991). Xanthine oxidase,

urate oxidase, and NAD(P)H oxidase generate superoxide as a consequence of oxidation of their substrates (McKersie, 1996).

A superoxide-generating NAD(P)H oxidase activity has been clearly identified in plasmalemma enriched fractions. Wounding, heat shock and xenobiotics transiently activate this superoxide generating enzyme, and consequently, it has been proposed that these superoxide generating reactions may serve as a signal in plant cells to elicit responses to biological, physical, or chemical stress (Doke *et al.,* 1991). In the cell wall, such a mechanism is also thought to be present. Some biosynthetic reactions (such as lignin biosynthesis) and oxidative enzymes (diamine oxidase, NADH oxidase) also lead to production of activated oxygen on the cell wall which may be signal for oxidative stress (McKersie, 1996).

Antioxidant defense systems

To detoxify the toxicity of active oxygen species, a highly efficient antioxidant defense system is present in plant cells. Antioxidants can be divided into two classes: 1) Non enzymatic system including glutathione, ascorbate (vitamin C), carotenoids, Q-tocopherol (vitamin E), and various phenylpropanoid derivatives (phenolic compounds) such as flavonoids, lignans, tannins, and lignins. 2) Enzymatic system, the enzymatic defense against the reactive oxygen species is essential for plants under biotic or abiotic stress. The antioxidative enzymes, superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), peroxidase (POD; EC 1.11.1.7), ascorbate peroxidase (APX; EC 1.1.1.11), glutathione reductase (GR; EC 1.6.4.2), and other ascorbate glutathione cycle enzymes (monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) and dehydroascorbate reductase (DHAR; EC 1.8.5.1)) catalyze the

synthesis, degradation, and recycling of antioxidant molecules and can directly catalyze the removal of free radicals from the cells.

Role of antioxidant system

Reactive oxygen species are thought to play an important role in NaCl stress. Plants tolerant to NaCl stress may evolve certain strategies to remove these ROS, thus reducing their toxic effects. Therefore, the expression patterns of the gene family encoding the H_2O_2 scavenging enzyme ascorbate peroxidase and glutathione reductase were analyzed in roots of etiolated rice (*Oryza sativa* L.) seedlings in response to NaCl stress (Hong *et al*., 2007; Hong *et al*., 2009). Tsai *et al*. (2004) examined the response of antioxidant systems to NaCl stress and the relative importance of Na^+ and Cl^- in NaCl induced antioxidant systems in roots of rice seedlings. NaCl treatment caused an increase in the activities of ascorbate peroxidase (APX) and glutathione reductase (GR) in roots of rice seedlings, but had no effect on the activities of superoxide dismutase (SOD) and catalase (CAT) (Tsai *et al*., 2004). Lin and Kao (2001) investigated the changes in cellwall peroxidase (POD) activity and H_2O_2 level in roots of NaCl stressed rice seedlings and their correlation with root growth. In the leaves of the rice plant, salt stress preferentially enhanced the content of H_2O_2 as well as the activities of the superoxide dismutase (SOD), ascorbate peroxidase (APX), and peroxidase specific to guaiacol, whereas it induced the decrease of catalase activity. On the other hand, salt stress had little effect on the activity levels of glutathione reductase (GR) (Lee *et al*., 2001). Dionisio-Sese and Tobita (1998) studied the possible involvement of activated oxygen species in the mechanism of damage by NaCl stress in leaves of four varieties of rice (*Oryza sativa*

L.) exhibiting different sensitivities to NaCl. Tsai *et al*. (2004) examined the response of antioxidant systems to NaCl stress and the relative importance of Na^+ and Cl^- in NaCl induced antioxidant systems in roots of rice seedlings. Shankhdhar *et al*., (2000) studied the embryogenic callus growth, plant regeneration, and proline and total protein contents under salt stress in six cultivars of rice (*Oryza sativa* L). Treatment with NaCl caused an increase in the activities of ascorbate peroxidase (APX) and glutathione reductase (GR) and the expression of OsAPX and OsGR in rice roots. Exogenously applied H_2O_2 also enhanced the activities of APX and GR and the expression of OsAPX and OsGR in rice roots (Tsai *et al*., 2005).

Effect of salt stress on enzymatic systems

The effect of salt stress on the antioxidant enzyme activities has been shown in many cases. It seems that salt stress strongly affects the oxidative defense mechanisms in plants, but the change in the activities of antioxidant enzymes depends on plant species, isozymes of antioxidant enzymes and their subcellular distributions. It has been shown in many studies that salt tolerant cultivar of a plant generally has enhanced antioxidant enzyme activities under salt stress when compared with the sensitive cultivars. This was observed in wheat (Meneguzzo *et al*., 1999), tomato (Gueta-Dahan *et al*., 1998; Rodriguez *et al*., 1999), soybean root nodules (Comba *et al*., 1997), tobacco (Benavides *et al*., 2000), Mulberry (Sudhakar *et al*., 2001), citrus (Gueta-Dahan *et al*., 1997), rice (Dionisio-Sese and Tobita, 1998), fox-tail millet (Sreenivasulu *et al*., 1999), potato (Mescht *et al*., 1998) and arabidopsis (Tsugane *et al*., 1999). It has been shown that salt stress increased the activity of antioxidant enzymes (SOD, GR, APX and CAT) of soybean root nodules in salt tolerant cultivar.

In contrast, salt sensitive cultivar responded to NaCl stress by decreasing the activities all antioxidant enzymes, except SOD (Comba *et al*., 1998). Responses of antioxidant enzymes to NaCl stress also differ with different subcellular locations of enzymes. In isolated chloroplasts and mitochondria of pea, under low NaCl stress the activities of all isozymes of SOD (chloroplastic and mitochondrial Cu/ZnSOD, FeSOD and MnSOD), APX, DHAR and GR elevated, however, chloroplastic DHAR, APX and GR as well as mitochondrial DHAR decreased to control levels under severe NaC1 stress. On the other hand, mitochondrial APX and MDHAR, and chloroplastic Cu/ZnSOD strongly enhanced under severe NaCl stress (Gomez *et al*., 1999). Impact of the salt stress on roots and shoots may also differ. This was observed in wheat Although the activity of antioxidant enzymes significantly increased in wheat shoots a general decrease in the activities of all antioxidant enzymes was observed in roots, which firstly suffer stress (Meneguzzo *et al.,* 1999).

Catalase

Catalase is a tetrameric, heme-containing enzyme with four iron atoms per molecule attached to protein and chelated to protoporphyrin IX. The catalytic center is at the iron atoms, where the Fe^{2+} performs the function of breaking a 2-electron oxidation into two energetically easier steps. These intermediate steps are easier than with Fe^{+2} alone. In plant, one subunit has molecular weight range of 54-59 kDa. Catalase is found in all aerobic eukaryotes and is important in the removal of hydrogen peroxide generated in peroxisomes (microbodies) by oxidases involved in β-oxidation of fatty acids, the glyoxylate cycle (photorespiration) and purine catabolism (McKersie, 1996).

Detoxification of H_2O_2 is mediated by catalase, which is mostly localized in peroxisomes. However, catalase possesses a very low affinity for H_2O_2 and its activity is either extremely low or no detectable in the cytosol, mitochondria and chloroplast (Halliwell, 1981). In plant cells, an alternative and more effective detoxification mechanism against H_2O_2 also exists, operating both in chloroplast and cytosol, called the ascorbate-glutathione or Halliwell- Asada cycle (Asada and Takahashi, 1987) (Figure 1.2). The pathway seems to be major H_2O_2 detoxification system both in cytosol and chloroplast, as well as in mitochondria. It is also important for the maintenance of ascorbate and glutathione pools in the reduced state.

In plant glyoxysomes, H_2O_2 is produced at high levels after germination as a result of β-oxidation of fatty acids (Figure 1.3). The glyoxysomes in plant cells are closely associated with the lipid bodies (spherosomes). The lipase activity, which is located either in the bounding outer glyoxysomal membrane or in the spherosomes, breaks down the stored triglycerides into fatty acids which then enter the glyoxysomes. Fatty acyl-CoA synthetase activates fatty acids into fatty acyl-CoA which is the initial substrate for β-oxidation. The H_2O_2 produced is scavenged by catalase while the acetyl-coA is used directly by the glyoxylate cycle in the same glyoxysomes (Tolbert, 1981).

H_2O_2 is also generated in leaves during photorespiration as a product of glycolate oxidase activity (Figure 1.3). The primary reaction in photorespiration is the oxygenation of ribulose 1,5-bisphosphate by Rubisco (ribulose 1,5-bisphosphate carboxylase/ oxygenase) producing phosphoglycolate (Somerville and Ogren, 1982). Phospho-glycolate is hydrolyzed to glycolate by phosphoglycolate phosphatase.

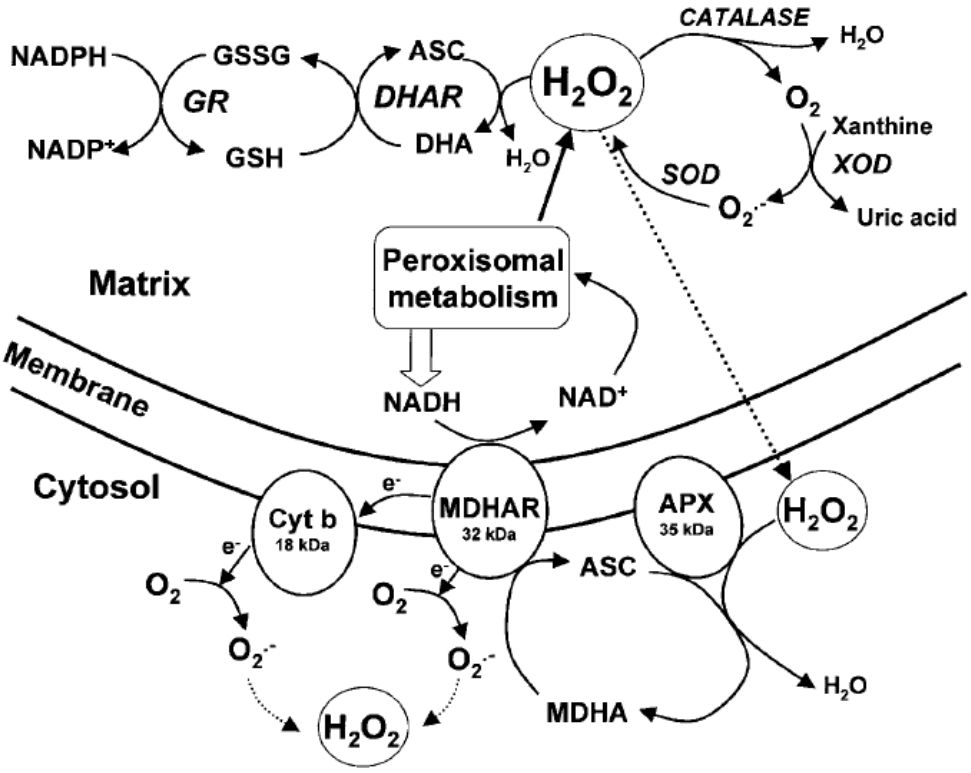


Figure 1.3 Model proposed for the function or the ascorbate-glutathione cycle in leaf peroxisomes. The model is based on results recently described and those previously reported on the characterization of PMPs from pea leaves and the NADH : MDHAR of glyoxysomal membranes from castor bean endosperm. ASC, ascorbate reduced form; DHA, ascorbate, oxidized form (dehydroascorbate); MDHAR, monodehydroascorbate reductase; MDHA; monodehydroascorbate; GR glutathione reductase; APX, ascorbate peroxidase; XOD, xanthine oxidase. (del Río *et al.,* 1998)

Glycolate leaves the chloroplast and diffuses to a peroxisome where it is oxidized by glycolate oxidase to glyoxylate and H_2O_2. The peroxide is destroyed by catalase, and the glyoxylate undergoes transamination to produce glycine. The glycine enters a mitochondrion and is converted to serine which moves back to a peroxisome. Here serine is finally converted to glycerate which re-enters the chloroplast to be phosphorylated into 3-phosphoglycerate. In C_3 plants, photorespiration may occur at 25-50% of the rate of photosynthesis.

Various forms of catalase have been described in many plants. In maize there are three isozymes localized separately in peroxisomes, cytosol and mitochondria (Scandalios, 1988). Catalase is very sensitive to light and has a rapid turnover rate. Regardless, stress conditions, which reduce the rate of protein turnover, such as salinity, heat shock or cold, cause the depletion of catalase activity (Hertwig *et al*., 1992). This may have significance in plant's ability to tolerate the oxidative components of these environmental stresses.

Relatively little is known about catalase activity and its regulation in plant cells and relatively tolerance to salt. Therefore, a major goal of the work presented in this thesis was to examine the activity of catalase of rice *Oryza sativa* L., a model plant for monocots and one of the most ecomonically important crops in Thailand and the world. In addition, three different cDNA clones encoding catalase were analyzed for their protein expression pattern and enzyme activity in various salts.

Objectives of the thesis

1. To clone and express rice *OsCat* genes in *E. coli* and characterize the recombinant proteins.
2. To determine CAT activity from rice *Oryza sativa* L. cultivar KDML105 under salt stress.
3. To determine the effect of salts on CAT activity.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Rice seeds

Rice *Oryza sativa* L. cultivar Khao Dok Ma Li 105 (KDML105)

2.1.2 cDNA clones

cDNA clones which were obtained from DNA Bank of the National Institute of Agrobiological Science (NIAS)

2.1.3 Instruments

Autoclave: Labo Autoclave MLS-3020 (Sanyo Electric Co., Ltd., Japan)

Automatic micropipette: Pipetman P2, P20, P100, P1000 (Gilson Medical Electronics S.A., France)

Balance: Sartorius CP423s (Scientific Promotion Co. USA)

Biophotometer (Eppendorf, Germany)

Centrifuge 5417C (Eppendorf, Germany)

-20 °C Freezer (Sharp, Japan)

-80 °C Freezer (Forma, Japan)

Gel documentation apparatus: Gel Doc™ (Syngene, England)

Gel electrophoresis apparatus: Gel mate 2000 (Toyobo, Japan)

Incubator: BM-600 (Memmert Gambh, Germany)

Incubator shaker: Innova™ 4000 (New Brunswick Scientific, UK)

Laminar flow: HS-124 (International Scientific Supply Co., Ltd., USA)

Luminescence Spectrometer LS55 (PerkinElmer, USA)

Magnetic stirrer: Fisherbrand (Fisher Scientific, USA)

Magnetic stirrer and heater: Cerastir (Clifton, USA)

Mastercycler gradient PCR system (Eppendorf, Germany)

Microcentrifuge: PMC-880 (Tomy Kogyo Co., Ltd., Japan)

Microwave oven (Panasonic, Japan)

Orbital shaker (Labinco, Taiwan)

pH meter: pH900 (Precisa, Germany)

PCR workstation Model#P-036 (Scientific Co., USA)

Power supply: Power PAC 1000 (Bio-RAD Laboratories, USA)

Refrigerated centrifuge: 5804R (Eppendorf, Germany)

Refrigerated centrifuge: 5417R (Eppendorf, Germany)

Sonicator: Vibra cell™ (SONICS & MATERIALS, USA)

Spectrophotometer: DU®640 (Beckman Coulter, USA)

UV transilluminator: 2001 microvue (San Gabriel California, USA)

Vortex mixer: Model K 550-GE (Scientific Inc., USA)

Water bath: Isotemp210 (Fisher Scientific, USA)

2.1.4 Inventory supplies

Centrifugal Filters (10,000 Daltons molecular weight cut-off) (Amicon, USA)

Filter paper: Whatman No.1 (Whatman International Ltd., England)

Microcentrifuge tube 0.6- and 1.5-ml (Axygen Hayward, USA)

Millipore membrane filter 0.22μm (Millipore, USA)

Nipro disposable syringe (Nissho, Japan)

Nitrocellulose membrane: Protran (Whatman, USA)

PCR thin wall microcentrifuge tube 0.2 ml (Eppendorf, Germany)

pipette tips 10-, 100-, 1000-μl (Axygen Hayward, USA)

PVDF membranes (Macherey-Nagel, Germany)

Rectangular quartz semi micro (Starna Pty Ltd., USA)

2.1.5 Chemicals and reagents

Absolute ethanol (BDH, England)

Adenosine 5′-triphosphate (ATP) disodium salt hydrate (Sigma Chemical Co., USA)

Agar (Merck, Germany)

Agarose (FMC Bioproducts, USA)

Alkaline phosphatase-conjugated rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.)

Ammoniun sulfate (Sigma Chemical Company Co., USA)

Ammonium persulfate: $(NH_4)_2S_2O_8$ (Sigma Chemical Co., USA)

Anti-His antiserum (GE Healthcare, USA)

Bacto agar (Difco, USA)

Bacto tryptone (Difco, USA)

Bacto yeast extract (Difco, USA)

BenchMark™ His-tagged Protein Standard (Invitrogen, USA)

Benzamidine hydrochloride (Sigma Chemical Co., USA)

Beta-mercaptoethanol (Fluka, Switzerland)

Boric acid (Merck, Germany)

Bovine Serum Albumin (Sigma Chemical Co., USA)

5-Bromo-4-chloro-3-indole- β -D-galactopyranoside; X-gal (Sigma Chemical Co., USA)

5-Bromo-4-chloro-indolyl phosphate: BCIP (Fermentas, USA)

Bromophenol blue (Merck, Germany)

Calcium chloride (Carlo Erba Reagenti, Italy)

Chloroform (Merck, Germany)

Coomassie brilliant blue R-250 (Bio Basic Inc., USA)

Coomassie brilliant blue G-250 (Fluka, Switzerland)

Copper sulfate (Carlo Erba Reagenti, Italy)

dATP, dCTP, dGTP, and dTTP (Fermentas Inc., USA)

Di-potassium hydrogen phosphate anhydrous (Carlo Erba Reagenti, Italy)

Di-Sodium hydrogen orthophosphate anhydrous (Carlo Erba Reagenti, Italy)

Dithiothreitol (Sigma Chemical Co., USA)

Ethidium Bromide (Sigma Chemical Co., USA)

Ethylene diamine tetraacetic acid: EDTA (Carlo Erba Reagenti, Italy)

Formaldehyde (Sigma Chemical Co., USA)

Glacial acetic acid (Carlo Erba Reagenti, Italy)

Glycerol (BDH, England)

Glycine (Sigma Chemical Co., USA)

Hydrochloric acid (Merck, Germany)

Hydrogen Peroxide 30% (Merck, Germany)

Imidazole (Affymetrix Inc., USA)

Iso-1-thio-β-D-thiogalactopyranoside: IPTG (Serva, Germany)

Isopropanol (Merck, Germany)

Lambda DNA (Promega Co., USA)

Magnesium sulfate (Sigma Chemical Co., USA)

Magnesium chloride (Sigma Chemical Co., USA)

Methanol (Merck, Germany)

Nickel(II) sulfate hexahydrate: $NiSO_4$ (Sigma Chemical Co., USA)

Ni-Sepharose resins Amersham Biosciences (Piscataway Co., USA)

Nitroblue tetrazolium: NBT (Fermentas, Inc., USA)

Phenol (BDH, England)

Phosphoric acid 85% (Lab Scan, Ireland)

Potassium chloride (Carlo Erba Reagenti, Italy)

Sodium acetate (Carlo Erba Reagenti, Italy)

Sodium chloride (Carlo Erba Reagenti, Italy)

Sodium dodecyl sulfate (Sigma Chemical Co., USA)

Sodium dihydrogen orthophosphate (Carlo Erba Reagenti, Italy)

Sodium hydroxide (Carlo Erba Reagenti, Italy)

TEMED $(CH_3)_2NCH_2CH_2N(CH_3)_2$ (Amresco, USA)

Tris-(hydroxyl methyl)-aminomethane (Fluka, Switzerland)

Triton X-100 (Merck, Germany)

Tween 20 (Bio-RAD Laboratories, USA)

2.1.6 Enzymes

DNA polymerase, large (Klenow) fragment (New England Biolabs, Inc., USA)

DyNAzyme II DNA polymerase (Finnzymes, Finland)

Restriction endonucleases: *Eco*RI, *Hin*dIII (Fermentas, Inc., USA), *Nde*I (New England Biolabs, Inc., USA)

RNase A (Promega)

T4 DNA ligase (New England Biolabs Inc., USA)

Taq DNA polymerase (Fermentus, Inc., USA)

Vent DNA polymerase (New England Biolabs Inc., USA)

2.1.7 Microorganisms

: *Escherichia coli* strain XLl-Blue (F′:: Tn*10 proA*$^+$*B*$^+$*lacI*^{q} Δ*(lacZ)M15/recA1 endA1 gyrA96 (Nal*^{r}*) thi hsdR17 (*r_k^- m_k^+*) supE44 relA1 lac*)

: *Escherichia coli* strain Rosetta-gami Δ(*ara-leu*)*7697* Δ*lacX74* Δ*phoA Pvu*II *phoR araD139 ahpC galE galK rpsL* F′[*lac*+ *lacI*^{q}

pro] *gor522*::Tn*10 trxB* pRARE (Cam^R, Kan^R, Str^R, Tet^R)

2.1.8 Kits and Plasmids

Geneaid™ Gel/PCR DNA Fragment Extraction kit

(Geneaid, Taiwan)

Geneaid™ High-Speed Plasmid Mini kit (Geneaid, Taiwan)

pET-21a vector system (Promega, USA), a vector for expression

(Appendix A)

pTZ-57R vector system (Promega, USA), a vector for cloning

(Appendix A)

2.1.9 Antibiotics

Ampicillin (Sigma Chemical Co., USA)

2.1.10 Oligonucleotide primers

The oligonucleotide primers were synthesized by Operon, Germany.

2.1.11 Softwares

BlastX (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi)

ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html)

ExPASy ProtParam (http://au.expasy.org/tools/protparam.html)

GENETYX version 7.0 program (Software Development Inc.)

SECentral (Scientific & Educational Software)

2.2 Bacterial growth medium

Luria-Bertani broth (LB medium) (Maniatis *et al*., 1982)

LB medium containing 1% peptone, 0.5% NaCl and 0.5% yeast extract was prepared and pH adjusted to 7.2 with NaOH. For agar plates, it was supplemented with 1.5% (w/v) agar. The medium was autoclaved for 20 minutes at 121° C, 1.2 kg/cm^2. If needed, the medium was then supplemented with a selective antibiotic drug.

2.3 Methods

2.3.1 Quantitative method for determination of DNA concentration

The concentration of DNA was determined by measuring the absorbance at 260 nm (A_{260}) and estimated in µg/ml using the equation: [DNA] (µg/ml) = A_{260} × dilution factor × 50 as an absorbance at 260 nm of 1.0 corresponds to 50 µg/ml of DNA (Sambrook *et al.,* 1989).

2.3.2 Sequence analysis

Sequences of the cDNA clones encoding putative OsCats (designated *OsCatA, OsCatB* and *OsCatC*) were obtained from the rice databases via the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/) and the Rice Annotation Project Database (RAP-DB) at the NIAS (http://rapdb.dna.affrc.go.jp/). Sequencing of the cDNA clones was carried out at Macrogen, Korea. Multiple sequence alignment was performed using ClustalW (http://www.ebi.ac. uk.clustalw).

2.3.3 Cloning of the *OsCat* genes into cloning vector (pTZ57R/T)

2.3.3.1 Primer design

The oligonucleotide primers were designed on the *OsCat* cDNA sequences. The genes (and their GenBank Accession numbers) used were as follows: *OsCatA* (AK065094); *OsCatB* (AK100019) and *OsCatC* (AK066378). Based on the cDNA sequences encoding the *OsCatA*, *OsCatB* and *OsCatC* genes, a pair of oligonucleotide primers for amplifying each coding region of the *OsCat* genes was designed using the SECentral program (Scientific & Educational Software) with *Nde*I and *Eco*RI restriction sites engineered at the 5′ and 3′ ends, respectively. The sequence and the length of all oligonucleotide primers are shown in Table 2.1.

Table 2.1 The sequences and the length of oligonucleotide primers used for PCR amplification.

