CLONING, EXPRESSION, AND USAGE OF CHITINASE FROM *LEUCAENA LEUCOCEPHALA* DE WIT

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy in Biochemistry

Suranaree University of Technology

Academic Year 2001

ISBN 974-533-049-3

ศึกษาการแสดงออกจากการโคลนเอนไซม์ไคติเนส จากกระถินบ้านและการนำไปใช้ประโยชน์

นายมานะ ขาวเมฆ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเคมี
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2544

ISBN 974-533-049-3

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(CLONING, EXPRESSION, AND USAGE OF CHITINASE FROM *LEUCAENA LEUCOCEPHALA* DE WIT)

อาจารย์ที่ปรึกษา : ผศ. ดร. เจมส์ อาร์ เกตุทัต-คาร์นส์, 195 หน้า. ISBN 974-533-049-3

เอนไซม์ไคติเนสที่ได้จากการทำบริสุทธิ์ต้นอ่อนกระถินบ้านมีมวลโมเลกุล 32 กิโลดาลตัน และค่า pI เท่ากับ 7.6 ซึ่งมีความคล้ายกันกับเอนไซม์ไคติเนส กลุ่มที่ 1 จากแหล่งต่าง ๆ ดังนั้นจึงมีการศึกษาการโคลนไคติเนส ด้วยวิธีการตรวจจับไคติเนสจากห้องสมุดสารพันธุกรรมนิวคลีโอไทด์และวิธี 5' RACE พบสารพันธุกรรม 2 ชนิด ที่มีลำดับกรดอะมิโน 323 และ 326 ตัว กรดอะมิโนทั้ง 2 ชนิด มีความเหมือนกันเอง 95% และมีความเหมือนกับไคติเนสจากถั่วแดงมากที่สุดถึง 74% นิวคลีโอไทด์ของกรดอะมิโน 326 ตัว ที่ปราศจากสัญญาณเปปไทด์ถูกนำมาแสดงออกในเซลล์แบคทีเรียที่รับเวคเตอร์ pET23d และ pET32a โปรตีนที่ได้จากเวคเตอร์ pET23d มีขนาด 32 กิโลดาลตัน ซึ่งส่วนใหญ่จะอยู่ในตัวเซลล์แบคทีเรีย ส่วนเวคเตอร์ pET32a จะผลิตรีคอมบิแนนท์โปรตีนที่มีขนาด 46 กิโลดาลตัน ประกอบด้วยไทโอรีดอกซิน 14 กิโลดาลตันและไคติเนส 32 กิโลดาลตัน รีคอมบีแนนท์โปรตีนนี้ถูกทำให้บริสุทธิ์ด้วยนิเกิลเอ็นทีเอคอลัมน์และทำให้เป็นเอ็นไซม์ไคติเนสที่บริสุทธิ์โดยการตัดด้วยเอ็นเทอโรไคเนส ไคติเนสบริสุทธิ์ที่ได้จากการตัดด้วยเอ็นเทอโรไคเนสมีมวลโมเลกุล 32 กิโลดาลตัน ค่า pI เท่ากับ 7.5 โดยมี pH และอุณหภูมิที่เหมาะสมในการทำปฏิกิริยาคือ 4.5 และ 55 องศาเซลเซียสตามลำดับ เอนไซม์นี้สามารถย่อยคอลลอยอัลดัลไคตินได้ดีที่สุด และจากการตรวจสอบชนิดของรีคอมบิแนนท์ไคติเนสพบว่าเป็นเอ็นโดไคติเนส มีค่า K_m และ k_{cat} ต่อการย่อยคอลลอยอัลดัลไคตินเท่ากับ 7.60 มิลลิกรัมแห้งของไคตินต่อมิลลิลิตรและ 8.28 ครั้งต่อนาที สำหรับพาราไนโตรเพนิลไตรอะซิติลกลูโคซามิไนด์เท่ากับ 48.78 ไมโครโมลาร์ และ 35.42 ครั้งต่อนาที และสำหรับเตตระอะซิติลไคโตเตตระโอสเท่ากับ 2.05 ไมโครโมลาร์ และ 95.22 ครั้งต่อนาทีตามลำดับ ในการทดลองการยับยั้งเชื้อราของรีคอมบีแนนท์โปรตีนพบว่าสามารถยับยั้งการเจริญเติบโตของเชื้อราได้ 13 ชนิด

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ลายมือชื่อนักศึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

MANA KAOMEK: CLONING, EXPRESSION, AND USAGE OF CHITINASE FROM *LEUCAENA LEUCOCEPHALA* DE WIT

THESIS ADVISOR: ASSISTANT PROFESSOR JAMES R. KETUDAT-CAIRNS, Ph.D., 195 PP. ISBN 974-533-049-3

Chitinase which was purified from *L. leucocephala* seedlings had a molecular weight of approximately 32 kDa and apparent pI of 7.6, similar to many class I chitinases. So cDNA were cloned using a combination of RT-PCR with homology-based 3′ RACE primers, λgt11 cDNA library screening and 5′ RACE to produce two closely related chitinase cDNA sequences. The derived chitinase precusor proteins (323 and 326 amino acids) exhibit 95% identity with each other and 74% identity with kidney bean chitinase precursor. The cDNA encoding a mature protein of 302 amino acids was expressed from pET23d and pET32a vectors in *E. coli* BL21 (DE3) and Origami (DE3), respectively. Induction of expression at 15°C resulted in an approximately 32 kDa protein from pET23d. The expressed protein was present in both insoluble inclusion bodies and soluble form. Using pET32a, the *E.coli* produced the recombinant protein of approximately 46 kDa, which contained 14 kDa of thioredoxin protein and 32 kDa of chitinase. It was purified using a Ni-NTA superflow column and was cleaved with enterokinase to produce free chitinase. The isoelectric point of free chitinase was 7.5. The enzyme showed an optimum pH of 4.5 and an optimum temperature 55°C. The best polysaccharide substrate was colloidal chitin. The recombinant chitinase displayed endochitinase like activities on *p*-nitrophenyl-triacetylchitotriose and the K_m and k_{cat} were 7.60 mg dry weight chitin/ml and 8.28 min^{-1} with colloidal chitin, 48.78 μM and 35.42 min^{-1} with *p*-nitrophenyl-*N,N′,N″*-triacetyl-chitotriose, 2.05 μM and 95.22 min^{-1} with *N,N′,N″,N‴*-tetraacetylchitotetraose respectively. The recombinant chitinase effectively inhibited growth of 13 fungal strains.

School of Biochemistry

Academic Year 2001

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Acknowledgements

I would like to express my deepest and sincere gratitude to my advisor, Asst. Prof. Dr. James Ketudat-Cairns for his kindly providing me a good opportunity to study in this field, supervision, laboratory facilities, encouragement, and valuable advice.

Deep appreciation and admiration to Assoc. Prof. Dr. Poonsook Sriyotha and Prof. Dr. Tatsuhito Fujimura for their valuable advice, use for their laboratory facilities, kindness and encouraging guidance.

I also wish to thank Prof. Dr. Jisnuson Svasti, Asst. Prof. Dr. Mariena Ketudat-Cairns, Dr. Kouichi Mizuno, Assoc. Dr. Misako Kato Mizuno, and for their guidance, use of their laboratory facilities and suggestions. I also would like to thank all of my friends in Prof. Dr. Fujimura's laboratory for their help and warmth toward me throughout. They were always nice and friendly. Special thanks are extended to Umezawa, Kato, Furuzawa, Yoshie, and Koga san for their kindness and help to using the automated DNA sequencing facility and isotope building.

I would like to thank the Plant Pathology and Microbiology Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok, Thailand and Department of Biology, Faculty of Science, Chiang Mai University, Chaing Mai, Thailand for fungal stains. I want to express my appreciation to Miss Kuntika Vechklang, Dr. Peiangpob Mounnumprang, Dr. Paladorn Suwannapho, Anirut Laudsong, and for their invaluable guidance and friendly encouragement.

I would like to express my deepest appreciation and sincere gratitude to my dear parents for their love, devotion, understanding and encouragement throughout my entire study.

Finally, I wish to express my special thanks to the Scholarship of Secondary Education Quality Improvement project of Rajabhat Institute Pecthburiwittayalongkorn for offering the scholarship which enabled me to continue my advanced studies in Suranaree University of Technology; the Association of International Education, Japan (AIEJ) for supporting research work at the University of Tsukuba, 1 September 1998-31 August 1999; and the Shell Centenary Scholarship Fund for offering a scholarship supporting my research work.