Gene	Primer	Sequence	Length
OsCatA	Forward	5' -ACTCATATGGATCCTTGCAAGTTCCGGCCG- 3'	30
	Reverse	5' -GTTTGAATTCATGCTTGGCTTCACGTTGAG- 3'	30
OsCatB	Forward	5' -CACCATATGGATCCCTACAAGCATCGGCCG- 3'	30
	Reverse	5' -GCCGGAATTCATGTTTGGTTTCAGGTTGAG- 3'	30
OsCatC	Forward	5' -TCACATATGGATCCCTACAAGCACCGCCCG- 3'	30
	Reverse	5' -ATCTGAATTCATGCTCGGCTTCGCGCTGAG- 3'	30

2.3.3.2 PCR amplification

The coding regions of *OsCatA, OsCatB* and *OsCatC* genes were amplified from *OsCat* cDNA clones, which were obtained from DNA Bank of the National Institute of Agrobiological Science (NIAS). The amplification reaction was performed in a 25-µl reaction that contained 1X NEB TermoPol buffer, 2.5 mM $MgCl_2$, 50-100 ng of DNA template, 200 µM each of dATP, dCTP, dGTP and dTTP, 1 µM of each primer and 5 units of *Vent* DNA polymerase (BioLab, Inc., USA). PCR amplification was performed as follows: pre-denaturation at 94 ºC for 5 minutes, started the cycle with denaturation at 94°C for 3 minutes, various annealing at 57-66.5°C for 1 minute and extension at 72°C for 2 minutes. After completion of 30 cycles, in the final extension step, 5 units of *Tag* DNA polymerase (Fermentus, Inc., USA) were added and reactions were continued at 72 ºC for 7 minutes for complete amplification.

2.3.3.3 Analysis of DNA fragment by agarose gel electrophoresis

Agarose gel electrophoresis was used to separate, identify, and purify DNA fragments. The concentration of agarose gel used has varied with the size of the DNA fragments to be separated. Appropriated amount of agarose powder was added to 100 ml Tris-acetate-EDTA (TAE) buffer in an Erlenmeyer flask and heated in the microwave oven until completely solubilized. The agarose solution was cooled down to below 60 ºC and air bubbles were completely eliminated. The solution was then poured into an electrophoresis mould. After the gel was completely set, the comb seal of mould was carefully removed. When ready, the DNA samples were run on an agarose gel in TAE buffer. To prepare samples, DNA was mixed with 10% (v/v) DNA gel loading buffer (0.1M EDTA/NaOH pH 7.5, 50% (v/v) of glycerol, 1% (w/v)

of SDS, 0.5 (w/v) of xylene cyanol FF, and 0.5 (w/v) of Bromophenol blue) and loaded into agarose gel. Electrophoresis was performed at a constant voltage of 100 volts until the bromophenol blue dye migrated to an appropiate distance through the gel. After electrophoresis, the gel was stained with ethidium bromide solution (5-10 μg/ml in distilled water) for 5 minutes and destained with distilled water for 10 minutes. DNA fragments on the agarose gel were visualized on the UV light transilluminator and photographed. The concentration and molecular weight of DNA fragments was determined by comparison of the band intensity and the relative mobility with those of the standard λ/*Hin*dIII DNA markers.

2.3.3.4 Elution of DNA fragments from agarose gel

The amplification products generated by PCR were purified from agarose gel using Geneaid gel extraction protocol (Geneaid, Taiwan). After electrophoresis, the desired DNA fragment was excised as gel slice from an agarose gel using a scalpel and transferred to a microcentrifuge tube. Five hundred μl of DF buffer was added and the mixture was incubated for 10 minutes at 55-60°C or until the gel slice has been completely dissolved. During incubation, the tube was inverted every 2-3 minutes. The mixture was then transferred into a DF column and the column was centrifuged at 15,000 xg for 1 minute. The flow through solution was discarded. Then 400 μl of W1 buffer was added and the column was centrifuged at 15,000 xg for 1 minute. The flow through was discarded and 600 μl of Wash buffer was added to the DF column. The column was let standing for 1 minute and centrifuged at 15,000 xg for 1 minute, then the flow through solution was discarded. The DF column was centrifuged again for 3 minutes to remove a trace element of the Wash buffer. The DF

column was then placed into a sterile 1.5 ml microcentrifuge tube. DNA was eluted by an addition of 15-50 µl of steriled water to the center of the DF column. The column was let standing for 2 minutes, and then centrifuged at 15,000 xg for 2 minutes. The recovered DNA was kept at 4 ºC until used and DNA concentration was determined by agarose gel electrophoresis.

2.3.3.5 Ligation of *OsCat* genes products to pTZ57R/T

The purified PCR products were ligated to pTZ57R/T vector (see in Appendix A). A suitable molecular ratio between vector and inserted DNA in a mixture of cohesive-end ligation is usually 1:3. To calculate the appropriate amount of the PCR product (insert) used in a ligation reaction, the following equation was used:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert : vector molar ratio} = \text{ng of insert}$$

The 20 µl of ligation mixture contained appropriate amounts of the vector DNA and the gene fragment, 1x ligation buffer and 10 U of T4 DNA ligase. The reaction was incubated overnight at 16^{o}C. The ligation products were used for transformation into *E. coli* XL1-Blue.

2.3.3.6 Transformation of ligated products to *E. coli* host cells by $CaCl_2$ method

a) Preparation of *E. coli* competent cells

Competent *E. coli* strain XL1-Blue was prepared according to the method of Sambrook *et al.* (1989). A single colony of *E. coli* strain XL1-Blue was inoculated in 3 ml LB broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl, pH

7.2) and incubated at 37 ºC with shaking at 250 rpm overnight. Fifty milliliters of LB medium were then inoculated with two percents of the starter culture and the culture was incubated at 37 ºC with shaking at 250 rpm for 3-4 hours until the optical density at 600 nm (OD_{600}) of the culture reached 0.45-0.55. The culture was chilled on ice slurry for 15-30 minutes and the cells were harvested by centrifugation at 8,000 xg at 4 ºC for 10 minutes. The supernatant was decanted and the cell pellet was washed with 25 ml of fresh ice-cold 150 mM $CaCl_2$, resuspended by gently mixing and centrifuged at 8,000 xg at 4 ºC for 10 minutes. The supernatant was discarded. The cells were resuspended with 25 ml of fresh ice-cold 150 mM $CaCl_2$ and incubated on ice for 30 minutes, then centrifuged at 8,000 xg at 4 ºC for 15 minutes. The supernatant was discarded and finally, the cells were resuspended in 1 ml of fresh ice-cold 150 mM $CaCl_2$ and incubated on ice for 1 hour. These cells were used as freshly prepared competent cells. (50 µl aliquot is sufficient for a transformation).

b) Transformation by $CaCl_2$ method

In this study, competent cells were transformed with the recombinant plasmids by $CaCl_2$ method. Sixty microliters of competent cells were mixed well with 10 µl of the ligation mixture and then placed on ice for 30 minutes. The cells were heat-shocked for 90 seconds in a waterbath set to 42 ^{o}C and quickly incubated on ice for 5 minutes. Five hundred µl of LB medium was added then the cell suspension was incubated at 37 ºC with shaking at 250 rpm for 1 hour and the cells were spun down to retain 200 µl. Finally, the cell suspension was spread onto LB agar plates containing 100 µg/ml ampicillin with 10 µl of 0.1 M Iso-1-thio-β-D-thiogalactopyranoside (IPTG), and 50 µl of 20 mg/ml 5-Bromo-4-chloro-3-indole-

beta-D-galactopyranoside (X-gal) spread on top of agar and incubated at 37 ºC overnight. The recombinant clones containing the inserted DNA were white while those without inserted DNA were blue. The white colonies which potentially contained the recombinant plasmids were selected and checked for the plasmids by restriction enzyme digestion.

2.3.3.7 Analysis of recombinant plasmids

a) Plasmid DNA isolation by alkaline lysis method

A single colony of recombinant cells was selected and grown in 5 ml of LB broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl) containing 100 µg/ml of ampicillin overnight at 37 ºC with shaking at 250 rpm. The cells were spun in a microcentrifuge at 8000×g for 10 minutes at 4 ºC. The cells were resuspended in 300 µl of Lysis buffer (50 mM of Tris base, 10 mM of $Na_2EDTA.H_2O$ and 100 µg/ml of RNaseA) and mixed by vortexing. The suspension was allowed to strand for 5 minutes at room temperature, then 300 µl of Alkaline-SDS solution (200mM NaOH and 1% SDS) was added and the suspension was inverted gently several times to mix and allowed to stand on ice for 5 minutes. Three hundred µl of High salt solution (3M of potassium acetate) was then added to the mixture. The suspension was mixed gently and allowed to stand for 10 minutes on ice. The insoluble salt-genomic DNA precipitate was then removed by centrifugation at 15,000 xg at 4 ºC for 15 minutes. The supernatant was transferred to a fresh microcentrifuge tube and the nucleic acid was precipitated by adding 480 µl (0.6 volumes) of isopropanol. The sample was mixed thoroughly and immediately centrifuged for 20 minutes to collect the precipitated DNA. The DNA pellet was resuspended in 90 µl of

sterile water and the suspension was vortexed gently. Ten µl of 3 M sodium acetate, pH 7.0 and 300 µl of cold absolute ethanol were added to the DNA solution, then mixed and chilled on ice for 30 minutes. The DNA was collected by centrifuging at 14,000xg for 20 minutes at 4 ºC. The pellet was rinsed with 300 µl of 70% ethanol and allowed to dry for 10-15 minutes. The plasmid DNA was resuspended with 30 µl of sterile water and stored at -20 ºC.

b) Restriction enzyme analysis

The recombinant plasmids isolated by the alkaline lysis method were analyzed for the presence of the interested cloned fragments by digestion with appropriate restriction endonucleases. The *OsCat* recombinant plasmids were digested with *Nde*I and *Eco*RI. All restriction digestion was performed in the conditions recommended by the enzyme manufacturer. Each of the reactions was carried out in a 10-µl mixture at 37 ºC. The DNA products were analyzed by 1% agarose gel electrophoresis. The size of DNA fragments was determined by comparing with the λ / *Hin*dIII marker.

2.3.4 Cloning of *OsCat* genes into expression vector pET21a(+)

2.3.4.1 Vector DNA preparation

The *E. coli* XL-1 Blue, which contained pET21a(+) plasmids was grown in 5 ml LB medium containing 100 µg/ml ampicillin at 37 ^{o}C with shaking at 250 rpm for 16 hours. The pET21a(+) vector was extracted using Alkaline lysis method as described above. The expression vector pET21a(+) was linearized with *Nde*I and *Eco*RI digestion using the conditions recommended by the enzyme manufacturer. The

reaction was incubated at 37 ^{o}C for 3-4 hours. The linearized pET21a(+) was harvested from agarose gel using Geneaid gel extraction protocol**.**

2.3.4.2 Preparation of *OsCat* gene fragments

After the *OsCat* genes were cloned into pTZ57R/T and the recombinant clones were checked with restriction enzyme digestion. The recombinant plasmids containing *OsCat* genes were extracted by using Alkaline lysis method as described in 2.3.3.7. The *OsCat* genes were digested with *Nde*I and *Eco*RI using the conditions recommended by the enzyme manufacturer. The reaction was incubated at 37^{o}C overnight. The *OsCat* gene fragments were harvested from agarose gel using Geneaid gel extraction protocol and DNA insert sizes were determined by comparing with the λ / *Hin*dIII marker.

2.3.4.3 Ligation of *OsCat* gene fragments to pET21a(+)

The gene fragment was ligated into pET21a(+) expression vector. A suitable molecular ratio between vector and inserted DNA in a mixture of 1:5 was used. The 20 µl of ligation mixture containing appropriate amounts of the vector DNA and the gene fragment, 1x ligation buffer and 10 U of T4 DNA ligase, was incubated overnight at 16^{o}C. The mixture was then used for transformation.

2.3.4.4 Transformation of *E. coli* XL-1Blue cell by $CaCl_2$ method

Competent cells were prepared as described in 2.3.3.6. To transform the *E. coli* cells, the ligation mixtures were mixed with the competent cells and then placed on ice for 30 minutes. The cells were heat-shocked for 90 seconds in a water bath set

to 42 ^{o}C and the cells were quickly incubated on ice for 5 minutes, 500 µl of LB medium was then added to the cells and the suspension was incubated at 37 ºC with shaking at 250 rpm for 1 hour and the cells were spin down to retain 200 µl. Finally, the cell suspension was spread onto the LB agar plates containing 100 µg/ml ampicillin and incubated at 37 ºC overnight. The recombinant clones which contained the pET21a(+) vector could growth on LB agar plates containing ampicillin. A single colony was selected and checked by restriction enzyme digestion.

2.3.4.5 Analysis of recombinant plasmids

A single colony of recombinant cells was selected and grown in 5 ml of LB broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl) containing 100 µg/ml of ampicillin overnight at 37 ºC with shaking at 250 rpm. The recombinant plasmids were extracted using alkaline lysis method. The recombinant plasmids were digested with *Nde*I and *Eco*RI. The reaction was incubated at 37^{o}C overnight and the products were analyzed by 1% agarose gel electrophoresis. The size of the DNA insert was determined by comparing with the λ / *Hin*dIII marker.

2.3.5 Nucleotide and amino acid sequences analysis

The recombinant plasmids were extracted by High Speed plasmid Mini kit (Geneaid, Taiwan) (see in Appendix B) and nucleotide sequences of the inserts were determined. DNA sequencing was carried out at Macrogen, Korea using T7 promoter and T7 terminator. The molecular weights of OsCATA, OsCATB and OsCATC were calculated from the deduced amino acid sequences using the program Compute Mw (http://expasy.org/tools/ pi_tool.html).

2.4 Expression of the *OsCat* genes

To produce recombinant proteins, the *OsCatA, OsCatB* and *OsCatC* genes that were cloned in the expression vector pET21a(+), were expressed in *E.coli* Rosetta-gami using the protocols described below.

2.4.1 Preparation of cell expression

The recombinant pET21a(+) plasmids containing *OsCat* genes were transformed into *E.coli* strain Rosetta-gami cells by the $CaCl_2$ method. The competent cells were prepared as described in 2.3.3.6. The recombinant plasmids were mixed with the competent cells and then placed on ice for 30 minutes. The cells were heat-shocked for 90 seconds in a water bath set to 42 ^{o}C and quickly incubated on ice for 5 minutes, 500 µl of LB medium was added, then the cell suspension was incubated at 37 ºC with shaking at 250 rpm for 1 hour. Finally, fifty µl of the cell suspension was spread onto the LB agar plates containing 100 µg/ml ampicillin and incubated at 37 ºC overnight.

2.4.2 Optimization for *OsCat* genes expression

A single colony of recombinant cells was grown overnight at 37^{o}C for 16 hours in 5 ml of LB medium, pH 7.0, containing 100 µg/ml ampicillin. The starter was diluted 1:100 into 200 ml of the same medium and cultured at 37^{o}C with shaking for 3 - 4 hours until the optical density at 600 nm (OD_{600}) of the culture reached 0.6. Production of recombinant proteins was induced by adding IPTG which was varied to a final concentration of 0, 0.2, 0.4, 0.6, 0.8 or 1.0 mM and the incubation was continued at 37^{o}C for 3 hours. The cells were harvested by centrifugation at 8000xg

for 15 minutes, and then the cell pellets were washed twice in cold wash buffer (50 mM Tris-HCl, pH 7.5) and the cells were collected again by centrifugation. The cell pellet was stored at -80^{o}C until it was sonicated. In crude extract preparation, the cell pellet was resuspended in 10 ml of cold lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM DTT), incubated on ice for 30 minutes and then broken by sonication on ice. Unbroken cells and cell debris were separated by centrifugation at 27,000 xg for 30 minutes. The supernatant and the pellet were kept at 4^{o}C until they were used.

2.4.3 Determination of protein concentration

Protein concentration was determined by the modified method of Bradford, M. M. (1976). The reaction mixture contained 100 µl of the protein sample and 1 ml of Bradford solution, which was prepared as described in (Appendix B). The combined solution was mixed by vortexing. Then, the protein concentration was determined by measuring the absorbance at 595 nm after letting the mixture stand at room temperature for 5 minutes but no longer than 1 hour. The protein concentration was calculated from the standard curve using bovine serum albumin as standard (0-20 µl of 1 mg/ml BSA).

2.4.4 SDS-polyacrylamide gel electrophoresis

The SDS-PAGE system was performed according to the method of Bollag *et al*., 1996. The slab gel system consisted of 0.1% SDS (w/v) in 12.5% seperating gel and 3.9% stacking gel. Tris-glycine (25 mM Tris, 192 mM glycine and 0.1% SDS), pH 8.3 was used as the electrode buffer. The gel preparartion was described in

Appendix B. The protein samples were mixed with 5x sample buffer (60 mM Tris-HCl pH 6.9, 79% glycerol, 2% SDS, 0.1% bromophenol blue and 14.4 mM β-mercaptoethanol) by the ratio of 5:1 and boiled for 10 minutes before loading to the gel. The electrophoresis was run from the cathode towards the anode at a constant current of 20 mA per gel at room temperature. The molecular weight marker proteins containing β-galactosidase (116,000 Da), bovine serum albumin (66,200 Da), ovalbumin (45,000 Da), lactate dehydrogenase (35,000 Da), restriction endonuclease Bsp98I (25,000 Da), β-lactoglobulin (18,400 Da) and lysozyme (14,400 Da) were used.

2.4.5 Protein staining

The gel was transferred to a box containing Coomassie staining solution (0.25% Coomassie Blue R-250, 50% methanol, and 7% glacial acetic acid). The gel was stained for 1 hour at room temperature with gentle shaking. After staining, the stain solution was poured out, and the gel was briefly rinsed with water and incubated with the Coomassie destaining solution (10% methanol and 7% glacial acetic acid). The gel was gently destained for several times until protein bands were readily visible.

2.5 Purification of the OsCAT proteins

2.5.1 Crude extract preparation

For the large scale protein expression, a single colony of recombinant cells was grown overnight at 37 ^{o}C for 16 hours in 25 ml of LB medium, pH 7.0, containing 100 μg/ml ampicillin. Then, one thousand ml of the same medium was

inoculated with 2.0% of the starter culture and was cultured at 37 ^{o}C with shaking for 3-4 hours until the optical density at 600 nm (OD_{600}) of the cell culture reached 0.6. Production of recombinant proteins was induced by adding IPTG and its level was highest at the final concentration of 0.4, 0.2 and 0.2 mM for the recombinant cell of *OsCatA, OsCat B* and *OsCatC*, respectively. Then, the incubation was continued at 37 ^{o}C for 4 hours. The cells were harvested by centrifugation at 8,000xg for 15 minutes, and then the cell pellet was washed twice in cold wash buffer (20 mM sodium phosphate buffer, pH 7.4) and collected again by centrifugation. The cell pellet was stored at -80 ^{o}C until it was sonicated. In crude extract preparation, the cell pellet was resuspended in 50 ml of cold lysis buffer (20 mM sodium phosphate buffer, pH 7.4, 1 mM EDTA, and 1 mM DTT), incubated on ice for 30 minutes and then the cells were broken by sonication on ice. Unbroken cells and cell debris were seperated by centrifugation at 20,000 xg for 30 minutes. The supernatant and the pellet were kept at 4 ^{o}C for further analysis and purification.

2.5.2 Preparation of inclusion bodies

The pellet containing the inclusion bodies from 2.5.1 was washed with 50 ml of Wash buffer I (20 mM sodium phosphate buffer (pH 7.4), 1% (v/v) Triton X-100) and washed twice with cold Wash buffer II (20 mM sodium phosphate buffer, pH 7.4). Then, the pellet was collected by centrifugation at 10,000 xg for 10 minutes at 4 ^{o}C. The inclusion bodies were kept at 4 ^{o}C for until further use.

2.5.3 Solubilization and refolding of inclusion bodies

The inclusion bodies were solubilized in 20 mM sodium phosphate buffer (pH 12.0). The resulting solution was incubated with agitation for 12 hours at 4 ^{o}C and the unsolubilized proteins were removed by centrifugation at 15,000 xg for 10 minutes at 4 ^{o}C. The remaining clear solution was dialyzed in 3 liters of 20 mM sodium phosphate buffer (pH 7.4), 1 mM EDTA and 1 mM DTT for overnight. The dialysate was purified by column chromatography using Ni-Sepharose column.

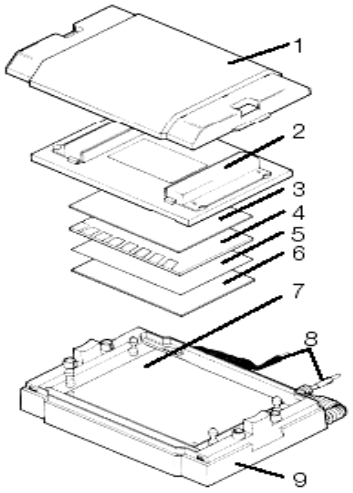
2.5.4 OsCAT proteins purification by Ni-Sepharose column

The high performance Ni-Sepharose was packed into a small column and washed with 5 column volumes of deionized water. Then, the column was equilibrated with Binding buffer (20 mM sodium phosphate buffer, pH 7.4, 30 mM imidazole and 0.5 M NaCl) of about 30 column volumes. Before applying the crude protein sample to the Ni-Sepharose column, imidazole and NaCl were added to a final concentration of 30 mM and 0.5 mM, respectively. The crude protein sample was applied into the Ni-Sepharose column at 4 ^{o}C. The flow through was collected by a gravity flow. The column was washed with binding buffer of about 10 column volumes to remove unbound proteins. After washing, the protein was eluted with elution buffer (20 mM sodium phosphate buffer, pH 7.4, 0.5 M NaCl and varying concentrations of 50-500 mM imidazole) of about 10 column volumes. The purified proteins were analyzed by SDS-polyacrylamide gel electrophoresis as described in 2.4.4. The imidazole was removed by dialysis in dialysis buffer (20 mM sodium phosphate buffer, pH 7.4, 1 mM EDTA, 1 mM DTT) for at least 4 hours at 4 ºC three times.

2.6 Western blot detection of the His-tagged protein

After running the SDS-PAGE, the SDS-gel slab was removed from the glass plates. The membrane, gel and filter paper were soaked in transfer buffer (25 mM Tris base, 150 mM glycine and 20% methanol) for 30 minutes before they were consequently laid on the Trans-Blot® SD (Bio-Rad) membrane. The filter paper was placed on the platform, followed by the membrane, the gel and the filter paper, respectively, as shown in Figure 2.1.

Protein transfer was performed at a constant current of 90 mA from the cathode towards the anode for 90 minutes. After transferring the proteins from the gel to the membrane, the orientation of the gel was marked on the membrane. The membrane was then transferred to an appropriate container (petri dish) and incubated in Blocking buffer (1× PBS buffer [10 mM sodium phosphate buffer, 150 mM NaCl, pH 7.4, 0.05% (v/v) Tween™-20 and 5% (w/v) non-fat dry milk]) at room temperature overnight with gentle shaking. The membrane was washed 3 times for 10 minutes each in Washing buffer (PBS-Tween buffer [1× PBS buffer (10 mM sodium phosphate buffer, 150 mM NaCl, pH 7.4, 0.025% (v/v) Tween™-20)] and incubated in an anti-His antibody solution (1 : 3000 dilution) in the washing buffer containing 1% (w/v) non-fat dry milk, at ambient temperature with gentle mixing for 3 hours. Then, the membrane was washed 3 times for 10 minutes each in washing buffer and then incubated in a secondary antibody solution (1 : 2500 dilution) in the washing buffer containing 1% (w/v) non-fat dry milk with agitation for 1 hour. The membrane was washed 3 times for 10 minutes each in washing buffer at room temperature. The bound antibody was detected by color development



1. Safety lid
2. Cathode assembly with latches
3. Filter paper
4. Gel
5. Membrane
6. Filter paper
7. Spring-loaded anode platform mounted On 4 guideposts
8. Power cables
9. Base

Figure 2.1 Exploded view of the Trans-Blot® SD (Bio-Rad).

using NBT/BCIP (Fermentas) dissolved in 100 mM Tris- HCl, 100 mM NaCl and 50 mM $MgCl_2$, pH 9.5 as substrate.

2.7 Determination of catalase activity

Catalase activity was measured by monitoring the decomposition in H_2O_2 at room temperature. The rate of H_2O_2 decomposition was measured by the decrease of absorbance at 240 nm at time intervals of 3 minutes, following the procedure of Aebi (1974). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.5) and 50 µg of enzyme solution. The reaction was started by the addition of 12.5 mM H_2O_2.