Mana Kaomek

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List of Abbreviations

A	Absorbance
Ac	Acetyl moiety
Amp	Ampicillin
ATP	Adenosine triphosphate
bp	Base pairs
BSA	Bovine serum albumin
°C	Degrees Celsius
cDNA	Complementary deoxynucleic acid
CRD	Cysteine-rich domain
CTE	Carboxy-terminal extension
CTP	Cytosine triphosphate
da	Degree of acetylation
DNA	Deoxyribonucleic acid
dNTPs	dATP, dCTP, dGTP and dTTP
EDTA	Ethylene diamine tetraacetic acid
g	Gravitational acceleration
GTP	Guanidine triphosphate
GlcN	Glucosamine
GlcNAc	*N*-acetyl-D-glucosamine
$(GlcNAc)_2$	*N*,*N*¢-diacetylchitobiose
$(GlcNAc)_3$	*N*,*N*¢,*N*¢¢-triacetylchitotriose
$(GlcNAc)_4$	*N*,*N*¢,*N*¢¢,*N*¢¢¢-tetraacetylchitotetraose
(m, μ, n) g	(Milli, Micro, Nano) gram
h	Hour
ha	Hectare
HPLC	High Performance Liquid Chromatography
HR	Hinge region (glycine-proline rich)

List of Abbreviations (Continued)

IEF	Isoelectric focusing
IPTG	Isopropyl-*b*-D-thio-glactopyranoside
(k) Da	(Kilo) dalton
kg	Kilogram
min	Minute
(m,μ, η) M	(Milli, Micro, Nano) Molar
(m, μ) l	(Milli, Micro) Liter
mRNA	messenger ribonucleic acid
MW	Molecular weight
Mur-NAc	*N*-acetylmuramic acid
4-Mu-GlcNAc	4-Methylumbelliferyl-*N*-acetyl-D-glucosamine
4-Mu- $(GlcNAc)_2$	4-Methylumbelliferyl-*N*,*N*¢-diacetylchitobiose
4-Mu- $(GlcNAc)_3$	4-Methylumbelliferyl-*N*,*N*¢,*N*¢¢-triacetyl-chitotriose
4-Mu- $(GlcNAc)_4$	4-Methylumbelliferyl-*N*,*N*¢,*N*¢¢,*N*¢¢¢-tetra-acetylchitotetraose
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
pI	Isoelectric point
pmol	Picomole
PMSF	Phenylmethylsulfonylfluoride
pGlcNAc	*p*-nitrophenyl-*N*-acetyl-D-glucosamine
pNP	*p*-nitrophenol
PVPP	Polyvinylpolypyrrolidone
RNase	Ribonuclease
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate

List of Abbreviations (Continued)

s, sec	Second
SP	Signal peptide
TEMED	*N*,*N*,*N*¢,*N*¢-tetramethylenediamine
Tris	Tris-(hydroxymethyl)-aminoethane
TTP	Thymidine triphosphate
UV	Ultraviolet
U	Unit, μmol/min
v/v	Volume/volume
w/v	Weight/volume
w/w	Weight/weight

Chapter I
Introduction

1.1 Chitin

Chitin is one of the most abundant polysaccharides in nature. Chitin was first discovered by Braconnot in 1811 from mushroom by treating *Agaricus valvaceus* with diluted warm alkali and then isolating (Muzzarelli, 1976). Natural chitin is major component of the exoskeleton of insects and other arthropods, and the cell walls of most fungus, such as the *Ascomycetes*, *Basidiomycetes* and *Deuteromycetes*. In fungi, chitin constitutes 3-60% of the cell wall (Bartnicki-Garcia, 1968; Wessels and Sietsma, 1981). Most chitin studies have been based on crab and shrimp chitin because these are widely available. Chitin exhibits various biological activities and has been used in agriculture, industry, and medicine (Nishimura *et al*., 1984; Suzuki *et al*., 1986).

1.1.1 Chitin structure and properties

Chitin is a linear β-1,4-linked polymer of *N*-acetyl-D-glucosamine ((2-acetamido-2-deoxy)-D-glucose or GlcNAc) units (Figure 1.1) which are arranged in an anti-parallel (α-chitin) (Figure 1.2), parallel (β-chitin) (Figure 1.3), or mixed stands (γ-chitin), with the α-conformation being the most abundant in nature. The difference among α-chitin, β-chitin, and γ-chitin is the hydrogen bonding in and between sheets. The α-chitin has hydrogen bonds between sheets to give a strong structure that has been found in fungi, insects, and crustaceans. The β-chitin has hydrogen bonds within the sheets (only) and has been found in the spines of *Aphrodite*, ink of *Luligo*, tubes of *Pogonophora*, and spines of diatom. The γ-chitin which is mixed with two to one parallel to anti-parallel, has been found in the stomach of *Luligo* (Gardner and Blackwell, 1975; Blackwell, 1988).

Chitin is soluble in anhydrous formic acid and is dispersed in concentrated aqueous solutions containing certain lithium or calcium salts. Chitin can also be dissolved with some depolymerization in concentrated mineral acids, but is insoluble in water, organic solvents, and ammonium reagents. Chitin is less reactive than cellulose because of its general insolubility. It is

slowly deacetylated to form products collectively termed chitosan (poly-*b*-1,4-(2-amino-2-deoxy)-D-glucose). Chitan, completely *N*-deacetylated chitin, is obtained by retreatment of chitosan with alkali or fractionational precipitation of aminopolymer as its hydrochloride salt. Chitin is difficult to acetylate or methylate by methods commonly used to form derivatives of cellulose, since it does not swell in the usual reaction media (Ward and Seib, 1970).