2.7.1 Effect of NaCl on OsCAT activity

The effect of NaCl on OsCAT activity using H_2O_2 as substrates which the final concentrations of NaCl in the reaction mixture were 0, 15, 30, 60, 125, 250, 500 and 1000 mM.

In the presence of catalase, the reaction mixture contained 50 mM potassium phosphate buffer pH 7.5, 50 µg of enzyme solution and varying concentrations of NaCl (0-1 M). The mixture was pre-incubated at 25^{o}C for 10 minutes. The catalase activity was assayed as described in 2.7.

2.7.2 Optimal pH

The reaction mixtures containing 50 µg of enzyme solution and various buffers (pH 3.0-6.0 using 50 mM citrate phosphate buffer; pH 6.0-7.5 using 50 mM potassium phosphate buffer; pH 7.5-9.0 using 50 mM Tris-HCl buffer and pH 9.0-

11.0 using carbonate buffer) were pre-incubated at room temperature for 10 minutes. The catalase activity was assayed as described in 2.7.

2.7.3 Optimal temperature

The reaction mixtures contained 50 mM of buffer which optimal of pH in each protein and 50 μg of enzyme solution which were pre-incubated at various temperatures (20, 25, 30, 35, 40, 45, 50, 55, 60, 65 and 70 ^{o}C for 10 minutes. The catalase activity was assayed as described in 2.7.

2.8 Determination of catalase from rice *Oryza sativa* L. cultivar Khao Dok Ma Li 105 (KDML105)

2.8.1 Preparation of rice seedlings

Seeds of the Khao Dok Ma Li 105 (KDML105) indica rice cultivar *Oryza sativa* L. were obtained from Kasetsart University. Healthy rice seeds were rinsed with deionized water and soaked for 20 minutes in 2.1% sodium hyperchlorite with shaking, then extensively washed with sterile deionized water for at least 3 times. Washed seeds were germinated in NB medium, which was prepared as described in (Appendix B) for 7 days under a 16-hr light/8-hr dark photoperiod. After 7 days, germinated seeds were transferred to 1X Limpinuntana's nutrient solution (Limpinuntana, 1978) (Appendix B) and grown for 2 weeks under a 16-hr light/8-hr dark photoperiod. To determine the effect of NaCl, 3-week old seedlings were treated with 150 mM NaCl in 1X Limpinuntana's nutrient solution for 0, 1, 2 and 3 days.

2.8.2 Preparation of crude enzyme extract

Enzyme extract for catalase was prepared by first freezing the weighted amount of leaf samples (0.5 g) in liquid nitrogen to prevent proteolytic activity followed by grinding with 5 ml of cold extraction buffer (0.1 M potassium phosphate buffer, pH 7.5, 0.5 mM EDTA and 5 mM DTT). The homogenated was filtered through four layers of cheesecloth, suspensions were centrifuged at 15,000 xg for 20 minutes at 4 ^{o}C. The supernatant was collected for measurement of catalase activity and stored at 4 ^{o}C for further analysis. Protein content was measured according to the method of Bradford (1976) with bovine serum albumin (BSA) as a standard. Then, determination of catalase activity was carried out as described in 2.7.

2.8.3 Effect of NaCl on CAT activity

The effect of NaCl on CAT activity using H_2O_2 which the final concentrations of NaCl in the reaction mixture were 0, 15, 30, 60, 125, 250, 500 and 1000 mM.

In the presence of catalase from rice, the reaction mixture contained 50 mM potassium phosphate buffer pH 7.5, 50 μg of enzyme solution and varying concentrations of NaCl (0-1 M). The mixture was pre-incubated at 25^{o}C for 10 minutes. The catalase activity was assayed as described in 2.7.

CHAPTER III

RESULTS

3.1 Catalase sequence analysis

Database searches for catalase (*Cat*) genes identified three putative *OsCats* in the rice *Oryza sativa* L. genome. We have named these genes *OsCatA, OsCatB* and *OsCatC*. The primary structures of OsCATA, OsCATB and OsCATC proteins were compared with those of AtCAT1, AtCAT2 and AtCAT3 from *Arabidopsis*. Based on pairwise alignments, OsCATA showed 72% and 75% identities with OsCATB and OsCATC, respectively, whereas OsCATB had 83% identity with OsCATC. The deduced amino acid sequence of AtCAT1 showed 72%, 80% and 79% identities with OsCATA, OsCATB and OsCATC, respectively. AtCAT2 had 70%, 82% and 84% identities with OsCATA, OsCATB and OsCATC, respectively. AtCAT3 also had 68%, 76% and 74% identities with OsCATA, OsCATB and OsCATC, respectively. Multiple sequence alignment of all proteins was performed and the result excluding the diverged N-terminal regions is shown in Figure 3.1.

3.2 Molecular cloning of the *OsCat* genes

Three *OsCat* genes: *OsCatA*, *OsCatB* and *OsCatC* were cloned. Based on the *OsCat* cDNA sequences obtained from the National Institute of Agrobiological Sciences, Japan, a pair of primers for amplifying each coding region of the *OsCat* genes was designed with the restriction endonuclease sites for *Nde*I and *Eco*RI engineered at its 5′- and 3′-ends, respectively. The sequences of the primers are shown

```
AtCAT1   MDPYRVRPSSAHDSPFFTTNSGAPVWNNNSSLTVGTRGPILLEDYHLLEKLANFDRERIP 60
AtCAT2   MDPYKYRPASSYNSPFFTTNSGAPVWNNNSSMTVGPRGLILLEDYHLVEKLANFDRERIP 60
AtCAT3   MDPYKYRPSSAYNAPFYTTNGGAPVSNNISSLTIGERGPVLLEDYHLIEKVANFTRERIP 60
OsCATB   MDPYKHRPSSGSNSTFWTTNSGAPVWNNNSALTVGERGPILLEDYHLIEKLAQFDRERIP 60
OsCATC   MDPYKHRPSSSFNGPLWSTNSGAPVWNNNNSLTVGSRGPILLEDYHLVEKLANFDRERIP 60
OsCATA   MDPCKFRPSSSFDTKTTTTNAGAPVWNDNEALTVGPRGPILLEDYHLIEKVAHFARERIP 60
         *** : **:*. :    :**.**** *: .::*:* ** :*******:**:*:* *****

AtCAT1   ERVVHARGASAKGFFEVTHDITQLTSADFLRGPGVQTPVIVRLSTVIHERGSPETLRDPR 120
AtCAT2   ERVVHARGASAKGFFEVTHDISNLTCADFLRAPGVQTPVIVRFSTVIHARGSPETLRDPR 120
AtCAT3   ERVVHARGISAKGFFEVTHDISNLTCADFLRAPGVQTPVIVRFSTVVHGRASPETMRDIR 120
OsCATB   ERVVHARGASAKGFFEVTHDISHLTCADFLRAPGVQTPVIVRFSTVVHERGSPETLRDPR 120
OsCATC   ERVVHARGASAKGFFEVTHDITHLTCADFLRAPGVQTPVIVRFSTVIHERGSPETLRDPR 120
OsCATA   ERVVHARGASAKGFFECTHDVTDITCADFLRSPGAQTPVIVRFSTVIHERGSPETIRDPR 120
         ******** ******* ***::.:*.*****.**.*******:***:* *.****:** *

AtCAT1   GFAVKFYTREGNFDLVGNNFPVFFVRDGMKFPDMVHALKPNPKSHIQENWRILDFFSHHP 180
AtCAT2   GFAVKFYTREGNFDLVGNNFPVFFIRDGMKFPDIVHALKPNPKSHIQENWRILDFFSHHP 180
AtCAT3   GFAVKFYTREGNFDLVGNNTPVFFIRDGIQFPDVVHALKPNRKTNIQEYWRILDYMSHLP 180
OsCATB   GFAVKFYTREGNFDLVGNNMPVFFIRDGMKFPDMVHAFKPSPKTNMQENWRIVDFFSHHP 180
OsCATC   GFAIKFYTREGNWDLVGNNFPVFFIRDGMKFPDMVHSLKPNPKSHVQENWRILDFFSHHP 180
OsCATA   GFAVKFYTREGNWDLLGNNFPVFFIRDGIKFPDVIHAFKPNPRSHVQEYWRVFDFLSHHP 180
         ***:********:**:*** ****:***::***::*::**. :::::** **:.*::** *

AtCAT1   ESLHMFSFLFDDLGIPQDYRHMEGAGVNTYMLINKAGKAHYVKFHWKPTCGIKCLSDEEA 240
AtCAT2   ESLNMFTFLFDDIGIPQDYRHMDGSGVNTYMLINKAGKAHYVKFHWKPTCGVKSLLEEDA 240
AtCAT3   ESLLTWCWMFDDVGIPQDYRHMEGFGVHTYTLIAKSGKVLFVKFHWKPTCGIKNLTDEEA 240
OsCATB   ESLHMFSFLFDDVGIPLNYRHMEGFGVNTYTLINKDGKPHLVKFHWKPTCGVKCLLDDEA 240
OsCATC   ESLHMFTFLFDDIGIPADYRHMDGSGVNTYTLVNRAGKSHYVKFHWKPTCGVKSLLDDEA 240
OsCATA   ESLHTFFFLFDDVGIPTDYRHMDGFGVNTYTFVTRDAKARYVKFHWKPTCGVSCLMDDEA 240
         ***  : ::***:*** :****:* **:** :: : .*   **********:. * :::*

AtCAT1   IRVGGANHSHATKDLYDSIAAGNYPQWNLFVQVMDPAHEDKFDFDPLDVTKIWPEDILPL 300
AtCAT2   IRLGGTNHSHATQDLYDSIAAGNYPEWKLFIQIIDPADEDKFDFDPLDVTKTWPEDILPL 300
AtCAT3   KVVGGANHSHATKDLHDAIASGNYPEWKLFIQTMDPADEDKFDFDPLDVTKIWPEDILPL 300
OsCATB   VTVGGTCHSHATKDLTDSIAAGNYPEWKLYIQTIDPDHEDRFDFDPLDVTKTWPEDIIPL 300
OsCATC   VTVGGTNHSHATQDLYDSIAAGNFPEWKLFIQTIDPDHEDRFDFDPLDVTKTWPEDIVPL 300
OsCATA   TLVGGKNHSHATQDLYDSIAAGNFPEWKLFVQVIDPEEEERFDFDPLDDTKTWPEDEVPL 300
           :**  *****:** *:**:**:*:*:*::* :** .*::******* ** **** :**

AtCAT1   QPVGRLVLNKNIDNFFNENEQIAFCPALVVPFIHYSDDNVLQTRIFSYADSQRHRLGPNY 360
AtCAT2   QPVGRMVLNKNIDNFFAENEQLAFCPAIIVPGIHYSDDKLLQTRVFSYADTQRHRLGPNY 360
AtCAT3   QPVGRLVLNRTIDNFFNETEQLAFNPGLVVPGIYYSDDKLLQCRIFAYGDTQRHRLGPNY 360
OsCATB   QPVGRMVLNKNIDNFFAENEQLAFCPAIIVPGIHYSDDKLLQTRIFSYADTQRHRLGPNY 360
OsCATC   QPVGRMVLNRNIDNFFSENEQLAFCPGIIVPGIYYSDDKLLQTRIFSYSDTQRHRLGPNY 360
OsCATA   RPVGRLVLNRNVDNFFNENEQLAFGPGLVVPGIYYSDDKMLQCRVFAYADTQRYRLGPNY 360
         :****:***:.:**** *.**:** *.::** *:****::** *:*:*.*:**:******

AtCAT1   LQLPVNAPKCAHHNNHHDGFMNFMHRDEEVNYFPSRLDPVRHAEKYPTTPIVCSGNREKC 420
AtCAT2   LQLPVNAPKCAHHNNHHEGFMNFMHRDEEVNYFPSRYDQVRHAEKYPTPPAVCSGKRERC 420
AtCAT3   LQLPVNAPKCAHHNNHHEGFMNFMHRDEEINYYPSKFDPVRCAEKVPTPTNSYTGIRTKC 420
OsCATB   LMLPVNAPKCAYHNNHHDGSMNFMHRDEEVNYFPSRFDAARHAEKVPIPPRVLTGCREKC 420
OsCATC   LLLPPNAPKCAHHNNHYDGFMNFMHRDEEVDYFPSRYDPAKHAPRYPIPSATLTGRREKV 420
OsCATA   LMLPVNAPKCAHHNNHYDGAMNFMHRDEEVDYYPSRHAPLRHAPPTPITPRPVVGRRQKA 420
         * ** ******:****::* *********::*:**:    : *   * ..    * * :

AtCAT1   FIRKENNFKQPGERYRSWDSDRQERFVKRFVEALWEPRVTHEIRSIWISYWSQADKSLGQ 480
AtCAT2   IIEKENNFKEPGERYRTFTPERQERFIQRWIDALSDPRITHEIRSIWISYWSQADKSLGQ 480
AtCAT3   VIKKENNFKQAGDRYRSWAPDRQDRFVKRWVEILSEPRLTHEIRGIWTSYWLKADRSLGQ 480
OsCATB   VIDKENNFQQAGERYRSFDPARQDRFLQRWVDALSDPRITHELRGIWISYWSQCDASLGQ 480
OsCATC   VIAKENNFKQPGERYRSWDPARQDRFIKRWIDALSDPRLTHEIRSIWLSYWSQADRSLGQ 480
OsCATA   TIHKQNDFKQPGERYRSWAPDRQERFIRRFAGELAHPKVSPELRAIWVNYLSQCDESLGV 480
          * *:*:*::.*:***:: . **:**::*:   * .*::: *:*.** .*  :.* ***
```

```
AtCAT1    KLATRLNVRPNF 492
AtCAT2    KLASRLNVRPSI 492
AtCAT3    KLASRLNVRPSI 492
OsCATB    KLASRLNLKPNM 492
OsCATC    KLASRLSAKPSM 492
OsCATA    KIANRLNVKPSM 492
          *:*.**. :*.:
```

Figure 3.1 Alignment of the deduced amino acid sequence of rice catalase (OsCATA, OsCATB and OsCATC) with *Arabidopsis* catalase (AtCAT1, AtCAT2 and AtCAT3). The putative heme-binding region, the heme-binding site are shown in green box and blue box, respectively and the peroxisomal targeting signal is shown in red letter; identical residues on the same column are indicated by an asterisk (*); conserved substitutions sequences are indicated by a colon (:), semi-conserved sequences are indicated by a dot (.).

in Table 2.1. The optimization of PCR was done to find an appropriate annealing temperature by varying the temperature at 57.0, 60.1, 63.6 and 66.5^{o}C. The amplified PCR products of approximately 1.49 kb which was the expected size of the *OsCatA*, *OsCatB* and *OsCatC* genes was obtained. For *OsCatA* gene, *OsCatB* and *OsCatC,* the optimal annealing temperature was 66.5^{o}C in which non-specific products were minimally amplified as shown in Figure 3.2, Figure 3.3 and Figure 3.4, respectively.

The amplified *OsCat* gene fragments were extended by adding dA at the 3′ end using *Taq* DNA polymerase and were purified from agarose gel using the Geneaid gel extraction kit. The purified *OsCatA, OsCatB* and *OsCatC* fragments were then ligated into pTZ57R/T vector by T4 DNA ligase. After competent *E. coli* strain XL1Blue cells were transformed with the ligation mixtures, the transformants were selected by blue/white colony screening on ampicillin agar plates containing X-gal and IPTG. White colonies were randomly picked and cultured in LB broth containing 100 µg/ml of ampicillin at 37 °C overnight and the cultures were subjected to plasmid extraction. To confirm insertion of the PCR product into the vector by restriction analysis, potential recombinant plasmids were digested with *Nde*I and *Eco*RI. Then, they were incubated at 37 °C overnight and analyzed by 1.0% agarose gel electrophoresis. The results showed that, in addition of the pTZ57R/T fragment of 2.9 kb, DNA fragments of approximately 1.49 kb in length were obtained as expected for the restriction patterns of the recombinant plasmids harboring *OsCatA*, *OsCatB* and *OsCatC* as shown in Figures 3.5 (lane 3, lane 4 and lane 5, respectively).To clone the *OsCat* genes into pET21a(+) expression vector, the recombinant pTZ57R/T plasmids containing *OsCat* genes were extracted and digested

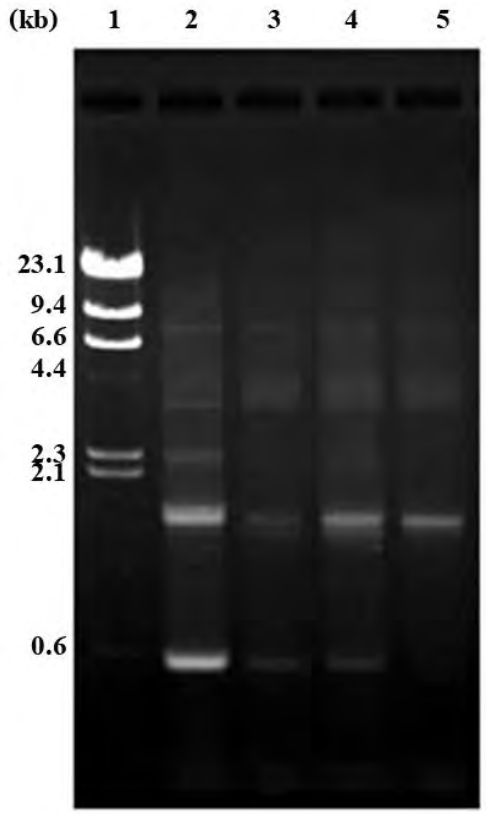


Figure 3.2 Agarose gel electrophoresis of the amplified fragment of the *OsCatA* gene at various annealing temperatures. The PCR product was separated on a 1.0 % agarose gel and visualized by ethidium bromide staining.

Lane 1 = λ-*Hind*III standard DNA marker

Lane 2 = PCR product of *OsCatA* gene using annealing temperature at 57.0 ^{o}C

Lane 3 = PCR product of *OsCatA* gene using annealing temperature at 60.1 ^{o}C

Lane 4 = PCR product of *OsCatA* gene using annealing temperature at 63.6 ^{o}C

Lane 5 = PCR product of *OsCatA* gene using annealing temperature at 66.5 ^{o}C

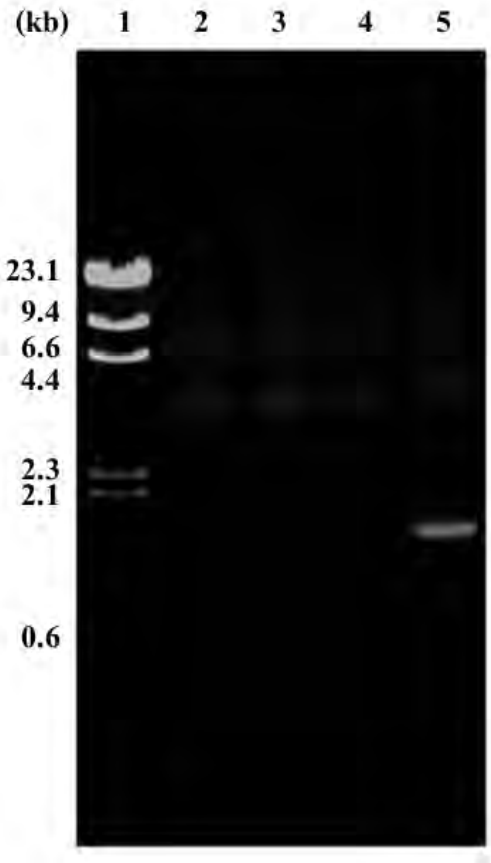


Figure 3.3 Agarose gel electrophoresis of the amplified fragment of the *OsCatB* gene at various annealing temperatures. The PCR product was separated on a 1.0 % agarose gel and visualized by ethidium bromide staining.

Lane 1 = λ-*Hind*III standard DNA marker

Lane 2 = PCR product of *OsCatB* gene using annealing temperature at 57.0 ^{o}C

Lane 3 = PCR product of *OsCatB* gene using annealing temperature at 60.1 ^{o}C

Lane 4 = PCR product of *OsCatB* gene using annealing temperature at 63.6 ^{o}C

Lane 5 = PCR product of *OsCatB* gene using annealing temperature at 66.5 ^{o}C

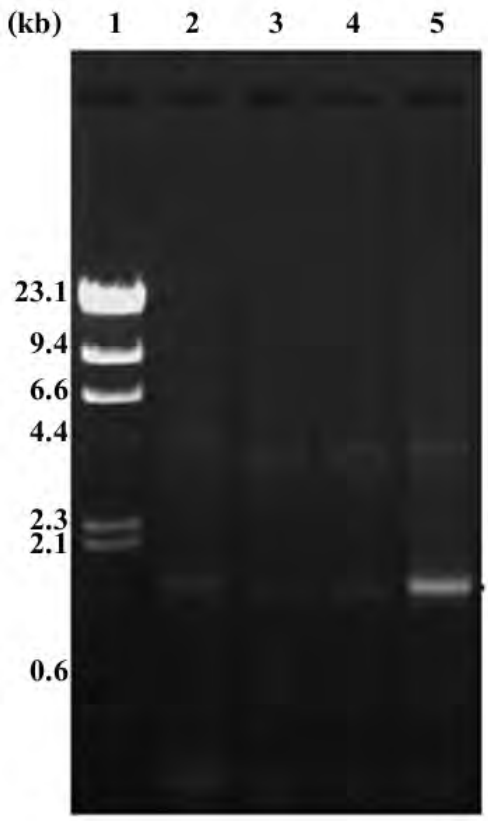


Figure 3.4 Agarose gel electrophoresis of the amplified fragment of the *OsCatC* gene at various annealing temperatures. The PCR product was separated on a 1.0 % agarose gel and visualized by ethidium bromide staining.

Lane 1 = λ-*Hind*III standard DNA marker

Lane 2 = PCR product of *OsCatC* gene using annealing temperature at 57.0 ^{o}C

Lane 3 = PCR product of *OsCatC* gene using annealing temperature at 60.1 ^{o}C

Lane 4 = PCR product of *OsCatC* gene using annealing temperature at 63.6 ^{o}C

Lane 5 = PCR product of *OsCatC* gene using annealing temperature at 66.5 ^{o}C

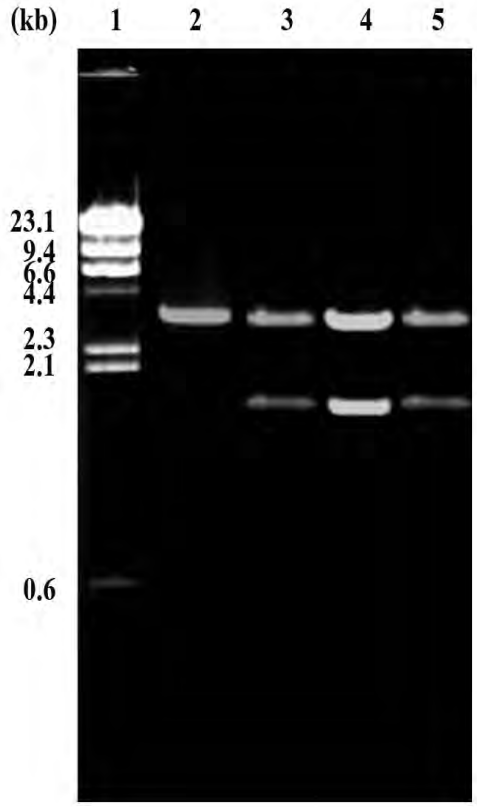


Figure 3.5 Analysis of the *OsCat* gene inserts in pTZ57R/T by digestion with *Nde*I and *Eco*RI restriction enzymes and separation in 1% agarose gel electrophoresis.

Lane 1 = λ-*Hind*III standard DNA marker

Lane 2 = pTZ57R/T

Lane 3 = *Nde*I-*Eco*RI digested *OsCatA*/pTZ57R/T

Lane 4 = *Nde*I-*Eco*RI digested *OsCatB*/pTZ57R/T

Lane 5 = *Nde*I-*Eco*RI digested *OsCatC*/pTZ57R/T

with *Nde*I and *Eco*RI. The *Nde*I and *Eco*RI-digested *OsCat* gene fragments were then ligated into the same restriction sites in the pET21a(+) vector. The ligation products were introduced into *E. coli* strain XL1-Blue. The transformants were selected by ampicillin resistance on LB agar. *E. coli* XL1-Blue single colonies were randomly picked for plasmid extraction and the plasmids were digested with *Nde*I and *Eco*RI. After digestion, a linear pET21a(+) of 5.4 kb and the inserted *OsCat* gene fragments: *OsCatA*, *OsCatB* and *OsCatC* were obtained as shown in Figures 3.6 (lanes 3, lane 4 and lane 5, respectively). The resulting recombinant plasmids are called pET21a(+)/*OsCatA*, pET21a(+)/*OsCatB* and pET21(+)/*OsCatC*, accordingly.

The *OsCat* gene fragments were inserted into pET21a(+) at the 5'-*Nde*I and 3'-*Eco*RI sites in frame with the C-terminal His6 tag. To confirm the nucleotide sequences of the *OsCat* genes inserted into pET21a(+), the recombinant plasmids were subjected to DNA sequencing using T7 promoter and T7 terminator primers. The results show that sequences of the *OsCatA*, *OsCatB* and *OsCatC* genes in the recombinant plasmids share 100 % identity with their respective cDNA sequences as shown in Figure 3.7, 3.8 and 3.9, respectively. Direction of the *OsCat* genes inserted into pET21a(+) is shown in Figure 3.10. The molecular weights of the recombinant OsCATA, OsCATB and OsCATC calculated from their deduced amino acid sequences are 58.8, 58.7 and 58.9 kDa, respectively.

3.3 Expression of the recombinant *OsCat* genes

The recombinant proteins were then expressed in an *Escherichia coli* strain Rosetta-gami for *OsCatA*, *OsCatB* and *OsCatC* genes. The *E. coli* transformants containing each of the three *OsCat* genes were grown and induced by IPTG at a final

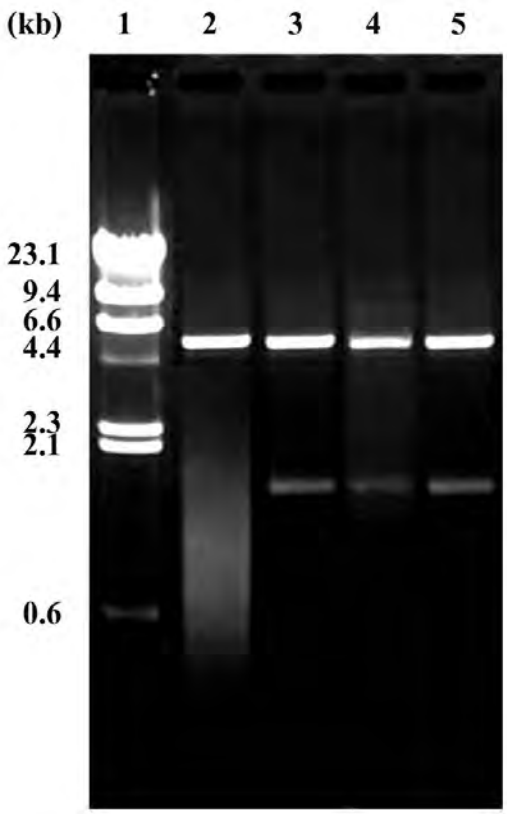


Figure 3.6 Analysis of the *OsCat* genes inserts in pET21a(+) by digestion with *Nde*I and *Eco*RI restriction enzymes and separation in 1% agarose gel electrophoresis.

Lane 1 = λ-*Hind*III standard DNA marker

Lane 2 = *Nde*I-*Eco*RI digested pET21a(+)

Lane 3 = *Nde*I-*Eco*RI digested *OsCatA*/pET21a(+)

Lane 4 = *Nde*I-*Eco*RI digested *OsCatB*/ pET21a(+)

Lane 5 = *Nde*I-*Eco*RI digested *OsCatC*/ pET21a(+)

```
ATGGATCCTTGCAAGTTCCGGCCGTCGAGCTCGTTCGACACGAAGACGACGACGACGAAC      60
M  D  P  C  K  F  R  P  S  S  S  F  D  T  K  T  T  T  T  N       20

GCGGGAGCTCCGGTGTGGAACGACAACGAGGCGCTGACAGTGGGGCCCAGGGGGCCGATC     120
A  G  A  P  V  W  N  D  N  E  A  L  T  V  G  P  R  G  P  I       40

CTCCTCGAGGACTACCACCTGATCGAGAAGGTGGCGCACTTCGCCCGGGAGCGCATCCCG     180
L  L  E  D  Y  H  L  I  E  K  V  A  H  F  A  R  E  R  I  P       60

GAGCGCGTGGTCCACGCCCGCGGCGCCTCCGCCAAGGGCTTCTTCGAGTGCACCCACGAC     240
E  R  V  V  H  A  R  G  A  S  A  K  G  F  F  E  C  T  H  D       80

GTCACCGACATCACCTGCGCCGACTTCCTCCGGTCCCCGGGCGCCCAGACCCCCGTCATC     300
V  T  D  I  T  C  A  D  F  L  R  S  P  G  A  Q  T  P  V  I      100

GTCCGCTTCTCCACCGTCATCCACGAGCGCGGCAGCCCGGAGACGATCCGCGACCCGCGC     360
V  R  F  S  T  V  I  H  E  R  G  S  P  E  T  I  R  D  P  R      120

GGGTTCGCCGTCAAGTTCTACACCCGCGAGGGCAACTGGGACCTCCTCGGCAACAACTTC     420
G  F  A  V  K  F  Y  T  R  E  G  N  W  D  L  L  G  N  N  F      140

CCCGTCTTCTTCATCCGCGACGGCATCAAGTTCCCCGACGTCATCCACGCCTTCAAGCCC     480
P  V  F  F  I  R  D  G  I  K  F  P  D  V  I  H  A  F  K  P      160

AACCCGCGCTCCCATGTCCAGGAGTACTGGAGGGTCTTCGACTTCTTGTCCCACCACCCC     540
N  P  R  S  H  V  Q  E  Y  W  R  V  F  D  F  L  S  H  H  P      180

GAGAGCCTCCACACCTTCTTCTTCCTCTTCGACGACGTCGGCATCCCCACCGATTACCGC     600
E  S  L  H  T  F  F  F  L  F  D  D  V  G  I  P  T  D  Y  R      200

CACATGGACGGCTTCGGCGTCAACACCTACACCTTCGTCACCCGCGACGCCAAGGCCAGG     660
H  M  D  G  F  G  V  N  T  Y  T  F  V  T  R  D  A  K  A  R      220

TACGTCAAGTTCCACTGGAAGCCCACCTGCGGCGTCAGCTGCTTGATGGACGACGAGGCC     720
Y  V  K  F  H  W  K  P  T  C  G  V  S  C  L  M  D  D  E  A      240

ACGCTCGTCGGCGGCAAGAACCACAGCCACGCCACCCAGGACCTCTACGACTCCATCGCC     780
T  L  V  G  G  K  N  H  S  H  A  T  Q  D  L  Y  D  S  I  A      260

GCCGGCAACTTCCCCGAGTGGAAGCTGTTCGTCCAGGTGATCGACCCGGAGGAGGAGGAG     840
A  G  N  F  P  E  W  K  L  F  V  Q  V  I  D  P  E  E  E  E      280

AGGTTCGACTTCGACCCGCTGGATGACACCAAGACATGGCCGGAGGACGAGGTGCCGCTC     900
R  F  D  F  D  P  L  D  D  T  K  T  W  P  E  D  E  V  P  L      300

CGGCCCGTGGGGCGCCTCGTTCTCAACCGCAACGTCGACAACTTCTTCAACGAGAACGAG     960
R  P  V  G  R  L  V  L  N  R  N  V  D  N  F  F  N  E  N  E      320

CAGCTGGCGTTCGGGCCGGGGCTGGTGGTGCCGGGGATCTACTACTCCGACGACAAGATG    1020

Q  L  A  F  G  P  G  L  V  V  P  G  I  Y  Y  S  D  D  K  M      340

CTGCAGTGCAGGGTGTTCGCGTACGCCGACACGCAGCGCTACAGGCTGGGGCCAAACTAC    1080
L  Q  C  R  V  F  A  Y  A  D  T  Q  R  Y  R  L  G  P  N  Y      360
```

```
CTGATGCTGCCGGTGAACGCGCCCAAGTGCGCCCACCACAACAACCACTACGACGGCGCC      1140
L  M  L  P  V  N  A  P  K  C  A  H  H  N  N  H  Y  D  G  A       380

ATGAACTTCATGCACCGGGACGAGGAGGTGGACTACTACCCATCGCGCCACGCGCCGCTC      1200
M  N  F  M  H  R  D  E  E  V  D  Y  Y  P  S  R  H  A  P  L       400

CGCCACGCGCCGCCGACGCCCATCACGCCGCGCCCCGTGGTGGGGAGGAGGCAGAAGGCG      1260
R  H  A  P  P  T  P  I  T  P  R  P  V  V  G  R  R  Q  K  A       420

ACGATACACAAGCAGAACGACTTCAAGCAGCCCGGGGAGAGGTACAGGTCGTGGGCGCCG      1320
T  I  H  K  Q  N  D  F  K  Q  P  G  E  R  Y  R  S  W  A  P       440

GATAGACAGGAGAGGTTCATCCGCCGCTTCGCCGGCGAGCTCGCGCACCCCAAGGTCTCC      1380
D  R  Q  E  R  F  I  R  R  F  A  G  E  L  A  H  P  K  V  S       460

CCTGAGCTCCGCGCCATCTGGGTCAACTACCTCTCCCAGTGTGATGAGTCGTTGGGGGTG      1440
P  E  L  R  A  I  W  V  N  Y  L  S  Q  C  D  E  S  L  G  V       480

AAGATTGCGAATAGGCTCAACGTGAAGCCAAGCATGAATTCGAGCTCCGTCGACAAGCTT      1500
K  I  A  N  R  L  N  V  K  P  S  M  N  S  S  S  V  D  K  L       500

GCGGCCGCACTCGAGCACCACCACCACCACCACTGA       1540
A  A  A  L  E  H  H  H  H  H  H  *         512
```

Figure 3.7 Nucleotide and deduced amino acid sequences of *OsCatA* gene. Numbers to the right refer to the catalase nucleotide sequence and its deduced amino acid residues. The sequences were aligned by the program ClustalW. The underlined amino acid sequence is a His_6 tag and the stop codon is marked with an asterisk (*).

```
ATGGATCCCTACAAGCATCGGCCGTCCAGCGGGAGCAATTCCACCTTCTGGACCACCAAC      60
M  D  P  Y  K  H  R  P  S  S  G  S  N  S  T  F  W  T  T  N       20

TCCGGCGCCCCCGTCTGGAACAACAACTCCGCCCTCACCGTCGGAGAGCGAGGCCCTATC      120
S  G  A  P  V  W  N  N  N  S  A  L  T  V  G  E  R  G  P  I       40

CTCCTTGAGGACTATCATCTGATTGAAAAGCTTGCACAGTTTGACAGGGAGCGTATCCCT      180
L  L  E  D  Y  H  L  I  E  K  L  A  Q  F  D  R  E  R  I  P       60

GAACGTGTCGTTCATGCAAGGGGAGCCAGTGCCAAGGGATTTTTTGAGGTTACTCATGAT      240
E  R  V  V  H  A  R  G  A  S  A  K  G  F  F  E  V  T  H  D       80

ATTTCTCACCTCACATGTGCTGATTTTCTCCGTGCTCCTGGTGTTCAGACCCCAGTTATT      300
I  S  H  L  T  C  A  D  F  L  R  A  P  G  V  Q  T  P  V  I      100

GTTCGGTTCTCCACAGTCGTGCATGAGCGTGGAAGCCCTGAGACATTGAGGGATCCACGT      360
V  R  F  S  T  V  V  H  E  R  G  S  P  E  T  L  R  D  P  R      120

GGTTTTGCTGTCAAGTTTTACACTAGAGAGGGTAATTTTGATCTTGTTGGGAACAATATG      420
G  F  A  V  K  F  Y  T  R  E  G  N  F  D  L  V  G  N  N  M      140

CCTGTCTTTTTTATCCGAGATGGGATGAAATTCCCTGACATGGTCCATGCTTTCAAGCCA      480
P  V  F  F  I  R  D  G  M  K  F  P  D  M  V  H  A  F  K  P      160

AGTCCAAAGACCAATATGCAGGAGAACTGGAGAATAGTTGATTTCTTTTCACACCACCCA      540
S  P  K  T  N  M  Q  E  N  W  R  I  V  D  F  F  S  H  H  P      180

GAGAGCCTGCACATGTTCTCCTTCCTCTTTGACGATGTAGGCATCCCACTCAACTACAGG      600
E  S  L  H  M  F  S  F  L  F  D  D  V  G  I  P  L  N  Y  R      200

CACATGGAGGGTTTTGGTGTCAACACCTACACCCTAATCAATAAGGATGGAAAGCCTCAC      660
H  M  E  G  F  G  V  N  T  Y  T  L  I  N  K  D  G  K  P  H      220

CTTGTCAAATTCCACTGGAAGCCTACCTGTGGTGTCAAATGCCTGTTGGATGATGAAGCT      720
L  V  K  F  H  W  K  P  T  C  G  V  K  C  L  L  D  D  E  A      240

GTGACTGTTGGCGGCACCTGCCACAGCCATGCCACGAAGGACTTGACTGATTCTATTGCA      780
V  T  V  G  G  T  C  H  S  H  A  T  K  D  L  T  D  S  I  A      260

GCAGGGAATTACCCAGAGTGGAAGCTTTACATCCAGACTATTGATCCTGATCATGAGGAC      840
A  G  N  Y  P  E  W  K  L  Y  I  Q  T  I  D  P  D  H  E  D      280

AGATTTGACTTCGATCCTCTTGATGTCACCAAGACATGGCCAGAGGATATCATCCCCCTG      900
R  F  D  F  D  P  L  D  V  T  K  T  W  P  E  D  I  I  P  L      300

CAGCCAGTTGGACGGATGGTCCTGAACAAAAACATTGATAACTTCTTTGCAGAAAATGAA      960
Q  P  V  G  R  M  V  L  N  K  N  I  D  N  F  F  A  E  N  E      320

CAGCTTGCTTTCTGCCCAGCGATAATTGTCCCTGGAATCCATTACTCTGATGATAAGCTG     1020
Q  L  A  F  C  P  A  I  I  V  P  G  I  H  Y  S  D  D  K  L      340

CTCCAGACAAGAATTTTCTCCTATGCTGATACCCAAAGGCACCGTCTTGGCCCAAACTAT     1080
L  Q  T  R  I  F  S  Y  A  D  T  Q  R  H  R  L  G  P  N  Y      360
```

```
TTGATGCTTCCTGTGAATGCACCAAAATGTGCATACCACAACAACCACCACGATGGCTCC      1140
L  M  L  P  V  N  A  P  K  C  A  Y  H  N  N  H  H  D  G  S       380

ATGAATTTCATGCACAGGGATGAAGAGGTTAACTACTTCCCTTCAAGGTTTGATGCTGCA      1200
M  N  F  M  H  R  D  E  E  V  N  Y  F  P  S  R  F  D  A  A       400

CGTCATGCTGAGAAGGTCCCTATTCCTCCTCGTGTTCTAACAGGCTGTCGGGAAAAGTGT      1260
R  H  A  E  K  V  P  I  P  P  R  V  L  T  G  C  R  E  K  C       420

GTCATTGACAAGGAGAACAATTTCCAACAGGCTGGTGAGAGATACCGGTCATTTGACCCT      1320
V  I  D  K  E  N  N  F  Q  Q  A  G  E  R  Y  R  S  F  D  P       440

GCCAGGCAAGATCGTTTTCTCCAGCGGTGGGTTGATGCTCTCTCAGATCCTCGTATTACA      1380
A  R  Q  D  R  F  L  Q  R  W  V  D  A  L  S  D  P  R  I  T       480

CATGAACTCCGTGGCATCTGGATCTCCTACTGGTCGCAGTGTGATGCGTCCCTTGGGCAG      1440
H  E  L  R  G  I  W  I  S  Y  W  S  Q  C  D  A  S  L  G  Q       500

AAGCTGGCTTCACGTCTCAACCTGAAACCAAACATGAATTCGAGCTCCGTCGACAAGCTT      1500
K  L  A  S  R  L  N  L  K  P  N  M  N  S  S  S  V  D  K  L       520

GCGGCCGCACTCGAGCACCACCACCACCACCACTGA                              1540
A  A  A  L  E  H  H  H  H  H  H  *                               512
```

Figure 3.8 Nucleotide and deduced amino acid sequences of *OsCatB* gene. Numbers to the right refer to the catalase nucleotide sequence and its deduced amino acid residues. The sequences were aligned by the program ClustalW. The underlined amino acid sequence is a His_6 tag and the stop codon is marked with an asterisk (*).

```
ATGGATCCCTACAAGCACCGCCCGTCGAGCTCGTTCAACGGCCCGCTGTGGAGCACCAAC      60
M  D  P  Y  K  H  R  P  S  S  S  F  N  G  P  L  W  S  T  N        20

TCCGGCGCCCCCCGTATGGAACAACAACAACTCGCTCACCGTCGGCTCCCGAGGCCCGATC      120
S  G  A  P  V  W  N  N  N  N  S  L  T  V  G  S  R  G  P  I        40

CTTCTGGAGGACTACCACCTGGTTGAGAAGCTGGCCAACTTCGACAGGGAGCGTATCCCG      180
L  L  E  D  Y  H  L  V  E  K  L  A  N  F  D  R  E  R  I  P        60

GAGCGCGTGGTGCACGCCCGCGGCGCCAGCGCCAAGGGCTTCTTCGAGGTCACCCACGAC      240
E  R  V  V  H  A  R  G  A  S  A  K  G  F  F  E  V  T  H  D        80

ATCACCCACCTCACCTGCGCCGACTTCCTCCGCGCCCCGGGCGTCCAGACCCCGGTCATC      280
I  T  H  L  T  C  A  D  F  L  R  A  P  G  V  Q  T  P  V  I        100

GTCCGCTTCTCCACCGTCATCCACGAGCGCGGCAGCCCGGAGACCCTCCGCGACCCGCGT      360
V  R  F  S  T  V  I  H  E  R  G  S  P  E  T  L  R  D  P  R        120

GGCTTCGCCATCAAGTTCTACACCCGGGAGGGCAACTGGGACCTCGTCGGCAACAACTTC      420
G  F  A  I  K  F  Y  T  R  E  G  N  W  D  L  V  G  N  N  F        140

CCCGTCTTCTTCATCCGCGACGGCATGAAGTTCCCGGACATGGTGCACTCGCTCAAGCCC      480
P  V  F  F  I  R  D  G  M  K  F  P  D  M  V  H  S  L  K  P        160

AACCCCAAGTCGCACGTCCAGGAGAACTGGCGCATCCTCGACTTCTTCTCCCACCACCCG      540
N  P  K  S  H  V  Q  E  N  W  R  I  L  D  F  F  S  H  H  P        180

GAGAGCCTCCACATGTTCACCTTCCTCTTCGATGACATCGGCATCCCCGCCGACTACCGC      600
E  S  L  H  M  F  T  F  L  F  D  D  I  G  I  P  A  D  Y  R        200

CACATGGACGGCTCCGGCGTCAACACCTACACGCTCGTCAACCGCGCCGGCAAGTCGCAC      660
H  M  D  G  S  G  V  N  T  Y  T  L  V  N  R  A  G  K  S  H        220

TACGTCAAGTTCCACTGGAAGCCCACCTGCGGCGTCAAGTCGCTGCTCGACGACGAGGCC      720
Y  V  K  F  H  W  K  P  T  C  G  V  K  S  L  L  D  D  E  A        240

GTCACCGTCGGCGGGACCAACCACAGCCACGCCACGCAGGACCTCTACGACTCCATCGCC      780
V  T  V  G  G  T  N  H  S  H  A  T  Q  D  L  Y  D  S  I  A        260

GCCGGCAACTTCCCGGAGTGGAAGCTGTTCATCCAGACCATCGACCCCGACCACGAGGAC      840
A  G  N  F  P  E  W  K  L  F  I  Q  T  I  D  P  D  H  E  D        280

CGCTTCGACTTCGACCCGCTCGACGTCACCAAGACGTGGCCCGAGGACATCGTCCCGCTG      900
R  F  D  F  D  P  L  D  V  T  K  T  W  P  E  D  I  V  P  L        300

CAGCCCGTGGGGAGGATGGTGCTCAACCGCAACATCGACAACTTCTTCTCGGAGAACGAG      960
Q  P  V  G  R  M  V  L  N  R  N  I  D  N  F  F  S  E  N  E        320

CAGCTGGCGTTCTGCCCCGGGATCATCGTGCCGGGGATCTACTACTCCGACGACAAGCTG      1020
Q  L  A  F  C  P  G  I  I  V  P  G  I  Y  Y  S  D  D  K  L        340

CTGCAGACGAGGATCTTCTCCTACTCCGACACGCAGCGCCACCGCCTCGGACCAAACTAC      1080
L  Q  T  R  I  F  S  Y  S  D  T  Q  R  H  R  L  G  P  N  Y        360

CTGCTGCTCCCGCCCAACGCGCCCAAGTGCGCCCACCACAACAACCACTACGACGGCTTC      1140
L  L  L  P  P  N  A  P  K  C  A  H  H  N  N  H  Y  D  G  F        380
```

```
ATGAACTTCATGCACCGCGACGAGGAGGTCGACTACTTCCCATCCCGCTACGATCCTGCC      1200
M  N  F  M  H  R  D  E  E  V  D  Y  F  P  S  R  Y  D  P  A       400

AAGCACGCCCCCCGCTACCCCATCCCCTCCGCCACCCTCACCGGCCGCCGCGAGAAGGTG      1260
K  H  A  P  R  Y  P  I  P  S  A  T  L  T  G  R  R  E  K  V       420

GTGATTGCCAAGGAGAACAACTTCAAGCAGCCAGGGGAGAGGTACCGTTCATGGGATCCG      1320
V  I  A  K  E  N  N  F  K  Q  P  G  E  R  Y  R  S  W  D  P       440

GCAAGGCAAGACCGGTTCATCAAGAGATGGATCGACGCACTCTCTGACCCTCGCCTCACC      1380
A  R  Q  D  R  F  I  K  R  W  I  D  A  L  S  D  P  R  L  T       460

CACGAGATCAGGAGCATCTGGCTCTCCTACTGGTCTCAGGCTGACAGGTCTCTGGGTCAG      1440
H  E  I  R  S  I  W  L  S  Y  W  S  Q  A  D  R  S  L  G  Q       480

AAACTGGCGAGCCGTCTCAGCGCGAAGCCGAGCATGAATTCGAGCTCCGTCGACAAGCTT      1500
K  L  A  S  R  L  S  A  K  P  S  M  N  S  S  S  V  D  K  L       500

GCGGCCGCACTCGAGCACCACCACCACCACCACTGA       1540
A  A  A  L  E  H  H  H  H  H  H  *        512
```

Figure 3.9 Nucleotide and deduced amino acid sequences of *OsCatC* gene. Numbers to the right refer to the catalase nucleotide sequence and its deduced amino acid residues. The sequences were aligned by the program ClustalW. The underlined amino acid sequence is a His_6 tag and the stop codon is marked with an asterisk (*).

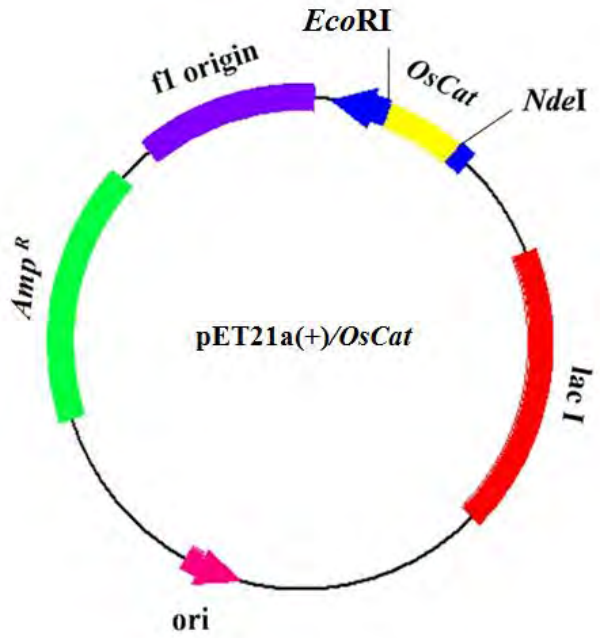


Figure 3.10 Direction of the *OsCat* gene inserted into pET21a(+)

concentration of 0, 0.2, 0.4, 0.6, 0.8 or 1 mM for 3 hours before cells were harvested as described in 2.4.2.