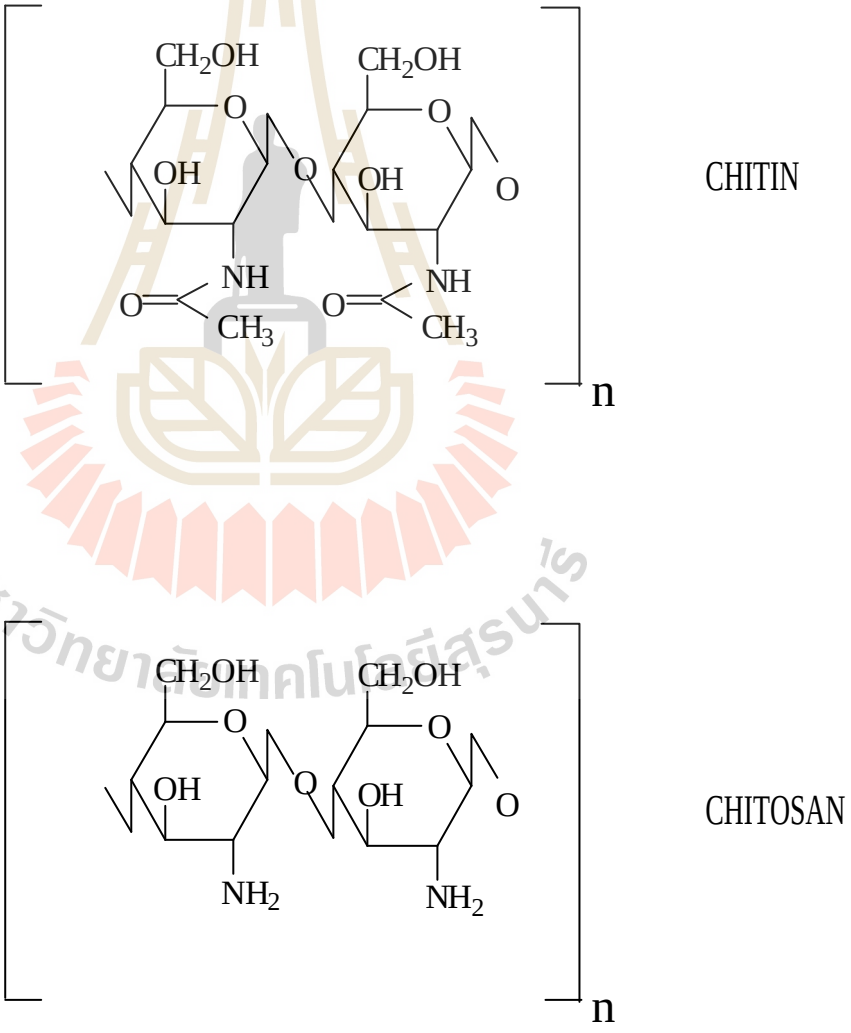

Figure 1.1 Structures of Chitin and Chitosan

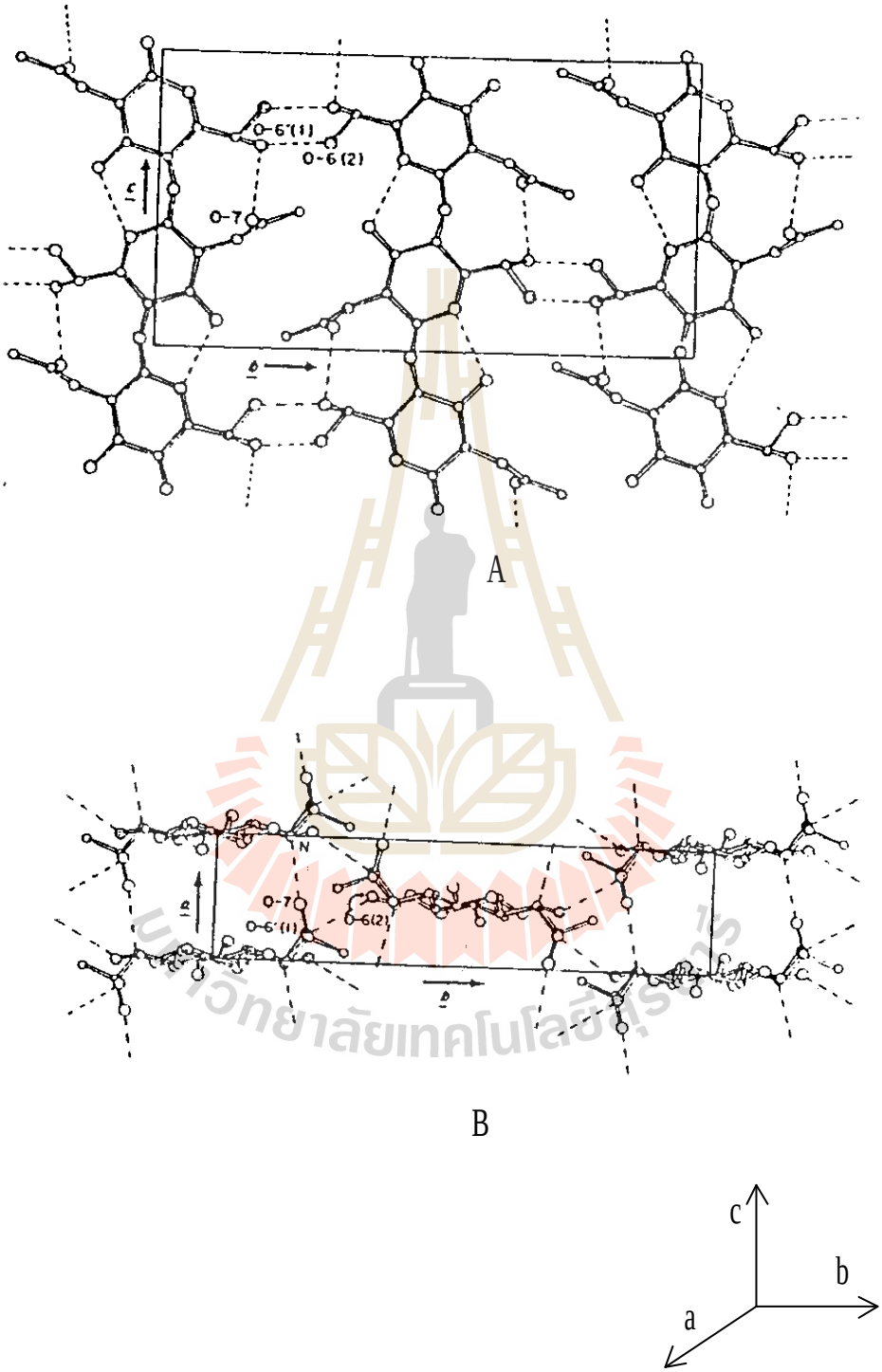

Figure 1.2 Structure of antiparallel α-chitin. A is the bc projection and B is the ab projection (Blackwell, 1988)

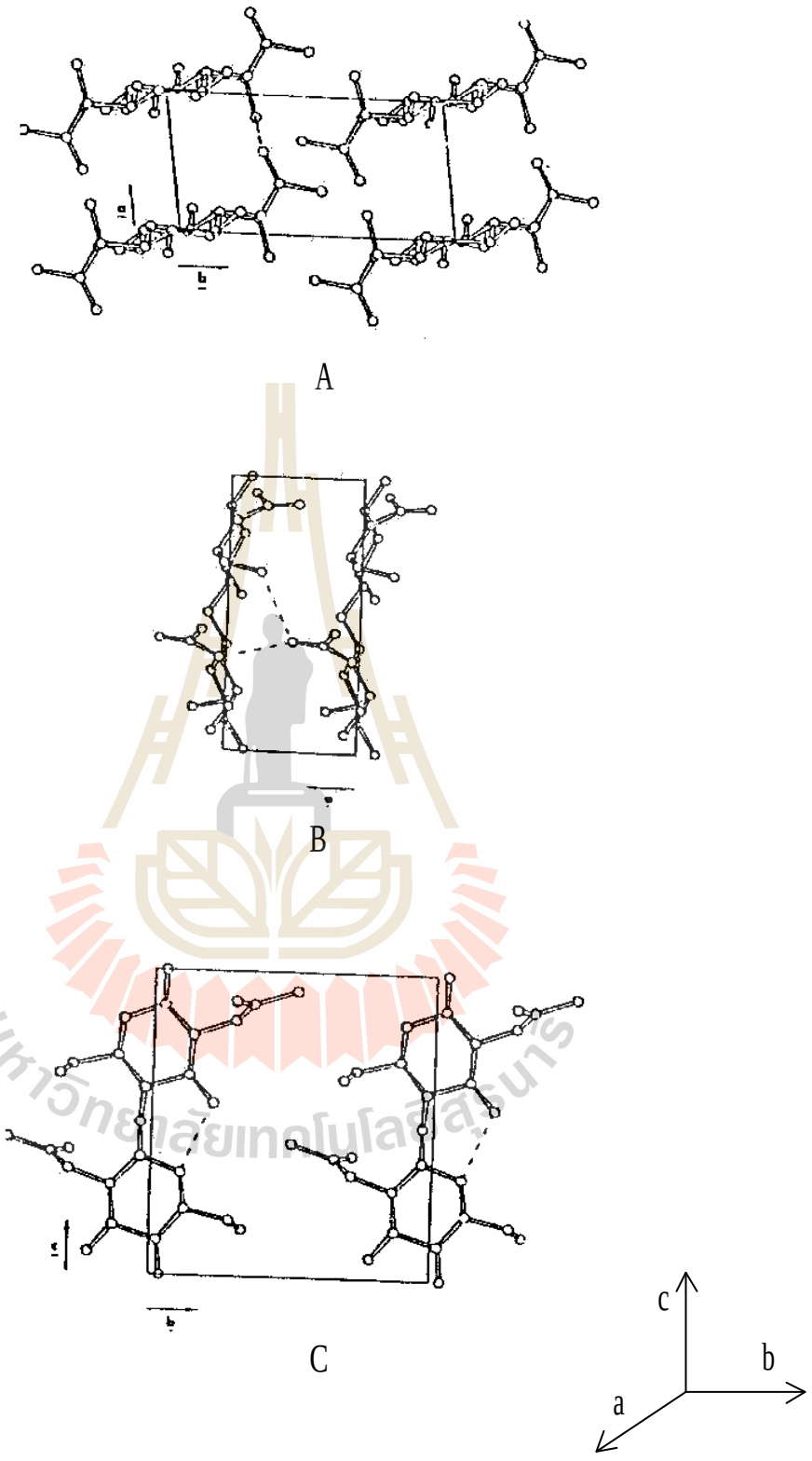

Figure 1.3 Structure of parallel β-chitin. A is the ab projection, B is the ac projection, and C is the bc projection. (Blackwell, 1988)

Both chitin and chitosan are copolymers of GlcNAc and glucosamine (GlcN). The copolymer is called chitin when it contains less than 7% nitrogen and chitosan when nitrogen content exceeds 7% (Davis and Hayes, 1988). The difference between chitin and chitosan is the degree of acetylation of D-glucosamine residues. Usually those of chitin are acetylated more than 60% and those of chitosan are acetylated less than 40%. In nature, chitin is covalently bonded to proteins which may also serve to link chitin to other carbohydrates. It may also be associated with salts, such as calcium carbonate in the shells of crustaceans.