Protein concentration of the crude extracts for each recombinant protein was determined by Bradford method as described in 2.4.3 and 20 μg proteins from the crude extracts were subjected to electrophoresis on 12 % SDS-polyacrylamide gel. The results in Figure 3.11-3.16 show protein expression patterns of the recombinant *E. coli* cells harboring *OsCatA*, *OsCatB* and *OsCatC* genes, respectively. The expression of OsCATA, OsCATB and OsCATC could not be detected in the soluble protein fraction (Figure 3.11, Figure 3.13 and Figure 3.15, respectively) but was detected in the insoluble fraction (Figure 3.12, Figure 3.14 and Figure 3.16, respectively) suggesting that each recombinant protein was aggregated as insoluble inclusion bodies in the cytoplasm. The protein band showed approximately of 58.8, 58.7 and 58.9 kDa after 0.4, 0.2 and 0.2 mM IPTG induction, respectively. Its levels were similar in all IPTG concentrations.

3.4 Purification and characterization of the recombinant OsCAT proteins

3.4.1 Preparation of OsCAT proteins from inclusion bodies

As shown in Figure 3.12, Figure 3.14 and Figure 3.16 that OsCATs from the recombinant cells were mostly expressed in the insoluble fraction as inclusion bodies, this fraction was subjected to protein purification.

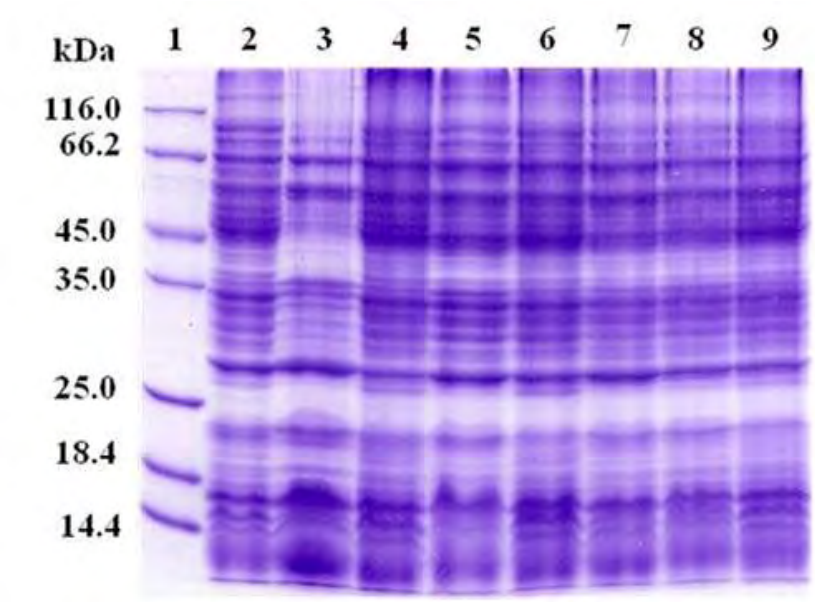


Figure 3.11 The expression pattern of OsCATA in soluble protein fraction of *E. coli* Rosetta-gami under various final concentrations of IPTG. Protein molecular mass marker (lane 1), soluble protein fraction of *E. coli* harboring pET21a(+) without or with 0.4 mM IPTG (lane 2 and 3, respectively), soluble protein fraction of *E. coli* harboring pET21a(+)/*OsCatA* at varying IPTG final concentration of 0, 0.2, 0.4, 0.6, 0.8 and 1 mM (lane 4 – 9, respectively).

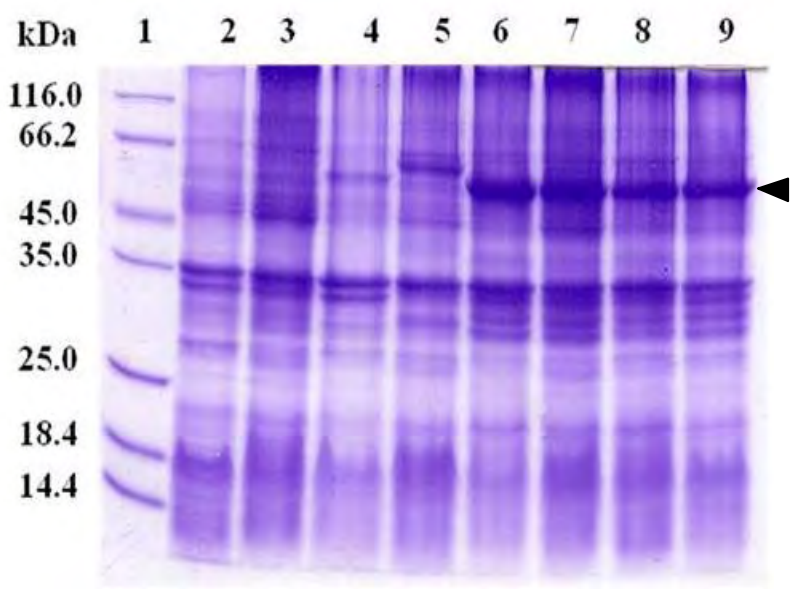


Figure 3.12 The expression pattern of OsCATA in insoluble protein fraction of *E. coli* Rosetta-gami under various final concentrations of IPTG. Protein molecular mass marker (lane 1), insoluble protein fraction of *E. coli* harboring pET21a(+) without or with 0.4 mM IPTG (lane 2 and 3, respectively), insoluble protein fraction of *E. coli* harboring pET21a(+)/*OsCatA* at varying IPTG final concentration of 0, 0.2, 0.4, 0.6, 0.8 and 1 mM (lane 4 – 9, respectively).

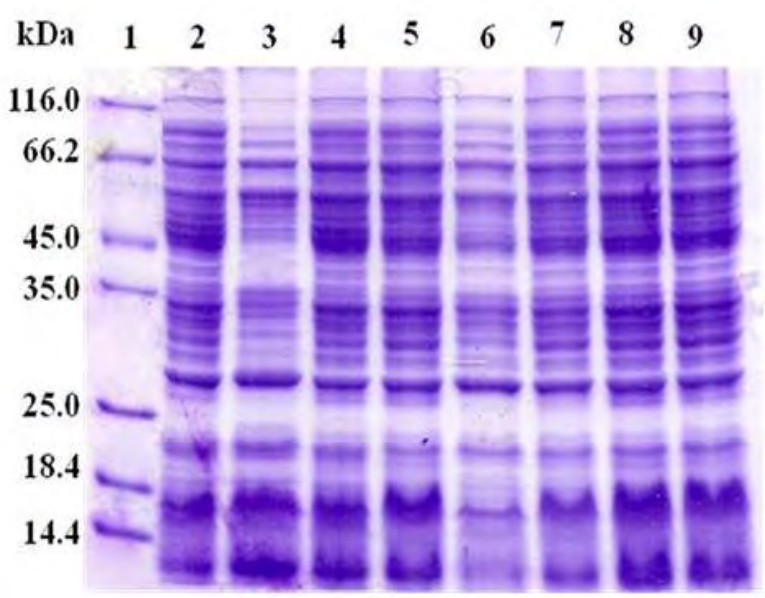


Figure 3.13 The expression pattern of OsCATB in soluble protein fraction of *E. coli* Rosetta-gami under various final concentrations of IPTG. Protein molecular mass marker (lane 1), soluble protein fraction of *E. coli* harboring pET21a(+) without or with 0.4 mM IPTG (lane 2 and 3, respectively), soluble protein fraction of *E. coli* harboring pET21a(+)/*OsCatB* at varying IPTG final concentration of 0, 0.2, 0.4, 0.6, 0.8 and 1 mM (lane 4 – 9, respectively).

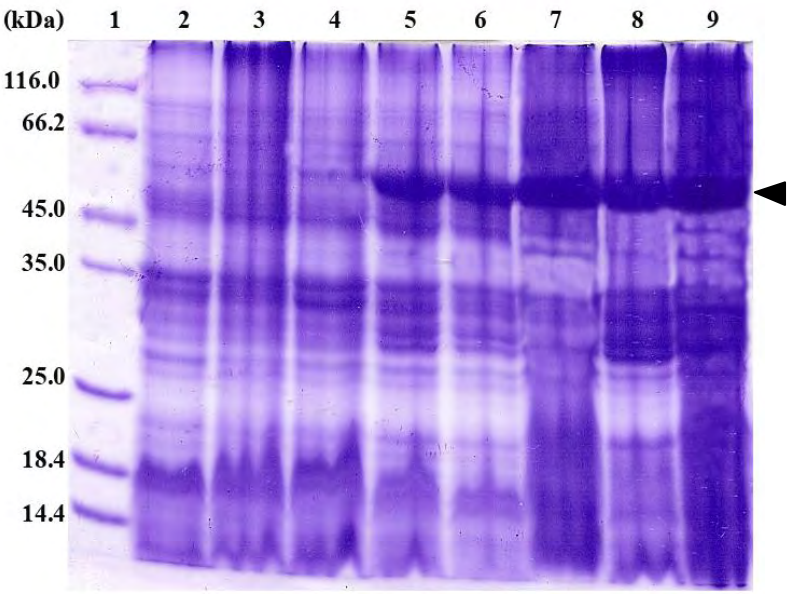


Figure 3.14 The expression pattern of OsCATB in insoluble protein fraction of *E. coli* Rosetta-gami under various final concentrations of IPTG. Protein molecular mass marker (lane 1), insoluble protein fraction of *E. coli* harboring pET21a(+) without or with 0.4 mM IPTG (lane 2 and 3, respectively), insoluble protein fraction of *E. coli* harboring pET21a(+)/*OsCatB* at varying IPTG final concentration of 0, 0.2, 0.4, 0.6, 0.8 and 1 mM (lane 4 – 9, respectively).

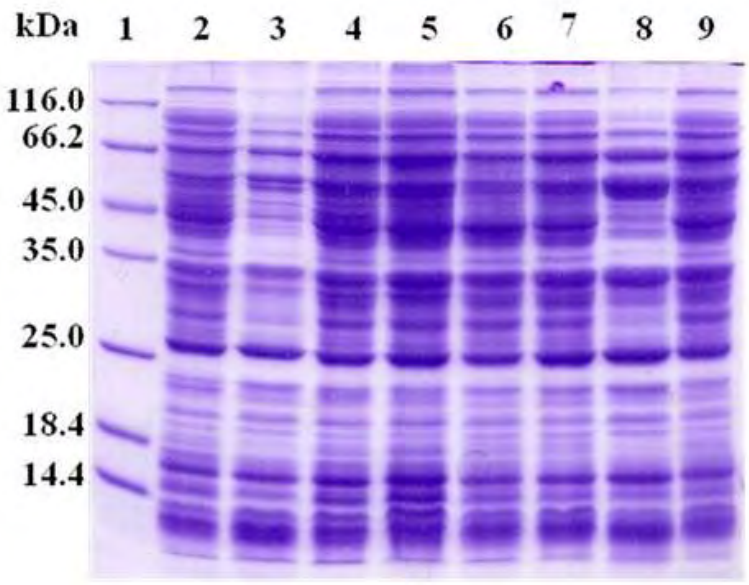


Figure 3.15 The expression pattern of OsCATC in soluble protein fraction of *E. coli* Rosetta-gami under various final concentrations of IPTG. Protein molecular mass marker (lane 1), soluble protein fraction of *E. coli* harboring pET21a(+) without or with 0.4 mM IPTG (lane 2 and 3, respectively), soluble protein fraction of *E. coli* harboring pET21a(+)/*OsCatC* at varying IPTG final concentration of 0, 0.2, 0.4, 0.6, 0.8 and 1 mM (lane 4 – 9, respectively).

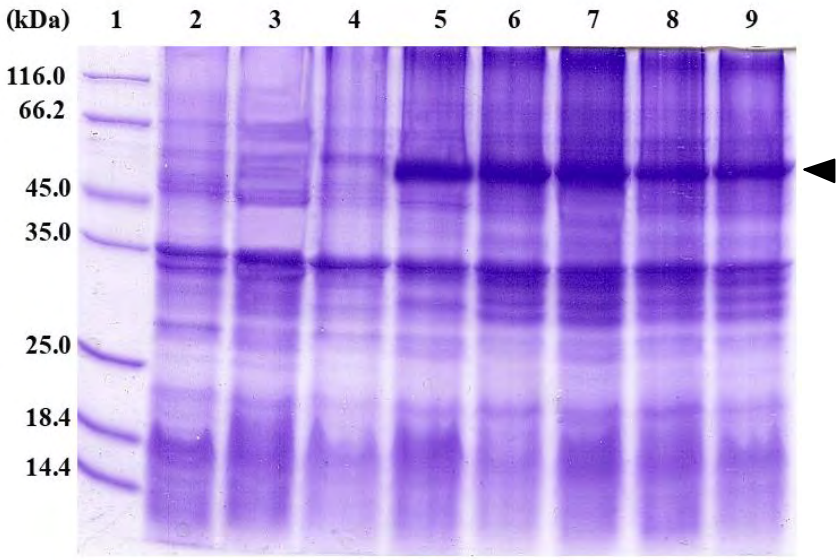


Figure 3.16 The expression pattern of OsCATC in insoluble protein fraction of *E. coli* Rosetta-gami under various final concentrations of IPTG. Protein molecular mass marker (lane 1), insoluble protein fraction of *E. coli* harboring pET21a(+) without or with 0.4 mM IPTG (lane 2 and 3, respectively), insoluble protein fraction of *E. coli* harboring pET21a(+)/*OsCatC* at vary IPTG final concentration of 0, 0.2, 0.4, 0.6, 0.8 and 1 mM (lane 4 – 9, respectively).

3.4.2 Solubilization and refolding of OsCAT from inclusion bodies

The inclusion bodies from crude protein extracts were solubilized and refolded to obtain protein activity. Solubilization of the inclusion bodies was achieved using 20 mM sodium phosphate buffer (pH 12.0) for 12 hours at 4^{o}C with shaking as described in 2.5.3. The remaining precipitates were removed by centrifugation at 13,000 xg for 10 minutes. Refolding was achieved and subsequently exthaustive dialysis in 20 mM sodium phosphate buffer, pH 7.4, 1 mM EDTA and 1 mM DTT. The cloudy, unrefolded protein was removed by centrifugation. SDS-PAGE analysis of proteins in the crude precipitates, solubilized and refolded preparations revealed the presence of a major protein band with the estimated molecular mass of about 58.8, 58.7 and 58.9 kDa, respectively. The final yield of the refolded OsCATA, OsCATB and OsCATC proteins were estimated as 20.1, 5.3 and 14.8 mg from the total volume of 10 ml.

3.4.3 Purification of the refolded OsCAT proteins by Ni-Sepharose column

The recombinant proteins produced were expected to contain a His_6 tag, which consists of six consecutive histidine residues, at their carboxyl terminal end, therefore a Ni-Sepharose was used to purify the proteins based on the principle that poly-His-fusion proteins bind to nickel ions.

All recombinant OsCAT proteins could be purified by Ni-Sepharose affinity chromatography. The flow through was collected by a gravity flow and the column was washed with the binding buffer containing 30 mM imidazole to remove unbound proteins. After washing, the OsCATA and OsCATC were eluted with the

elution buffer containing 200 mM imidazole while the OsCATB was eluted with elution buffer containing 100 mM imidazole.

Proteins obtained from each step of purification were analyzed for the identity and purity by 12% SDS-PAGE. The molecular weights of OsCATA, OsCATB and OsCATC were estimated to be 58.8 kDa (Figure 3.17), 58.7 kDa (Figure 3.18) and 58.9 kDa (Figure 3.19), respectively, by its mobility in SDS-PAGE when compared with those of standard proteins.

3.4.4 Western blot detection of the His-tagged protein

To verify the presence of a His_6 tag within the recombinant OsCAT proteins, western blot analysis using an anti-His_6 tag antibody was conducted. After proteins of interest were separated by SDS-PAGE and transferred from the gel to the PVDF membrane, detection of His_6-tagged proteins was carried out with the Anti·His primary antibody (anti-His antibody: mouse anti-His Ab from mouse ascites fluid; product code 27-4710-01, Amersham Biosciences) with the dilution of 1:3000, followed by the secondary antibody, alkaline phosphatase (AP)-conjugated rabbit anti-mouse IgG (Jackson ImmunoResearch) with the dilution of 1:2500. The result was visualized by assaying the alkaline phosphatase activity using NBT/BCIP (Figure 3.20).

Data describing the purification of the recombinant proteins OsCATs from the insoluble protein fraction were obtained as presented in Table 3.1, Table 3.2 and Table 3.3, respectively. Overall, through the Ni-Sepharose high performance column, a 4, 2.2 and 2.2 -purification fold were obtained.

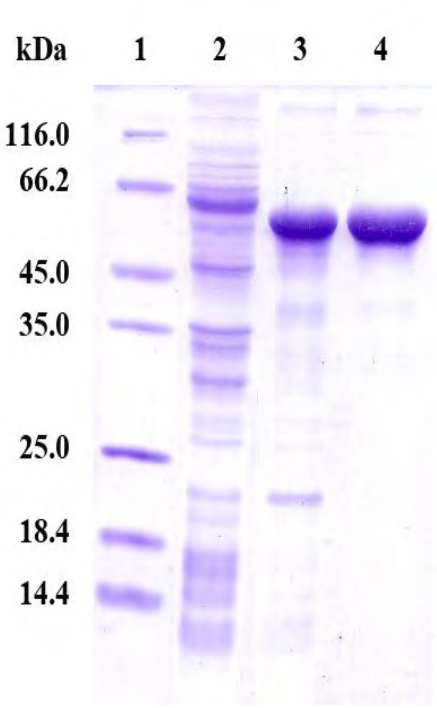


Figure 3.17 Purification of his-tagged recombinant *OsCatA* expressed in *E. coli* Rosetta-gami using Nickel-Sepharose. Proteins were run on 12% SDS-polyacrylamide gel and visualized by Coomassie blue staining. Protein molecular mass marker (lane 1), crude extract of *E. coli* harboring pET21a(+)/Os*CatA* (lane 2), inclusion bodies refolding of *E. coli* harboring pET21a(+)/Os*CatA* (lane 3), and purified OsCATA using nickel sepharose after elution with 200 mM imidazole.

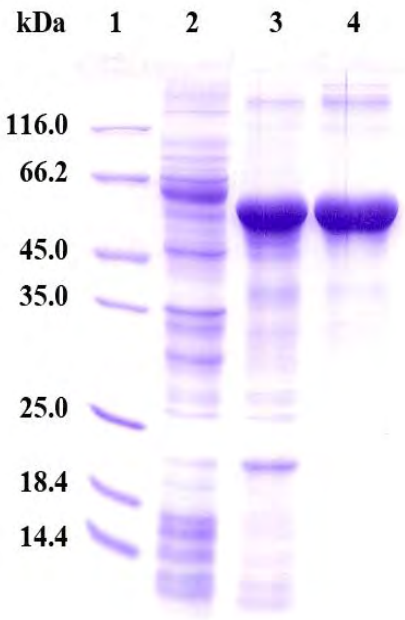


Figure 3.18 Purification of his-tagged recombinant *OsCatB* expressed in *E. coli* Rosetta-gami using Nickel-Sepharose. Proteins were run on 12% SDS-polyacrylamide gel and visualized by Coomassie blue staining. Protein molecular mass marker (lane 1), crude extract of *E. coli* harboring pET21a(+)/Os*CatB* (lane 2), inclusion bodies refolding of *E. coli* harboring pET21a(+)/Os*CatB* (lane 3), and purified OsCATB using nickel sepharose after elution with 100 mM imidazole.

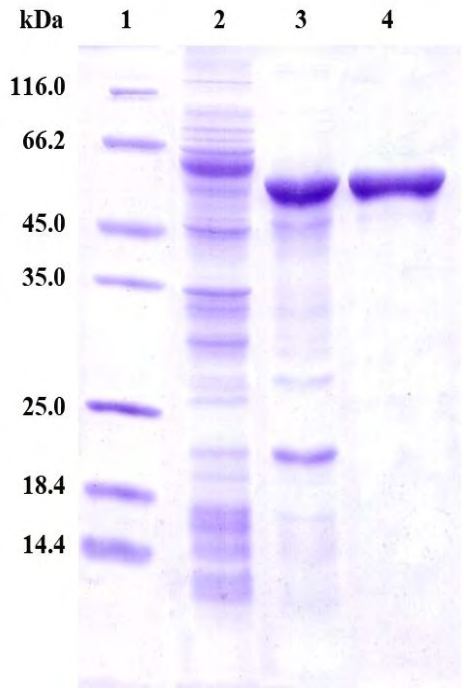


Figure 3.19 Purification of his-tagged recombinant *OsCatC* expressed in *E. coli* Rosetta-gami using Nickel-Sepharose. Proteins were run on 12% SDS-polyacrylamide gel and visualized by Coomassie blue staining. Protein molecular mass marker (lane 1), crude extract of *E. coli* harboring pET21a(+)/Os*CatC* (lane 2), inclusion bodies refolding of *E. coli* harboring pET21a(+)/Os*CatC* (lane 3), and purified OsCATC using nickel sepharose after elution with 200 mM imidazole.

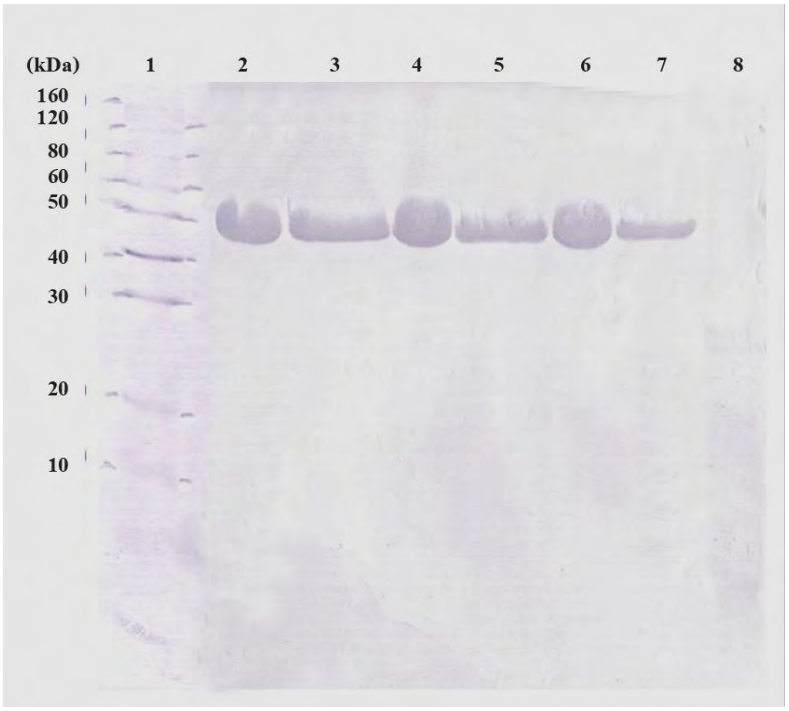


Figure 3.20 Western blot analysis of recombinant proteins from *E. coli* Rosetta gami harboring *OsCat* genes. Proteins were run on 12% SDS-polyacrylamide gel and visualized by Coomassie blue staining. His-tagged Protein Standard (lane 1), inclusion bodies refolding protein of cells harboring pET21a(+)/*OsCatA*, pET21a(+)/*OsCatB* or pET21a(+)/*OsCatC* (lane 2, 4 and 6, respectively) and purified OsCATA, OsCATB or OsCATC proteins using nickel sepharose after elution with 200, 100 and 200 mM imidazole (lane 3, 5 and 7, respectively) and insoluble protein of cells harboring pET21a(+) (lane 8).