1.1.2 Sources of chitin

Chitin is found in fungal cell walls, insect exoskeletons, shells of crustaceans, mycelial yeasts, green algae, and several species of brown and red algae. In plants, it may serve as an alternative to cellulose and, in animals, as an alternative to collagen. Chitin is widespread in the animal kingdom, occurring in the form of sheets as in the cuticles of arthropods, molluscs, and annelids, or in the form of well-oriented fibres as in the mandibular tendon of lobsters (Ward and Seib, 1970). Chitin is not present in bacteria, *Actinomycetes*, and higher plants (Flach *et al*., 1992).

1.1.3 Enzymatic hydrolysis of chitin and chitosan

The enzyme which hydrolyzes chitin is chitinase, and for chitosan is chitosanase. The difference of two enzymes are not well clarified at the molecular level (Ando *et al*., 1992). Only zygomycetous fungi have cell walls composed of both chitin and chitosan (Yabuki *et al*., 1988).

Enzymatic hydrolysis of chitin to free monomer is formed by the binary hydrolases; chitinase (poly-*b*-1,4-(2-acetamido-2-deoxy)-D-glucoside glycanohydrolase or chitin glycanohydrolase) and *N*-acetylglucosaminidase (*N*,*N*¢-acetylchitobiase) or chitobiase. These enzymes are found in a wide variety of microorganism, plant and animal species. Chitin is first hydrolyzed by chitinase to low-molecular weight oligosaccharides (Fukamizo *et al*., 1986). Those oligomers are hydrolyzed to the products *N*,*N*¢-diacetylchitobiose (chitobiose) with small amount of GlcNAc products. Endo-*N*-acetylglucosaminidase splits chitobiose into two molecules of GlcNAc. Exo-*N*-acetylglucosaminidase also hydrolyzes glycoside bonds of oligosaccharides from their terminal non-reducing ends and releases GlcNAc products. Alternatively, chitin can be

degraded by deacetylation and further processing. Three enzyme systems, endo-chitosanase, exo-chitobiohydrolase (exo-chitosanase), and chitobiase (endo-glucosaminidase) or exo-glucosaminidase are involved in this pathway. Chitosan is hydrolyzed by chitosanase to low-molecular weight oligosaccharide. Those oligomers are hydrolyzed to the products chitobiose with small amount of GlcN products. Endo-glucosaminidase splits chitobiose into two molecules of GlcN. Exo-glucosaminidase also hydrolyzes glycoside bonds of oligosacharides from their terminal non-reducing ends and release GlcN products (Figure 1.4).

1.2 Overview of chitinases

Chitinases (EC 3.2.1.14) are enzymes that catalyze the hydrolysis of the β-1,4-*N*-acetyl-D-glucosamine linkages of the polysaccharide chitin. Chitinases are made by chitin-producing organisms in which they have essential functions during growth and molting (Kramer *et al*., 1993), but also by bacteria, which degrade chitin for use as a carbon and energy source (Morgavi *et al*., 1994), and by plants, which produce chitinase to defend themselves against pathogenic fungi (Boller, 1988; Bowles, 1990; Linthorst, 1991). Chitinases have two types, endochitinase and exochitinase. Endochitinase activity results in random cleavage at internal points in the chitin chain to multimers of *N*-acetyl-D-glucosamine that can dissolve in water, such as chitobiose, chitotriose, and chitotetraose (Figure 1.5), while exochitinase (chitobiosidase) activity is a progressive action at non-reducing ends with the subsequent release of diacetylchitobiose units (Figure 1.6) (Robbins *et al*., 1988). Chitobiase hydrolyzes chitobiose. Most plant chitinases are endo-type chitinases (which randomly hydrolyze internal β-1,4-*N*-acetyl-D-glucosidic linkages of chitin), producing chitooligosaccharides from chitin.