Table 3.1 Purification table of the recombinant OsCATA protein from *E. coli* Rosetta-gami

Purification step	Total volume (ml)	Total protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min/mg)	Purification fold	Yield (%)
Crude extract	10	20.1	272.3	13.5	1	100
Ni-column purified	2	1.8	101.4	54.9	4	37.1

Table 3.2 Purification table of the recombinant OsCATB protein from *E. coli* Rosetta-gami

Purification step	Total volume (ml)	Total protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min/mg)	Purification fold	Yield (%)
Crude extract	10	5.3	314.2	59.3	1	100
Ni-column purified	2	0.7	91.5	130.8	2.2	29.1

Table 3.3 Purification table of the recombinant OsCATC protein from *E. coli* Rosetta-gami

Purification step	Total volume (ml)	Total protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min/mg)	Purification fold	Yield (%)
Crude extract	10	14.8	22.2	1.5	1	100
Ni-column purified	2	1.0	3.4	3.4	2.2	15.3

3.5 Biochemical characterization of purified catalases

The catalase activity was assayed at various temperatures and pH values using the purified catalase. For OsCATA, the optimal pH was 8.0 and there was no activity at and below pH 5.5 and at pH 11.0 (Figure 3.21) while the maximal activity was at 30 ^{o}C (Figure 3.22). For OsCATB, the optimal pH was 7.5 and there was no activity at and below pH 6.0 and at and above pH 10.5 (Figure 3.23) while the maximal activity was 25 ^{o}C (Figure 3.24) and the activity was decreased when the temperature was increased. For OsCATC, The optimal pH was 7.0 and there was no activity at and below pH 5.5 and at and above pH 8.5 (Figure 3.25) while the maximal activity was 30 ^{o}C (Figure 3.26) and the activity was decreased when the temperature was increased and there was no activity at and over 50 ^{o}C. To examine the kinetic properties of the purified OsCATs, initial velocities of OsCATs were measured in the presence of various concentrations of the H_2O_2 as substrate. The substrate saturation curves of OsCATA, OsCATB and OsCATC were generated and plotted. For OsCATA, the effect on the initial velocity of varying concentrations of H_2O_2 (0-100 mM) is shown in Figure 3.27. The K_m and V_{max} values of the OsCATA for H_2O_2 were 80.0 mM and 0.33 μmol min^{-1}. For OsCATB, the effect on the initial velocity of varying concentration of H_2O_2 (0-20 mM) is shown in Figure 3.28. The K_m and V_{max} values of the OsCATB for H_2O_2 were 66.67 mM and 1.0 μmol min^{-1}. For OsCATC shows the different concentration of H_2O_2 (0-20 mM) as shown in Figure 3.29. The K_m and V_{max} values of OsCATC for H_2O_2 were 40.0 mM and 0.03 μmol min^{-1}. The observe V_{max}/K_m of OsCATA, OsCATB and OsCATC, there were 4.12 x 10^{-3}, 14.99 x 10^{-3} and 0.75 x 10^{-3} μmol min^{-1}mM^{-1}, respectively. The k_{cat} values were

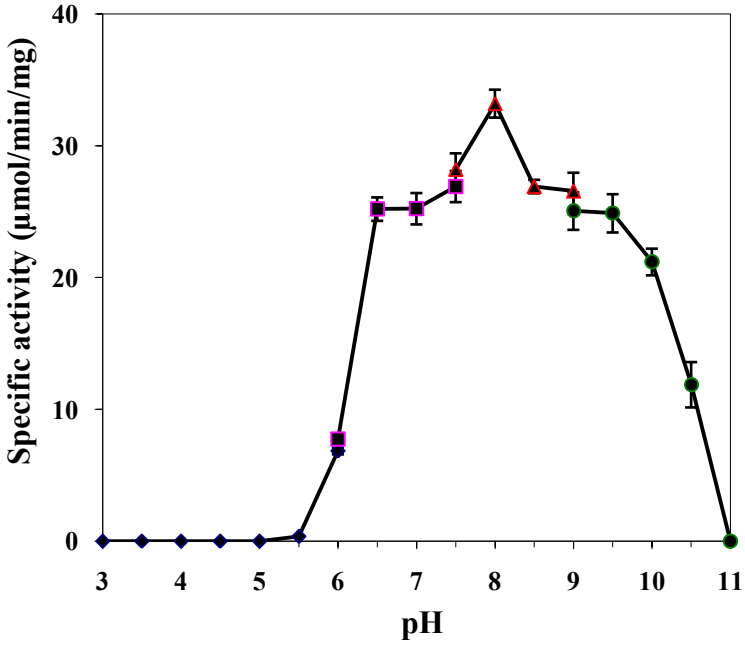


Figure 3.21 The effect of pH on OsCATA activity. The enzymes in buffers at various pHs ranged from 3.0–11.0 were incubated at 25^{o}C for 10 minutes. The 50 mM buffers used were citrate phosphate buffer (pH 3.0-6.0; ◆), potassium phosphate (pH 6.0-7.5; ■), Tris-HCl buffer (pH 7.5-9.0; ▲) and carbonate buffer (pH 9.0-11.0; ●). Error bars represent the standard error of mean for three replicates.

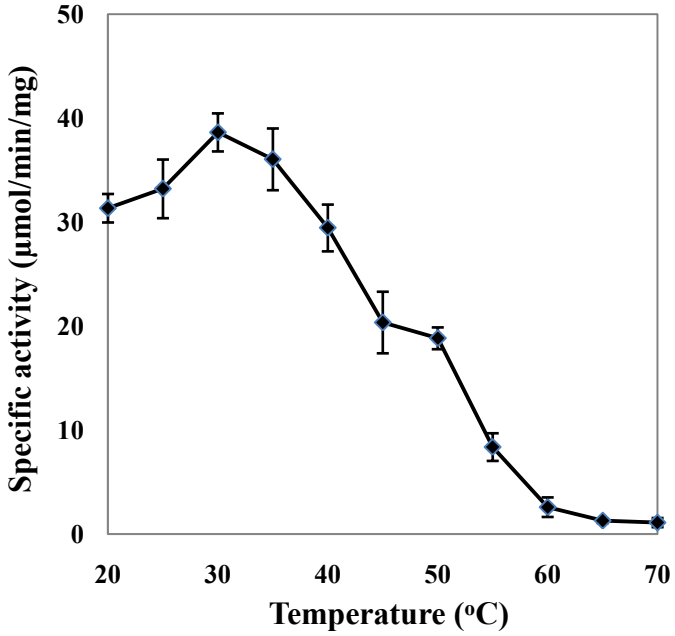


Figure 3.22 The effect of temperature on OsCATA activity. The enzyme activity was preformed at 20 to 70°C for 10 minutes. Error bars represent the standard error of mean for three replicates.

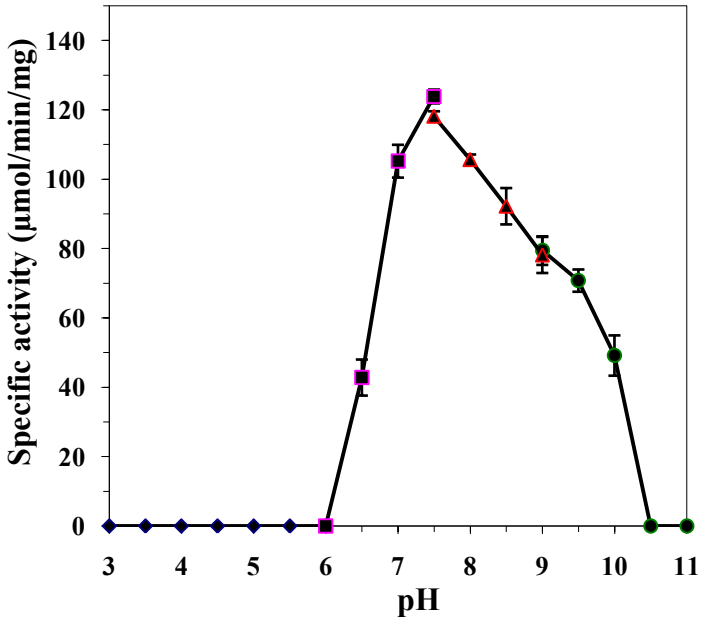


Figure 3.23 The effect of pH on OsCATB activity. The enzymes in buffers at various pHs ranged from 3.0–11.0 were incubated at 25°C for 10 minutes. The 50 mM buffers used were citrate phosphate buffer (pH 3.0-6.0; ◆), potassium phosphate (pH 6.0-7.5; ■), Tris-HCl buffer (pH 7.5-9.0; ▲) and carbonate buffer (pH 9.0-11.0; ●). Error bars represent the standard error of mean for three replicates.

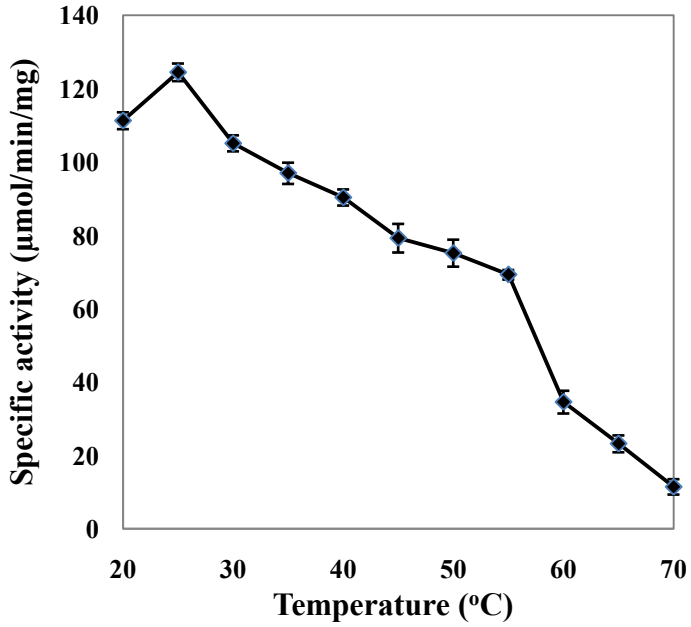


Figure 3.24 The effect of temperature on OsCATB activity. The enzyme activity was preformed at 20 to 70°C for 10 minutes. Error bars represent the standard error of mean for three replicates.

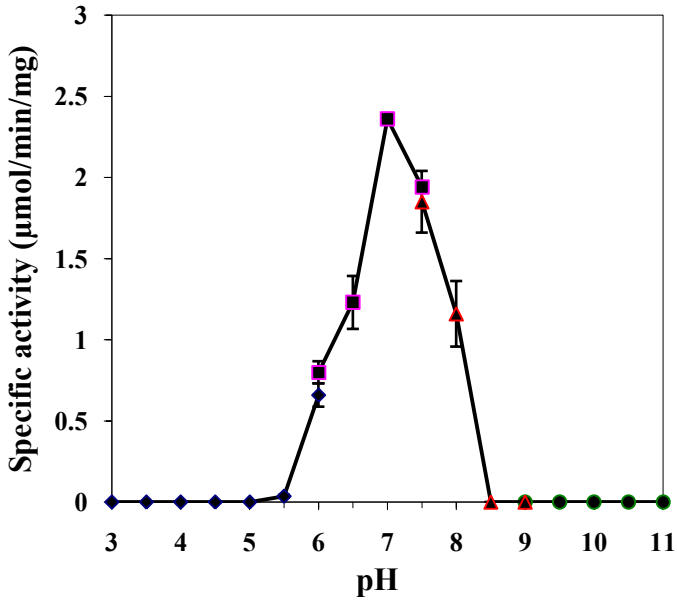


Figure 3.25 The effect of pH on OsCATC activity. The enzymes in buffers at various pHs ranged from 3.0–11.0 were incubated at 25°C for 10 minutes. The 50 mM buffers used were citrate phosphate buffer (pH 3.0-6.0; ◆), potassium phosphate (pH 6.0-7.5; ■), Tris-HCl buffer (pH 7.5-9.0; ▲) and carbonate buffer (pH 9.0-11.0; ●). Error bars represent the standard error of mean for three replicates.

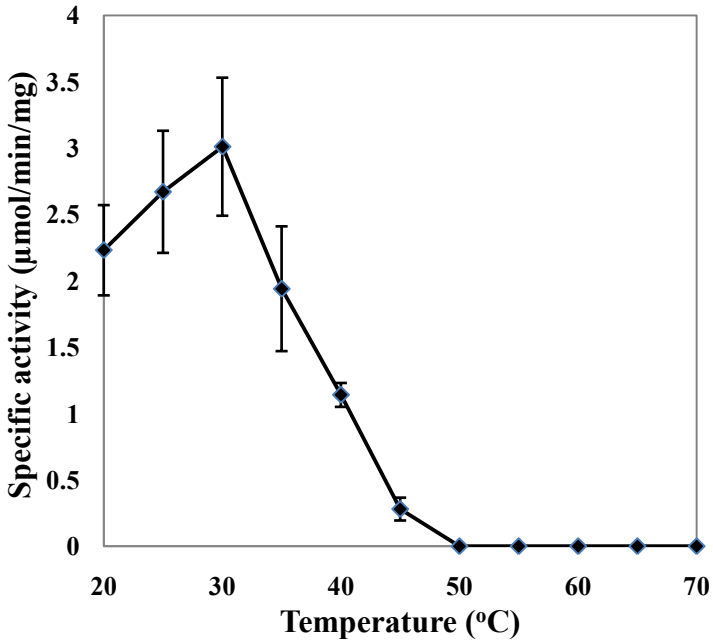


Figure 3.26 The effect of temperature on OsCATC activity. The enzyme activity was preformed at 20 to 70°C for 10 minutes. Error bars represent the standard error of mean for three replicates.

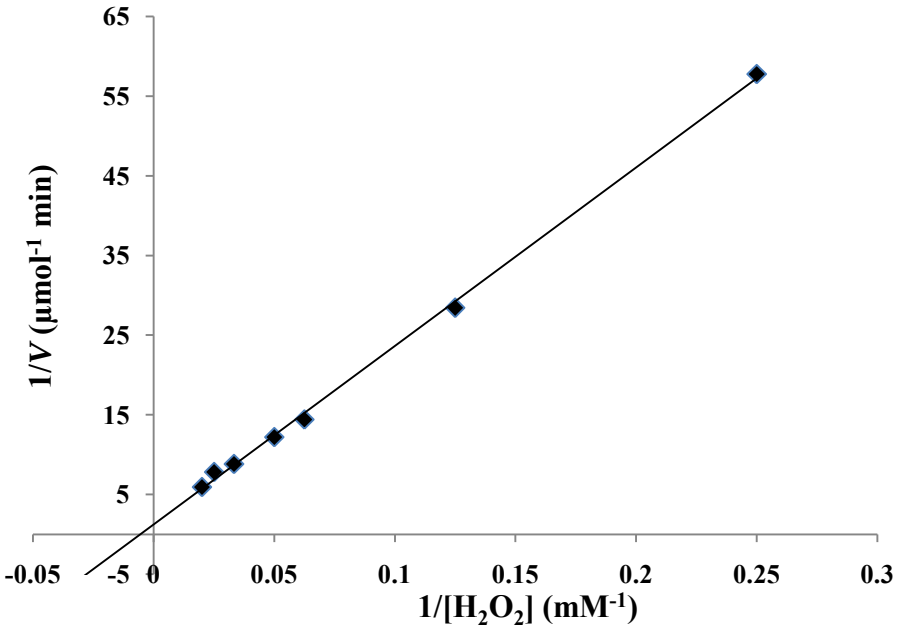


Figure 3.27 The apparent Lineweaver-Burk plot of OsCATA with H_2O_2 of different concentration (0 to 100 mM) as substrate.

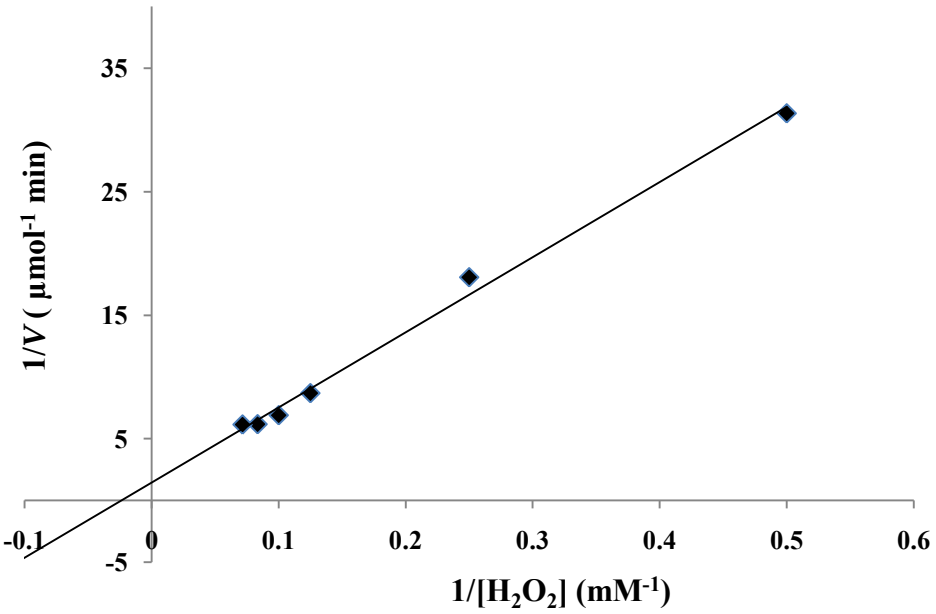


Figure 3.28 The apparent Lineweaver-Burk plot of OsCATB with H_2O_2 of different concentration (0 to 20 mM) as substrate.

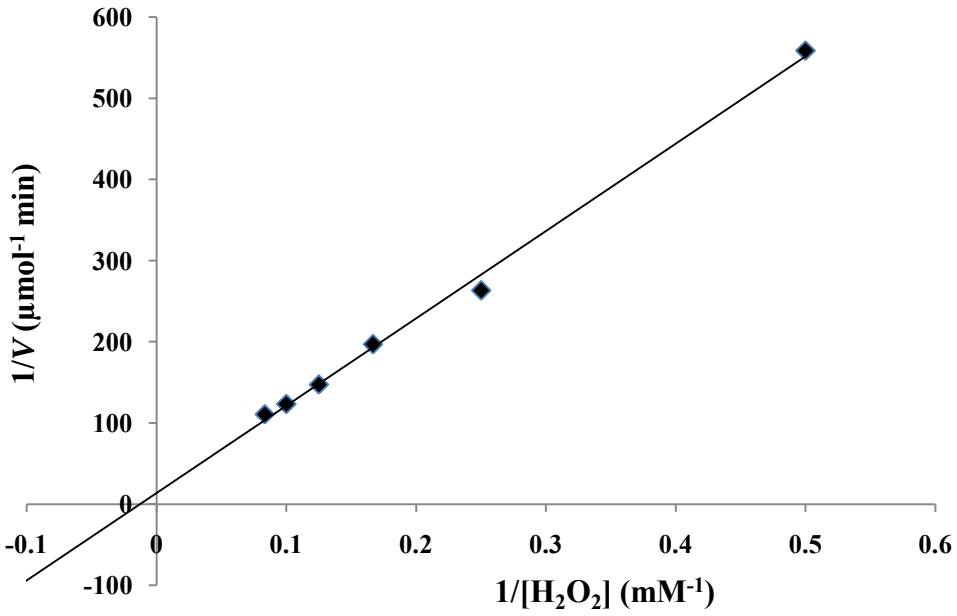


Figure 3.29 The apparent Lineweaver-Burk plot of OsCATC with H_2O_2 of different concentration (0 to 20 mM) as substrate.

calculated to be 6.60 x 10^{-3}, 20.0 x 10^{-3} and 0.46 x 10^{-3} min^{-1}, respectively. The observed of k_{cat}/K_m were 13.20 x 10^{-5}, 2.99 x 10^{-5} and 1.50 x 10^{-5} min^{-1} mM^{-1}, respectively. (Table 3.4)

3.6 Effect of NaCl on OsCAT activity

OsCAT activities in the presence of NaCl at final concentrations of 0, 15, 30, 60, 125, 250, 500 and 1000 mM were determined. The results obtained were plotted as relative enzyme activity with various NaCl concentrations (Figure 3.30). The reference value was the enzyme activity determined in the absence of added NaCl.

The activity of OsCATA, OsCATB and OsCATC were slightly decreased at 60, 30 and 60 mM, respectively. The results show similar pattern of sensitivity in enzyme response to all NaCl concentrations for OsCATA and OsCATB proteins. Interestingly, the activity of OsCATC decreased significantly at high NaCl concentration (250 mM).

3.7 CAT activity of rice *Oryza sativa* L. cultivar Khao Dok Ma Li 105 (KDML105)

3.7.1 Measurement of CAT activity in rice grown under salt stress condition

CAT activity was assayed in the crude extracts from leaves of KDML105 rice seedlings grown under salt stress. After 1 week of germination, rice seedlings were transferred to 1X Limpinuntana's nutrient solution and grown for 2 weeks under a 16-hr light/8-hr dark photoperiod. Then, the 3-week old seedlings were treated with 150 mM NaCl in 1X Limpinuntana's nutrient solution for 0, 1, 2 or 3 days. Then, the

Table 3.4 Kinetic parameters of recombinant OsCAT proteins from *E. coli* Rosetta-gami

Enzyme	K_m (mM)	V_{max} (μmol min^{-1})	V_{max}/K_m (μmol min^{-1} mM^{-1}) (10^{-3})	K_{cat} (min^{-1}) (10^{-3})	K_{cat}/K_m ($min^{-1}mM^{-1}$) (10^{-5})
OsCATA	80.0	0.33	4.12	6.60	13.20
OsCATB	66.67	1.0	14.99	20.0	29.99
OsCATC	40.0	0.03	0.75	0.46	1.50

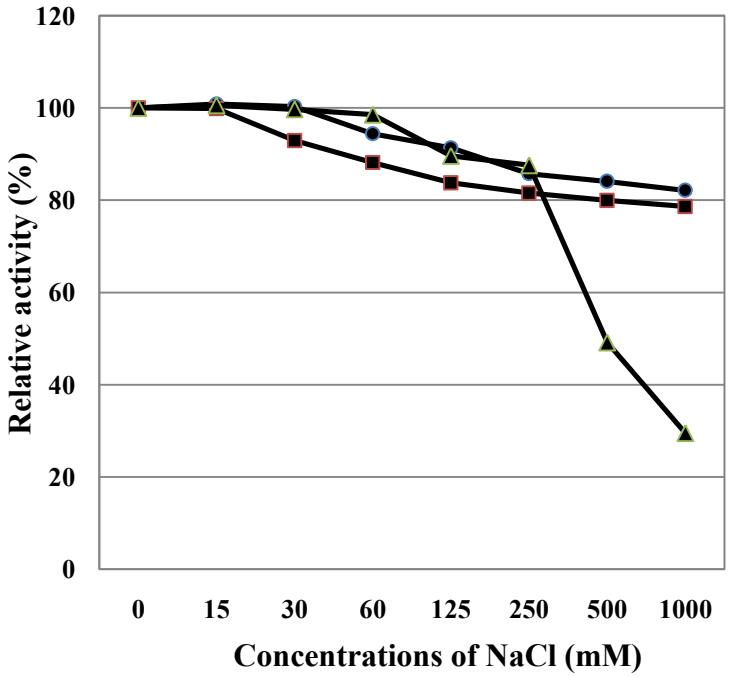


Figure 3.30 Effect of NaCl on OsCAT activity. The activity was assayed in the presence of NaCl at various concentrations varying from 0 to 1000 mM; OsCATA (●), OsCATB (■) and OsCATC (▲). Error bars represent the standard error of mean for three replicates.

3-week old seedlings were treated with 150 mM NaCl in 1X Limpinuntana's nutrient solution for 0, 1, 2 or 3 days. In control, no change occurred in catalase activity during the experimental period. However, the catalase activity showed a significant decrease under salt stress, especially, on the first and second day of salinity but the catalase activity was slightly decreased on third day after salt stress treatment (Figure 3.31).

3.7.2 Effect of NaCl on CAT activity in rice

CAT activity from rice in the presence of NaCl at final concentrations of 0, 15, 30, 60, 125, 250, 500 and 1000 mM were determined. The results obtained were plotted as relative enzyme activity with various NaCl concentrations (Figure 3.32). The reference value was the enzyme activity determined in the absence of added NaCl. The CAT activity was stimulated by low concentrations of NaCl (15-60 mM) but it was inhibited at high concentration of NaCl (125-1000 mM).

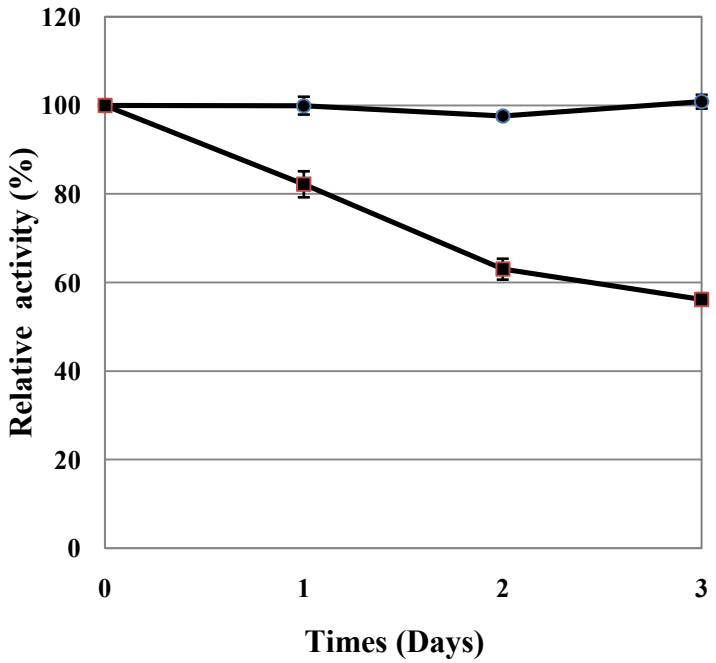


Figure 3.31 Effect of salt stress on CAT activities of KDML105 rice seedling leaves at 0, 1, 2 and 3 days after salt stress treatments; control: untreated (●) and 150 mM NaCl treatments (■). Error bars represent the standard error of mean for three replicates.

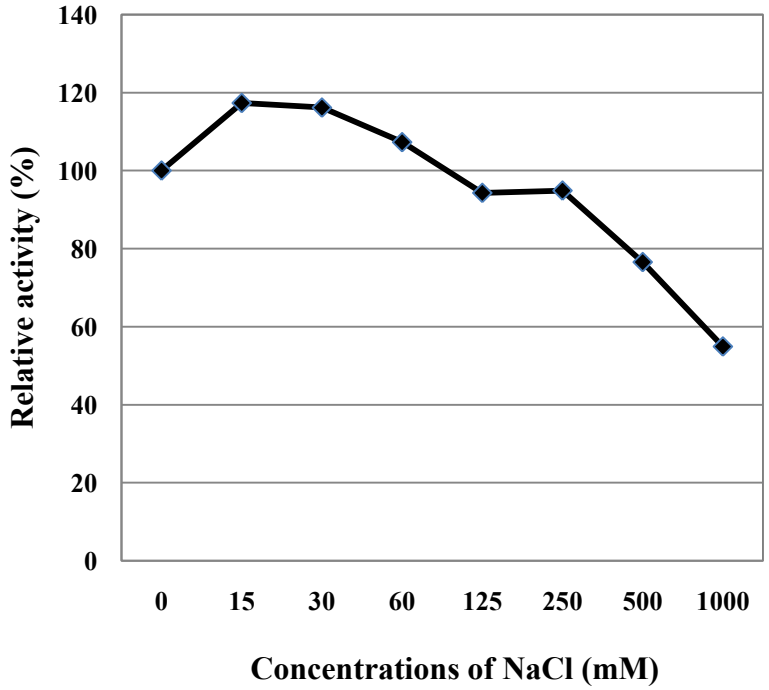


Figure 3.32 Effect of NaCl on CAT activity of KDML105 rice seedlings leaves. The activity was assayed in the presence of NaCl at various concentrations varying from 0 to 1000 mM. Error bars represent the standard error of mean for three replicates.

CHAPTER IV

DISCUSSION

4.1 Cloning and expression of *OsCat* genes

In this research, three *OsCat* genes (*OsCatA*, *OsCatB* and *OsCatC*) were amplified by PCR using the full-length corresponding cDNA clones as templates. The sense primer contained *Nde*I site, while the antisense primer consisted of *Eco*RI site for further cloning purpose. The *Nde*I site within the sense primer allowed each gene fragment to be placed at an appropriate distance behind the T7 promoter and the ribosome binding site from the phage T7 major capsid protein in pET-21a (Novagen, 2002). The antisense primer, which contained an *Eco*RI site, was designed to replace the stop codon in these genes with an amino acid and allow the gene fragment to fuse in-frame with the sequence encoding His-tag within pET-21a.

DNA fragments of *OsCat* gene from rice *Oryza sativa* L. *OsCatA*, *OsCatB* and *OsCatC* were ligated into a TA vector, pTZ57 R/T and introduced by transformation into *E. coli* XL1-Blue. The *lacZ* reading frame is interrupted resulting in the appearance of white colonies. The *OsCat* gene fragments were ligated with the *Nde*I-*Eco*RI sites of pET-21a in frame with the sequence encoding a 6x His tag to produce a fusion protein. The His-tag behind the interested genes were used to facilitate the purification and detection of the recombinant proteins.

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-gami. Protein expression was achieved by IPTG induction of the gene cassette integrated within the *E. coli* chromosome in which the T7 RNA polymerase was

expressed from the lacUV5 promoter and then polymerase would specifically bind the T7 promoter in the expression vector. pET also carry the natural promoter and coding sequence for the *lac* repressor (*lacI*), oriented so that the T7*lac* and *lacI* promoters diverge. However, the host system would supply the eukaryotic codon usage. For this reason, *E. coli* Rosetta-gami was selected since the Rosetta-gami strain is a powerful combination of the Rosetta and Origami strains, encompassing rare codon usage and disulfide bond formation which combines the enhanced disulfide bond formation with the Rosetta characteristics resulting from the *trx*B/*gor* mutations with enhanced expression of eukaryotic proteins. Analyzed by SDS-PAGE, the expression of OsCAT A, OsCATB and OsCATC were observed in the inclusion bodies.

4.2 Solubilization and refolding of OsCAT from inclusion bodies

The inclusion bodies from crude protein extracts were solubilized and refolded to obtain active proteins activity. It is possible that the overexpressed protein may result in an accumulated misfolding protein leading to formation of inactive aggregates of protein known as inclusion bodies. The process of obtaining activity of this protein often involves a lot trial and error strategies for suitable renaturation. The active yields are often low to avoid the problem from inclusion body formation. In general, addition of detergents such as Triton X-100 (non-ionic detergent) for washing the inclusion body preparation will allow the removal of cellular contaminants in microbial cell (Cabrita and Bottomley, 2004). In this study phosphate buffer pH 12.0 was chosen. The solubilization of inclusion body results in soluble protein that is devoid of its native conformation. During this period, more often than not, misfolding occurs more.

4.3 Purification and characterization of recombinant OsCAT proteins

The host *E.coli* cells were collected and the cell wall was disrupted by ultrasonication or high pressure sound waves, which causes cell breakage by cavitations and shear forces. Proteins were extracted in extraction buffer containing ethylenediamine tetraacetic acid (EDTA) to inhibit metalloproteases by chelating the divalent cations necessary for their activity. This reagent protected the desired protein from the degradation by proteolytic enzymes. Addition of a reagent containing a thiol group such as dithiothreitol (DTT) and also a chelating agent such as EDTA to chelate metal ions in the extraction buffer would minimize the oxidation damage (Bollag and Edelstein, 1991). The recombinant OsCATA, OsCATB and OsCATC were successfully purified by Ni-sepharose affinity chromatography from the solubilized inclusion bodies. The $(His)_6$ fusion protein can easily be bound with Ni-Sepharose and then eluted with buffer containing imidazole. Western blot analysis using an anti-His_6 tag antibody confirmed identity of the recombinant proteins produced by the pET expression system.

Overall, through the Ni-Sepharose high performance column, 4.0, 2.2 and 2.2-purification fold was obtained for the purification of the recombinant OsCATA, OsCATB and OsCATC, respectively.

4.4 Biochemical characterization of catalase purified

Temperature plays a significant role in enzyme performance. The influence of temperature on an enzymatic reaction is resulted from two opposing effects, an increase in rate and an increase in denaturation, in which a conformational alteration occurs resulting in the loss of biological activity. If deactivation were the only factor

influencing optimal enzyme use parameters, lower temperatures would be preferable. However, inactivation by heat must be balanced with a temperature dependent increase in the enzymatic rate of catalysis that accompanies increasing temperature up to an optimum temperature, which is often a temperature where deactivation of the enzyme. The optimum temperatures of OsCATA, OsCATB and OsCATC were 30^{o}C, 25^{o}C and 30^{o}C, respectively. For other catalases, such as CATs from *Rhodospirillum rubrum* (Lee *et al.,* 2007), *Vibrio rumoiensis* (Yumoto *et al.,* 2000), *Zantedeschia aetgiopica* (Trindade *et al.,*1998), and apple (*Malus domestica*) (Yoruk *et al.,* 2005) the optimum temperatures have been reported to be 30 ^{o}C, 40 ^{o}C, 40 ^{o}C, and 50 ^{o}C, respectively.

pH is a measure of the concentration of hydrogen ions in the solution. Most enzymes function efficiently over a narrow pH range. A change in pH above or below this range reduces the rate of enzyme reaction considerably. Changes in pH lead to the breaking of the ionic bonds that hold the structure of enzyme in place. When the enzyme begins to lose its functional shape, particularly the shape of the active site, such that the substrate will no longer fit into it, the enzyme is said to be denatured. Also changes in pH affect the charges on the amino acids within the active site such that the enzyme will not be able to form an enzyme-substrate complex. In this study, the optimum pH values of OsCATA, OsCATB and OsCATC were 8.0, 7.5 and 7.0, respectively. On other catalases such as CAT from apple *Malus domestica* (Yoruk *et al.,* 2005), CAT from Calla lily *Zantedeschia aethiopica* (Trindade *et al.,*1998), KatA from bacterium *Xanthomonas campestris* (Chauvatcharin *et al.,* 2003), CAT from photosynthetic bacterium *Rhodospirillum rubrum* (Kang *et al.*, 2006) and CAT1 and CAT2 from wheat *Triticum aestivum* L. (Garcia *et al.,* 2000) the optimum pH have

been reported to be 5.0, 7.0, 7.0, 7.5, 7.0 and 7.0, respectively.

The kinetic mechanism of the recombinant OsCAT proteins were determined by initial velocity studies**.** Based on a Lineweaver-Burk plot, initial velocities of OsCATA, OsCATB and OsCATC were measured in the presence of various concentrations of the H_2O_2 as substrate. The calculated K_m (mM) values are 80.0, 66.67 and 40.0, respectively. The V_{max} (μmol min^{-1}) values were calculated to be 0.33, 1.0 and 0.03, respectively.

Catalases generally have low substrate affinities with apparent K_m values for H_2O_2 of 10.6 and 44.7 mM for KatB and KatA from *Pseudomonas aeruginosa* (Brown *et al.,* 1995), respectively, and 60 mM for CatF from *Pseudomonas syringae* (Klotz *et al.,* 1995)

4.5 Effect of NaCl on activity of recombinant OsCAT proteins

OsCAT activity was determined in the presence of the series of NaCl. The salt solution was buffered with 50 mM potassium phosphate buffer at pH 7.5. The obtained results are plotted as relative enzyme activity against various NaCl concentrations. The reference value was enzyme activity determined in the absence of added salt. Our results showed that the activity of OsCATs were inhibited under high NaCl condition. In addition, OsCATC was sensitive in enzyme response to NaCl more than OsCATA and OsCATB. It is consistant with those obtained by previous investigations (Lanyi and Stevenson, 1969) who showed that the catalase activity of *Halobacterium cutirubrum* was inhibited at high NaCl concentration (2-6 M). Thus, the possibility that decrease in catalase activity due to salt is associated with enzyme denaturation and the influence of salt on the enzyme may be due to binding of water

by the salts which changes the structure of water around the enzyme and thus affects its configuration and activity (Lanyi and Stevenson, 1969).

4.6 CAT activity of rice (*Oryza sativa* L.) cultivar Khao Dok Ma Li 105 (KDML105)

4.6.1 Measurement of CAT activity in rice which grown under salt stress condition

The present CAT activity of the leaves in rice *Oryza sativa* L. was stressed by NaCl treatment. The CAT activity was reduced by salt stress treatment and this decrease became more severe with time after the salt treatment. The reduction of CAT activity showed that catalase might be a limiting factor in the antioxidative system of rice. CAT enzyme probably did not get activated in rice seedlings under salt stress. This enzyme may not contribute to the tolerance of the rice cultivar against salt stress.

These results are consistent with the results of Benavides *et al*., (2000) in potato, Lin and Kao (2000) in rice *Oryza sativa* L., cv. Taichung Native 1 and Shim *et al*., (2003) in wheat which CAT activity significantly was decreased during salt stress treatment. On the other hand, CAT activity has been found to increase under salt stress in tomato (Rodriguez-Rosales *et al*., 1999), soybean (Comba *et al*., 1998), tobacco (Bueano *et al*., 1998; Savoure *et al*., 1999), cucumber (Lechno *et al*., 1997) and mulberry (Sudhakar *et al*., 2001).

4.6.2 Effects of NaCl on CAT activity of rice seedlings leaves

CAT activity from rice in the presence of NaCl, the results obtained were plotted as relative enzyme activity with various NaCl concentrations. In the inhibitory

effect, and to some extent the stimulation, is salt specific. Thus, stimulation of enzyme activity was observed when NaCl was added at its optimal concentration. These results are agreement with the results of Lanyi and Stevenson (1969), which *Halobacterium cutirubrum* catalase was activated with 0.5 to 1.5 M NaCl and it was inhibited by NaCl concentration higher than 1.5 M NaCl. The activation and inactivation of the enzyme may therefore be related to the affinity of the enzyme for specific ion pairs rather than individual cations or anions whose effect would be independent of one another. The correlation of the inhibition of the enzyme by the salts and the effectiveness of the salts in binding water molecules by the lowering of vapor pressure indicates that the binding of the salts by the protein and the binding of water by the salts may be similar and appear to be influenced by the same properties of the salts. (Lanyi and Stevenson, 1969).

CHAPTER V

CONCLUSIONS

1. Alignments of their deduced amino acid sequences, OsCATA, OsCATB and OsCATC shared OsCATA showed 72% and 75% identities with OsCATB and OsCATC, respectively, whereas OsCATB had 83% identity with OsCATC. All amino acid sequence of OsCAT proteins have peroxisomal targeting signal in their conserved C-terminal region.

2. cDNA sequences of *OsCatA*, *OsCatB* and *OsCatC* were engineered by PCR amplification to facilitate cloning in the T7-based expression plasmid, pET-21a(+). The recombinant expression plasmids were introduced into *E. coli* strain Rosetta-gami cells and successfully used to produce the recombinant OsCATA, OsCATB and OsCATC proteins which encode 512 amino acids with a predicted mass of roughly 58.8, 58.7 and 58.9 kDa, respectively.

3. The recombinant *OsCat* genes were expressed in the inclusion bodies in *E. coli* Rosetta-gami which were solubilized, refolded to obtain active activity at the condition used. The fusion protein with 6x His-tag could be detected by western blot analysis and purification by using Ni-column chromatography.

4. The soluble recombinant OsCATA, OsCATB and OsCATC proteins were purified using Ni-Sepharose with 4, 2.2 and 2.2 -purification fold, respectively. The following kinetic properties; the calculated K_m (mM) values are 80.0, 66.67 and 40.0 respectively. The k_{cat} (min^{-1}) values were calculated to be 6.60×10^{-3}, 20.0×10^{-3} and 0.46×10^{-3}, respectively.

5. The optimum pH of OsCATA, OsCATB and OsCATC were 8.0, 7.5 and 7.0, respectively.

6. The optimum temperature of OsCATA, OsCATB and OsCATC were 30 ^{o}C, 25 ^{o}C and 30 ^{o}C, respectively.

7. OsCATC was sensitive to the NaCl at high concentration much more than OsCATA and OsCATB.

8. CAT activities of rice seedling leaves **(***Oryza sativa* L.) was deactivated by salt stress.

9. CAT activities from rice seedling leaves in the presence of NaCl which was stimulated catalase activity at its optimal concentration and deactivated at high concentrations of NaCl.

REFERENCES

Apse, M. P., Aharon, G. S., Snedden W. A., and Blumwald, E. (1999). Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in *Arabidopsis*. *Science.* 285: 1256-1258.

Asada, K. (2006). Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiology.* 141: 391-396.

Asada, K. and Takahashi, M. (1987). Production and scavenging of active oxygen in Photosynthesis. In: Eds. Kyle DJ, Osmond CB, Amtzen DJ, Photoinhibition. *Elsevier*. 227-287.

Ashrafuzzaman, M., Khan, M. H., and Shahidullah, S. M. (2002). Vegetative growth of maize (*Zea mays*) as affected by a range of salinity. *Crop Res. Hisar*. 24: 286-291.

Ashraf M. and Harris P. J. C. (2004). Potential biochemical indicators of salinity tolerance in plants. *Plant Science*. 166: 3-16.

Benavides, M. P., Marconi, P. L., Gallego, S. M., Combo, M. E., and Tomaro, M. L. (2000). Relationship between antioxidant defense system and salt tolerance in *Solanum tuberosum*. *J. Plant Physiol*. 27: 273-278.

Blum, A. and Ebercon, A. (1976). Genotypic responses in sorghum to drought stress. Free proline accumulation and drought resistance. *Crop Science*. 16: 361-367.

Bollag, D. M. and Edelstein, S. J. (1991). Concentrating protein solutions: Preparative methods, *Protein methods*. 23: 79-82.

Brown, S. M., Howell, M. L., Vasil, M. L., Anderson, A., and Hassett, D. J. (1995). Cloning and characterization of *katB* gene of *Pseudomonas aeruginosa*

encoding a hydrogen peroxide-inducible catalase: purification of KatB, cellular localization, and demonstration that it is essential for optimal resistance to hydrogen peroxide. *J. Bacteriol.* 177: 6536-6544.

Bueno, P., Piqueras, A., Kurepa, J., Savoure, A., Verbruggen, N., Montagu, V. M., and Inze, D. (1998). Expression of antioxidant enzymes in response to abscisic acid and high osmoticum in tobacco BY-2 cell cultures. *Plant Science*: 138: 27-34.

Cabria, L. D. and Bottomley, S. P. (2004). Protein expression and refolding: A practical guide to getting the most out of inclusion bodies. *Biotechnology annual review.* 10: 1387-2656.

Chauvatcharin, N., Vattanaviboon, P., Switala, J., Loewen, P. C., and Mongkolsuk, S. (2003). Cloning and characterization of *katA*, encoding the major monofunctional Catalase from *Xanthomonas campestris* pv. phaseoli and characterization of the encoded catalase KatA. *Current Microbiology*. 46: 83-87.

Cha-um, S., Supaibulwatana, K., and Kirdmanee, C. (2006). Water relation, photosynthetic ability and growth of thai jasmine rice (*Oryza sativa* L. ssp. *indica* cv. KDML 105) to salt stress by application of exogenous glycinebetaine and choline. *J. Agronomy & Crop Sci.* 192: 25-36.

Comba, M. E., Benavides, M. P., and Tomaro, M. L. (1998). Effect of salt stress on the antioxidant defense system of soybean root nodules. *J. Plant Physiol*. 25: 665-671.

Dat, J., Vandenabeele, S., Vranova, E., Van Montagu, M., Inze, D., and Van Breusengem, F. (2000). Dual action of the active oxygen species during plant stress responses. *Cellular and Molecular Life Sciences*. 57: 779-795.

Del Rio, L. A., Corpas, F. J., Sandalio, L. M., Gomez, J. M., and Barroso, J. B. (2002). Reactive oxygen species, antioxidant systems and nitric oxide in peroxisames. *Journal of Experimental Botany*. 53: 1257-1272.

Dionisio-Sese, M. L. and Tobita, S. (1998). Antioxidant responses of rice seedlings to salinity stress. *Plant Sci*. 135: 1-9.

Doke, N., Miura, Y., Chai, H-B., and Kawakita, K. (1991). Involvement of active oxygen in induction of plant defense against infection and injury. *Plant Physiol*. 69: 84-96.

Elstner, E. F. (1991). Mechanism of oxygen activation in different compartments of plant cells. *Plant Physiol*. 15: 13-25.

Elstner, E. F. (1987). Metabolism of activated oxygen species. *Plant Physiol*. 11: 253-315.

Feierabend, J. and Dehne, S. (1996). Fate of the porphyrin cofactors during the light-dependent turnover of catalase and of the photosystem II reaction-center protein D1 in mature rye leaves. *Planta.* 198: 413-422.

Foyer, C. H. and Halliwell, B. (1976). The presence of glutathione glutathione reduclase in chloroplasts: a proposed role in ascorbic acid metabolism. *Planta*. 133: 21-25.

Garcia, R., Kaid, N., Vignaud, C., and Nicolas, J. (2000). Purification and some properties of catalase from wheat germ (*Triticum aestivum* L.). *J. Agric Food Chem.* 48: 1050-1057.

Gueta-Dahan, Y., Yaniv, Z., Zilinskas, B. A., and Ben-Hayyim, G. (1998). The effect of salt stress on lipid peroxidation and antioxidants in the leaf of the cultivated tomato and its wild salt-tolerant relative *Lycopersicon Pennellii*. *Physiologia Plantarum*. 104: 169-174.

Gueta-Dahan, Y., Yaniv, Z., Zilinskas, B. A., and Ben-Hayyim, G. (1997). Salt and oxidative stress: similar and specific responses and their relation to salt tolerance in Citrus. *Planta*. 203: 460-469.

Gomez, J. M., Hernandez, J. A., Jimenez, A., Del Rio, L. A., and Sevilla, F. (1999). Differential responses of antioxidant enzymes of chloroplasts and mitochondria to long-term NaCl stress of pea plants. *Free Radical Research*. 31: 11-18.

Halliwell, B. (1981). Free radicals, oxygen toxicity and aging. In: Sohal RS, ed. Age Pigments. *Elsevier*: 1-62.

Hasegawa, P. M., Bressen, R. A., Zhu, J. K., and Bohnert, H. J. (2000). Plant cellular and molecular responses to high salinity. *Annu Rev Plant Physiol Plant Mol Biol.* 51: 463-499.

Hayward, H. E. and Bernstein, L. (1958). Plant-growth relationships on salt-affected soils. *Bot. Rev*. 24: 584-635.

Hayward, H. E. and Wadleigh, C. H. (1949). Plant growth on saline and alkali soils. *Advances Agron*. 1: 1-38.

Hong, C. Y., Chao, Y., Yang, M. S., Cho, C. S., and Kao. C. H. (2009). NaCl-induced expression of glutathione reductase in roots of rice (*Oryza sativa* L.) seedlings is mediated through hydrogen peroxide but not abscisic acid. *Plant Soil*. 320: 103-115.

Hong, C. Y., Hsu, Y., Tsai, Y. C., and Kao, C. H. (2007). Expression of ascorbate peroxidase in roots of rice (Oryza sativa L.) seedlings in response to NaCl. *J. Exp. Bot*. 58: 3273-3283.

Kang, Y. S., Lee, D. H., Yoon, B. J., and Oh, D. C. (2006). Purification and characterization of a catalase from photosynthetic bacterium *Rhodospirillum rubrum* S1 grown under anaerobic conditions. *J. Microbiol.* 44(2): 185-191.

Karakas, B., Ozias-Akins, P., Stushnoff, C., Suefferheld, M., and Rieger M. (1997). Salinity and drought tolerance of mannitol accumulating transgenic tobacco. *Plant Cell Environ.* 20: 609-616.

Klotz, M. G., Kim, Y. C., Katsuwon, J., and Anderson, A. J. (1995). Cloning, characterization and phenotypic expression in *Escherichia coli* of *catF*, which encodes the catalytic subunit of catalaase isozyme CatF of *Pseudomonas syringae. Appl Microbiol Biotechnol.* 43: 656-666.

Lanyi, J. K. and Stevenson, J. (1969). Effect of salts and organic solvents on the activity of *Halobacterium cutirubrum* catalase. *Journal of bacteriology*. 98(2): 611-616.

Lechno, S., Zamski, E., and Telor, E. (1997). Salt stress-induced responses in cucumber plants. *J. Plant Physiology*. 150: 206-211.

Lee, D. H., Kim, Y. S., and Lee, C. B. (2001). The inductive responses of the antioxidant enzymes by salt stress in the rice (*Oryza sativa* L.). *J. Plant Physiol*. 158: 737-745.

Lee, D. H., Oh, D. C., Oh, Y. S., Malinverni J. C., Kukor, J. J., and Kahng, H. Y. (2007). Cloning and characterization of monofunctional catalase from

photosynthetic bacterium *Rhodospirillum rubrum* S1. *J. Microbiol.* 17(9): 1460-1468.

Lin, C. C. and Kao, C. H. (2000). Effect of NaCl on H_2O_2 metabolism in rice leaves. *Plant Growth Regulation*. 30: 151-155.

Lin, C. C. and Kao, C. H. (2001). Cell wall peroxidase activity, hydrogen peroxide level and NaCl-inhibited root growth of rice seedlings. *Plant Soil*. 230: 135-143.

Lindqvist, Y., Branden, C. L., Mathews, F. S., and Lederer, F. (1991). Spinach glycolate oxidase and yeast flavocytochrome are strueturaly homologous and evolutionary related enzymes with distinctly different function and flavin mononucleotide binding. *Journal of Biological Chemistry*, 266: 3198-3207.

Magistad, O. C. (1943). Effect of salt concentration, kind of salt and climate on plant growth in sand cultures. *Plant Physiol*. 18: 151-166.

Meneguzzo, S., Navario-Jzzo, F., and Izzo, R. (1999). Antioxidative responses of shoots and roots of wheat to increasing NaCl concentrations. *Journal of Plant Physiology*. 155: 274-280.

Mescht, A. V. D., De Ronde, J. A., and Rossouw, F. T. (1998). Cu/Zn superoxide dismutase, glutathione reductase and ascorbate peroxidase levels during drought stress in potato. *South African Journal of Science*. 94: 496-499.

Nieman, R. H. (1962). Some effects of sodium chloride on growth, photosynthesis, and respiration of 12 crop plants. *Bot. Gaz*. 123: 279-285.

Osmond, C. B. and Avadhani, P. N. (1968). Acid metabolism in Atriplex. Oxalate synthesis during acid metabolism in the dark. *J. Biol. Sci*. 21: 917-927.

Polle, A. (1997). Defense against photooxidative damage in plants. In: Scandalios J(ed) Oxidative Stress and the Molecular Biology of Antioxidant Defenses. *Cold Spring Harbor Laboratory Press*, 785-813.

Rabinowitch, H. D. and Fridovich, I. (1983). Superoxide radicals, superoxide dismutases and oxygen toxicity in plants. *Phytochem. Photobiol*. 37: 679-690.

Richardson, T. and Hyslop, D. B. (1985). Enzymes. In: *Food Chemistry,* 3rd edn, ed. O. R. Fennema. 420-421.

Rodriguez-Rosales, M. P., Kerkeb, L., Bueno, P., and Donaire, J. P. (1999). Changes induced by NaCl in lipid content and composition, lipoxygenase, plasma membrane H^+-ATPase and antioxidant enzyme activities of tomato *(Lycopersicon esculentum,* Mill). *Plant Science*. 143: 143-150.

Sairam, R. K. and Tyagi, A. (2004). Physiology and molecular biology of salinity stress tolerance in plants. *Current science*. 86: 407-421.

Sandalio, L. M. and Del Rio, L. A. (1988). Intraorganellar distribution of superoxide dismutase in plant peroxisomes. *Plant Physiol*. 88: 1215-1218.

Savoure, A., Thorin, D., Davey, M., Xue-Jun Hua, Mauro, S., Van Montagu, M., Inze, D., and Verbruggen, N. (1999). NaCl and $CuSO_4$ treatments trigger distinct oxidative defense mechanisms in *Nicotiana plumbaginifolia* L. *Plant Cell and Environment*. 22: 387-396.

Scandalias, J. G. (1993). Responses of plant antioxidant defense genes to environmental stress. *Adv. Genet*. 28: 1-41.

Shankhdhar, D., Mani, S. C., and Pant, R. C. (2000). *In vitro* selection for salt tolerance in rice. *Biol. Plant*., 43: 477-480.

Shim, I. S., Momose, Y., Yamamoto, A., Kim, D. W., and Usui, K. (2003). Inhibition of catalase activity by oxidative stress and its relationship to salicylic acid accumulation in plants. *Biotechnology Annual Review.* 10: 1387-2656.

Somerville C. R. and Ogren, W. L. (1982). Genetic modification of photorespiration. *Trends Biochem Sciences.* 7: 171-174.

Sreenivasulu, N., Ramanjulu, S., Ramachandra-Kini, K., Prakash, H. S., Shekar-Shetty, H., Savithri, H. S., and Sudhakar, C. (1999). Total peroxidase activity and peroxidase isoforms as modified by salt stress in two cultivars of fox-tail millet with differential salt tolerance. *Plant Science*. 141: 1-9.

Sudhakar, C., Lakshmi, A., and Giridarakumar, S. (2001). Changes in the antioxidant enzyme efficacy in two high yielding genotypes of mulberry *(Morus alba* L.) under NaCl salinity. *Plant Science*. 161: 613-619.

Tanji, K. K. (1990). Nature and extend of agricultural salinity, Chapter I in Agricultural Salinity Assessment and Management, *American Society of Civil Engineers*. 1: 1-18.

Thiyagarajan, T. M. and R. Selvaraju, (2001). Water saving in rice cultivation in India. Proceedings of an International Workshop on Water Saving Rice Production Systems. Nanjing University, China: 15-45.

Trindade, H., Karmali, A., and Pais, S. M. (1998). One- step purification and properties of catalase from *Zantedeschia aethiopica*. *Biochimie*. 40: 1759-1764.

Tolbert, N. E. (1981). Metabolic pathways in peroxisomes and glyoxysomes. *Ann. Rev. Biochem.* 50: 133-157.

Tsai, Y., Hong, C., Liu, L., and Kao, C. H. (2004). Relative importance of Na^+ and Cl^- in NaCl-induced antioxidant systems in roots of rice seedlings. *Physiol. Plant*. 122: 86-94.

Tsugane, K., Kobayashi, K., Niwa, Y., Ohba, Y., Wada, K., and Kobayashi, H. (l999). A recessive arabidopsis mutant that grows photoautotrophically under salt stress shows enhanced active oxygen detoxification. *The Plant Cell*. 11: 1195-1206.

Turrens, J. F., Freeman, B. A., and Crapo, J. D. (1982). Hyperoxia increases H_2O_2 release by lung mitochondria and microsomes" *Arch. Biochem. Biophys*. 217: 411- 421.

Waisel, Y. (1972). Biology of halophytes. *Academic Press*. 13: 395-406.

Williams, M. C. (1960). Effect of sodium and potassium salts on growth and oxalate content of Halogeton. *Plant Physiol*. 35: 500-505.

Wilson, C., Liu, X., and Zeng, L. (2005). Elevated CO_2 influences salt tolerance of rice. Proceedings of the International Salinity Forum, managing saline soils and water: science, technology and soil issues. Riverside, CA. 481-484.

Winston, G. W. and Cederbaum, A. I. (1983). Oxyradical production by purified components of the liver microsomal mixed function oxidase system I: Oxydation of hydroxyl radical scavenging agents. *Journal of Biological Chemistry*. 258: 1508-1513.

Wu, C. J., Cheng, Z. Q., Huang, X. Q., Yin, S. H., Cao, K. M., and Sun, C. R. (2004). Genetic diversity among and within populations of rice granulate from Yunnan of China revealed by RAPD and ISSR markers: Implications for conservation of the endangered species. *Plant Sci*. 167: 35-42.

Xujun, M., Qian, Q., and Dahai, Z. (2005). Expression of a calcineurin gene improves salt stress tolerance in transgenic rice. *Plant Mol Biol*. 58: 483-495.

Xiong, L., Lee, H., Ishitani, M., and Zhu, J. K. (2002). Regulation of osmotic stress responsive gene expression by the *LOS6/ABA1* locus in *Arabidopsis*. *J. Biol Chem.* 277: 8588-8569.

Xiong, L., Schumaker, K. S., and Zhu, J. K. (2002). Cell Signaling during Cold, Drought, and Salt Stress. *Plant Cell*. 67: 165-183.

Yoruk, I. H., Demir, H., Ekici, K., and Savran, A. (2005). Purification and Properties of Catalase from Van Apple (Golden Delicious). *J. Nutritional.* 4(1): 8-10.

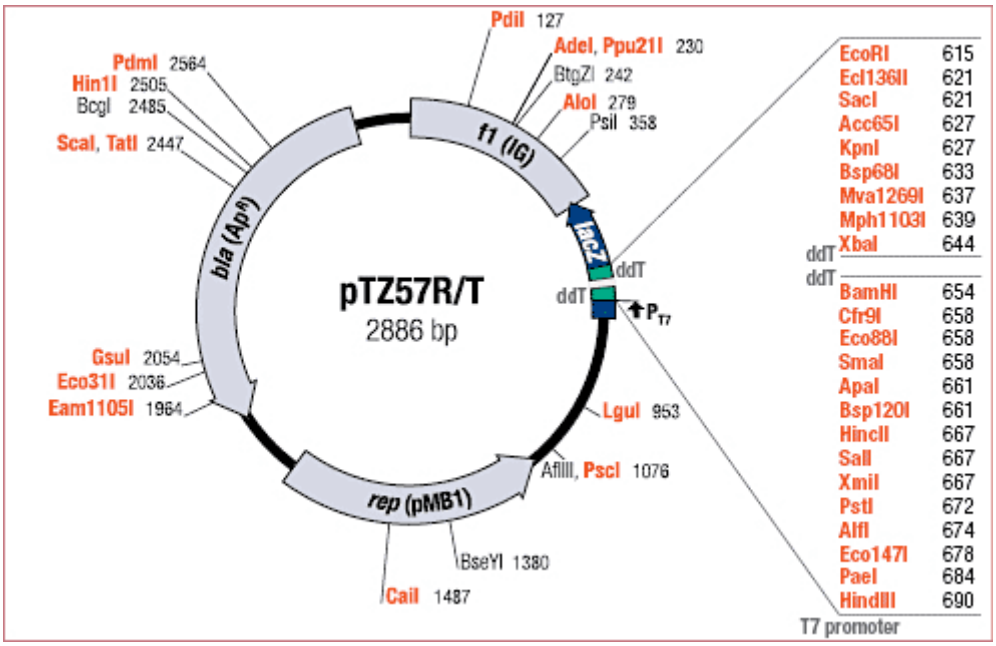
Yumoto, I., Ichihashi, D., Iwata, H., Istokovics, A., Ichise, N., Matsuyama, H., Okuyama, H., and Kawasaki, K. (2000). 9. *J. Bacteriol.* 182(7): 1903-1909.

Zhu, J. K. (2001). Salt and drought stress signal transduction plants. *Annu Rev Plant Biol.* 53: 247-273.

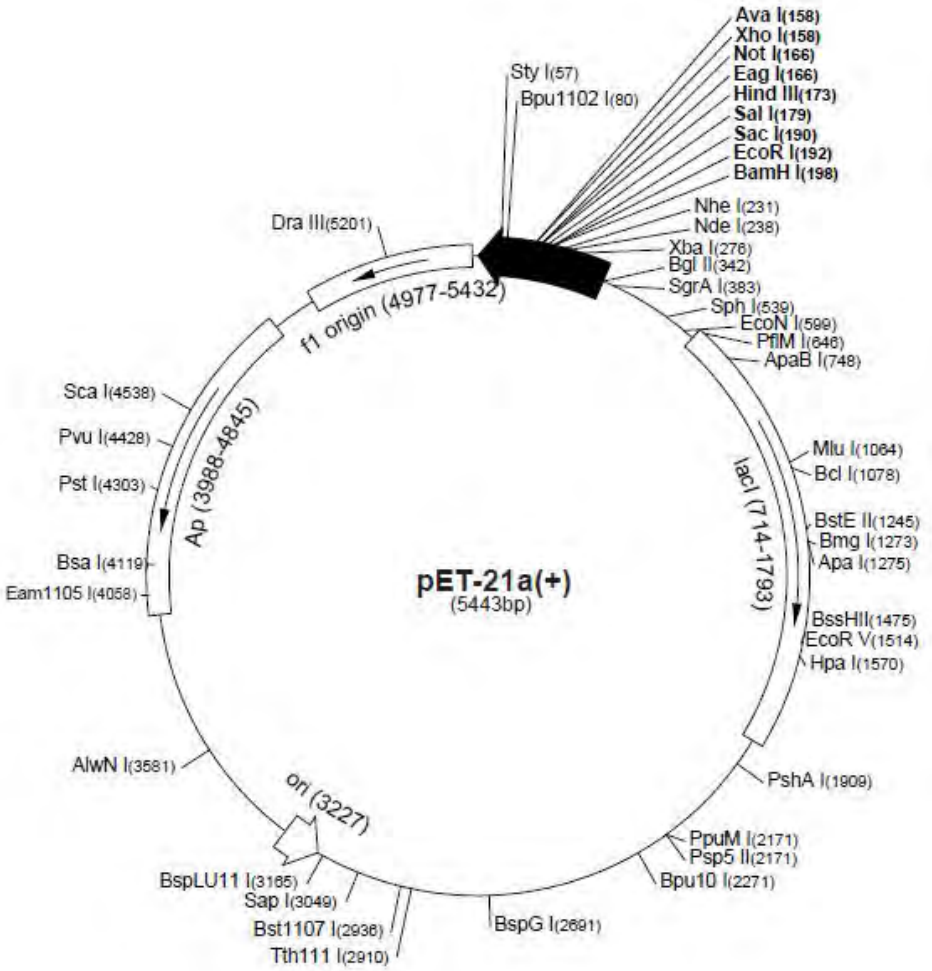
APPENDICES

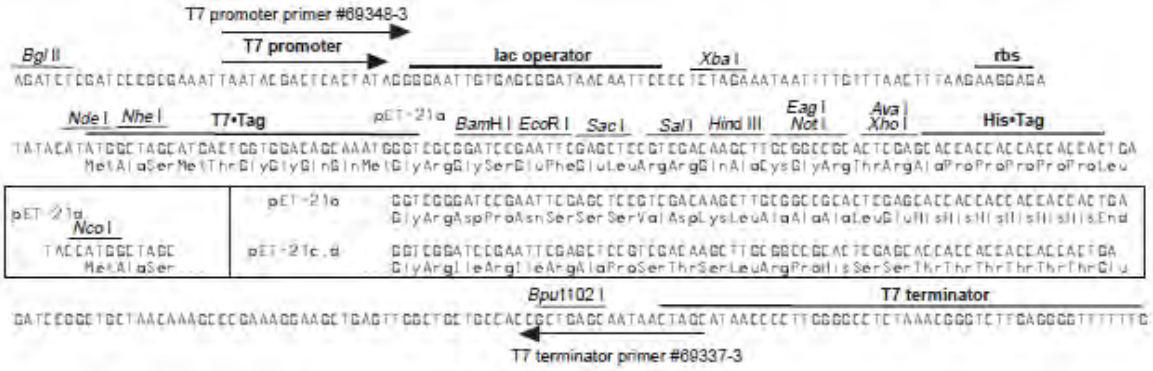
APPENDIX A

Restriction map of pTZ57R/T



Restriction map of pET-21a (+)





APPENDIX B

High Speed plasmid Mini kit protocol (Geneaid, USA)

1. Transfer 1.5 ml of cultured bacterial cells to a microcentrifuge tube. Centrifuge for 1 minute and discard the supernatant
2. Add 200 µl of PD1 Buffer (RNase A added) to the tube and resuspend the cell pellet by vortex or pipetting.
3. Add 200 µl of PD2 Buffer and mix gently by inverting the tube 10 times. Do not vortex to avoid shearing the genomic DNA. Let stand at room temperature for 2 minutes or until the lysate is homologous.
4. Add 300 µl of PD3 Buffer and mix immediately by inverting the tube 10 times. Centrifuge for 10 minutes and place a PD column in a 1.5 ml collection tube. Add the supernatant to the PD column and centrifuge for 1 minute. Discard the flow-through and place the PD column back in the collection tube.
5. Add 400 µl of W1 Buffer into the PD column. Centrifuge for 1 minute and discard the flow-through and place the PD column back in the collection tube. Add 600 µl of Wash Buffer (ethanol added) into the PD column.
6. Centrifuge for 1 minute and discard the flow-through and place the PD column back in the collection tube. Centrifuge again for 3 minutes to dry the column matrix.
7. Transfer the dried PD column to a new microcentrifuge tube. Add 50 µl of Elution Buffer or H_2O into the center of the column matrix. Let stand for 2 minutes and centrifuge for 2 minutes to elute the DNA.

APPENDIX C

Chemical solution

1. Plasmid DNA isolation by alkaline lysis method

Lysis buffer (Buffer P1)

Tris base	1.51 g (50 mM)
$Na_2EDTA \cdot 2H_2O$	0.93 g (10 mM)
H_2O	200 ml

Adjust pH to 8.0 with HCl, adjust the volume to 250 ml with distilled water then add 25 mg (100 μg/ml) RNaseA and store at 4°C

Alkaline-SDS solution (Buffer P2)

1 M NaOH	50 ml (200 mM)
H_2O	150 ml

Mix well and add 12.5 ml of 20% SDS then adjust volume to 250 ml with distilled water and store at room temperature

High salt buffer (Buffer P3)

Potassium acetate	73.6 g (3 M)
H_2O	150 ml

Adjust pH to 5.5 with glacial acetic acid and adjust the volume to 250 ml with distilled water and autoclave for 20 minutes at 121 °C

2. Preparation for protein determination by Bradford, M. M. (1976)

Bradford solution

Coomassie Brilliant Blue (G250)	100 mg
Absolute ethanol	50 ml

Stir the solution in a container protected from light for 2 hours and adjust 100 of 85% phosphoric acid. Bring the volume to 1 L with distilled water and filter

through a sterile, 0.22 μm nitrocellulose filter. Store the solution in a brown glass bottle (usable for several weeks).

3. Preparation for polyacrylamide gel electrophoresis

Resolving gel buffer 8X

Tris base	181.7 g
H_2O	250 ml

Adjust pH to 8.8 with conc. HCl, bring the volume to 500 ml with distilled water. Filter through a 0.22 μm nitrocellulose and store at room temperature.

Stacking gel buffer 4X

Tris base	15.1 g
H_2O	150 ml

Adjust pH to 6.8 with conc. HCl, bring the volume to 250 ml with distilled water. Filter through a 0.22 μm nitrocellulose and store at room temperature.

Acrylamide, 30% A: 0.8% B

Acrylamide	150 g
Bisacrylamide	4 g

Dissolve in final volume of 500 ml in distilled water. Filter through a 0.22 μm nitrocellulose and store at 4 °C in a dark bottle.

Reservoir buffer 10X

Tris base	60.5 g
Glycine	288 g

Dissolve in final volume of 2 L in distilled water. Store at room temperature. Before use, add 2 g of SDS (0.1% SDS).

20% (w/v) SDS

Sodium dodecylsulfate	20 g

Adjust the volumn to 100 ml with distilled water. Stir rapidly to dissolve completely and filter through a sterile, 0.22 μm nitrocellulose filter.

Sample buffer 5X

1M Tris-HCl, pH 6.9	60 μl
20% (w/v) SDS	100 μl
Glycerol	790 μl
2-mercaptoethanol	50 μl
Bromophenol blue	1 mg

Adjust the volume to 1 ml with distilled water. Store at -20 °C in 1 ml aliquots.

10% (w/v) Ammonium Persulfate

Ammonium persulfate	0.5 g

Dissolve in a final volume of 5 ml in H_2O. Store at 4 °C. Prepare fresh every two weeks.

SDS-PAGE

12.5% Separating gel

Acrylamide, 30% A: 0.8% B	5 ml
Separating gel buffer 8X	1.5 ml
20% (w/v) SDS	30 μl
Distilled water	5.4 ml
10% (w/v) Ammonium persulfate	60 μl
TEMED	6 μl

4% Stacking gel

Acrylamide, 30% A: 0.8% B	0.65 ml
Stacking gel buffer 4X	1.25 ml
20% (w/v) SDS	12.5 µl
Distilled water	3 ml
10% (w/v) Ammonium persulfate	37.5 µl
TEMED	2.5 µl

4. Protein staining solution

Staining solution, 1 litre

Coomassie Brilliant Blue R250	2.5 g
Methanol	500 ml
Glacial acetic acid	70 ml
H_2O	430 ml

Dissolve with rapid stirring and store at room temperature.

Destaining solution

Methanol	100 ml
Glacial acetc acid	70 ml
H_2O	830 ml

Dissolve with rapid stirring and store at room temperature.

5. Preparation of NB medium

Reagents:

		Final concentrations (1X)
100X NB Nitrate stock 1 L		
$(NH_4)_2SO_4$	46.3 g	(463 mg/l)

KNO_3	283.0 g	(2,830 mg/l)

100X NB Sulfate stock 1 L

$MgSO_4.7H_2O$	18.5 g	(185 mg/l)
$MnSO_4.H_2O$	1.0 g	(10 mg/l)
$ZnSO_4.7H_2O$	200 mg	(2 mg/l)
$CuSO_4.5H_2O$	2.5 mg	(0.025 mg/l)

100X NB Halide stock 1 L

$CaCl_2.2H_2O$	16.6 g	(166 mg/l)
KI	75.0 mg	(0.75 mg/l)
$CoCl_2.6H_2O$	2.5 mg	(0.025 mg/l)

100X NB PBMO stock 1 L

$KH_2.PO_4$	46.0 g	(460 mg/l)
H_3BO_3	300 mg	(3 mg/l)
Na_2MoO_4	25.0 mg	(0.25 mg/l)

100X NB NaFeEDTA stock 1 L

$FeSO_4.7H_2O$	2.78 g	(27.8 mg/l)
Na_2EDTA	3.78 g	(37.8 mg/l)

Nicotinic acid stock (1 mg/ml)

Dissolve 100 mg of nicotinic acid in sterilized deionized H_2O, adjust volume to 100 ml. Filter sterilize the solution.

Pyridoxine stock (1 mg/ml)

Dissolve 100 mg of pyridoxine in sterilized deionized H_2O, adjust volume to 100 ml. Filter sterilize the solution.

Thiamine stock (10 mg/ml)

Dissolve 1 g of thiamine in sterilized deionized H_2O, adjust volume to 100 ml. Filter sterilize the solution.

Mix the followings:

		Final concentrations (1X)
100X NB Nitrate stock		10 ml
100X NB Sulfate stock		10 ml
100X NB Halide stock		10 ml
100X NB PBMO stock		10 ml
100X NB NaFeEDTA stock		10 ml
Myo-inositol	100 mg	(100 mg/l)
Nicotinic acid stock (1 mg/ml)	1 ml	(1 mg/l)
Pyridoxine stock (1 mg/ml)	1 ml	(1 mg/l)
Thiamine stock (10 mg/ml)	1 ml	(10 mg/l)
Casein hydrolysate	300 mg	(300 mg/l)
L-Proline	500 mg	(500 mg/l)
L-Glutamine	500 mg	(500 mg/l)
Sucrose	30 g	(30 g/l)

Adjust pH to 5.8 and adjust the volume to 1 ml with distilled water. Finally, autoclave and autoclave for 20 minutes at 121 °C.

6. Limpinuntana's nutrient solution

Growing rice

Reagents:

Limpinuntana's solution

		Final concentrations (1X)
300X Solution A (1 L)		
KNO_3	30.333 g	(0.10 g/L)
$Ca(NO_3)_2.4H_2O$	47.230 g	(0.16 g/L)
300X Solution B (1 L)		
$MgSO_4.7H_2O$	12.324 g	(41 mg/L)
$NH_4H_2PO_4$	11.502 g	(38 mg/L)
NaCl	16.577 g	(55 mg/L)
300X Solution C (1 L)		
$FeSO_4.7H_2O$	6 g	(20 mg/Ll)
Na_2EDTA	8 g	(27 mg/L)
300X Solution D (1 L)		
$MnCl_2.4H_2O$	0.4323 g	(1.44 mg/L)
H_3BO_3	0.342 g	(1.14 mg/L)
$Na_2MoO_4.2H_2O$	0.0075 g	(0.025 mg/L)
$ZnSO_4.7H_2O$	0.0264 g	(0.088 mg/L)
$CuSO_4.5H_2O$	0.0117 g	(0.039 mg/L)

1X Limpinuntana's solution (300 ml)

Mix solution A, B, C and D together (1 ml each), then add deionized water to 300 ml and autoclave for 20 minutes at 121 °C.

BIOGRAPHY

Miss Suntareeya Boonkomrat was born on August 21, 1985 in Nakhonratchasima. She graduated with the degree of Bachelor of Science from the Department of Bioprocess, Faculty of Technology and Management, Prince of Songkla University in 2006. She keeps on studying for Master degree of Science at the Biotechnology Program, Faculty of Science at Chulalongkorn University.