Some chitinases also display more or less pronounced lysozyme activity (EC 3.2.1.17), corresponding to cleavage of glycosidic bond between the C1 of *N*-acetylmuramic acid (Mur-NAc), and the C4 of *N*-acetylglucosamine in the bacterial peptidoglycan (Jolles and Jolles, 1984).

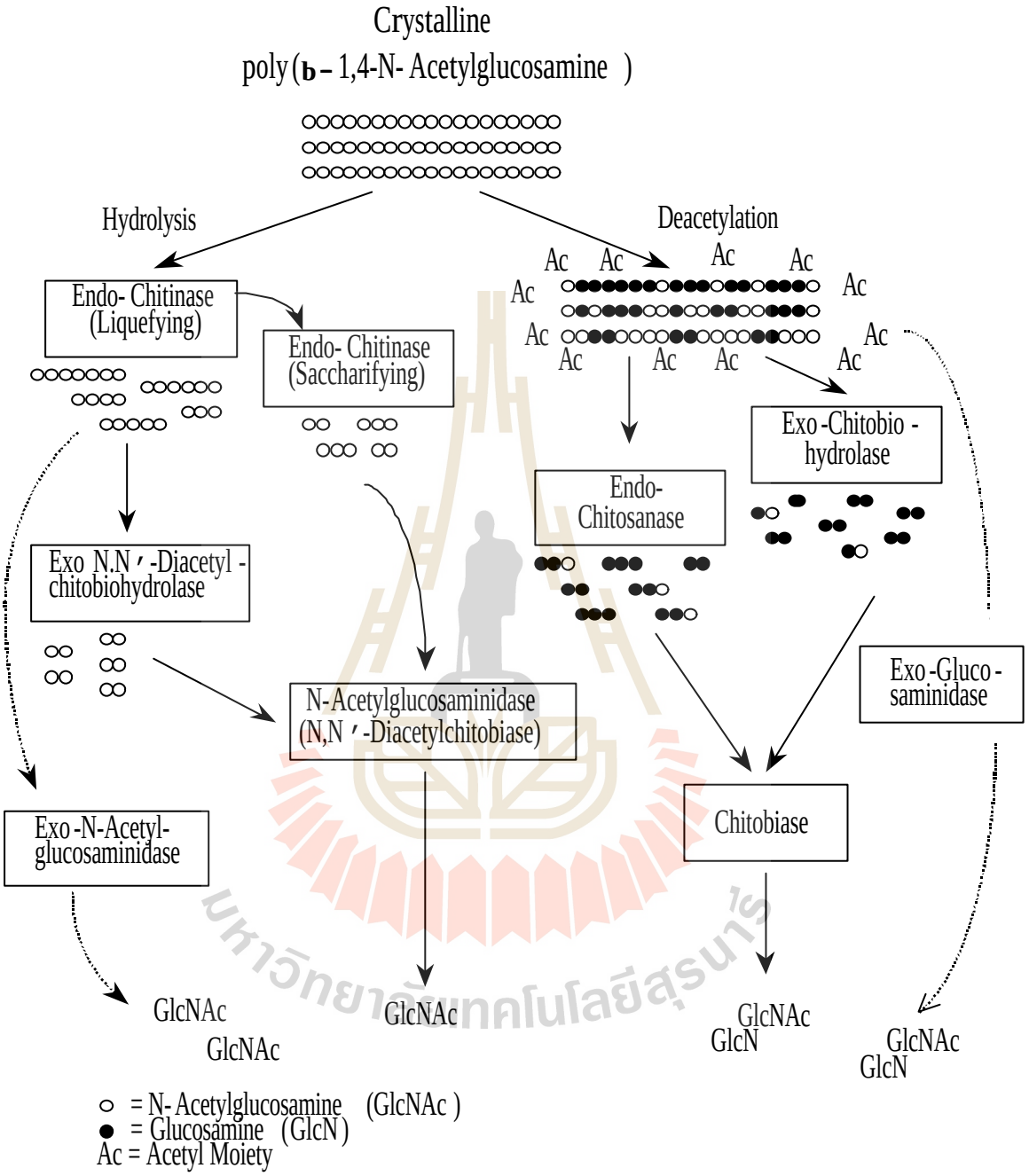

Figure 1.4 Paths for enzymatic degradation of chitin. Black lines are the confirmed enzyme steps and dotted lines are the hypothetical hydrolysis (Davis and Eveleigh, 1984).

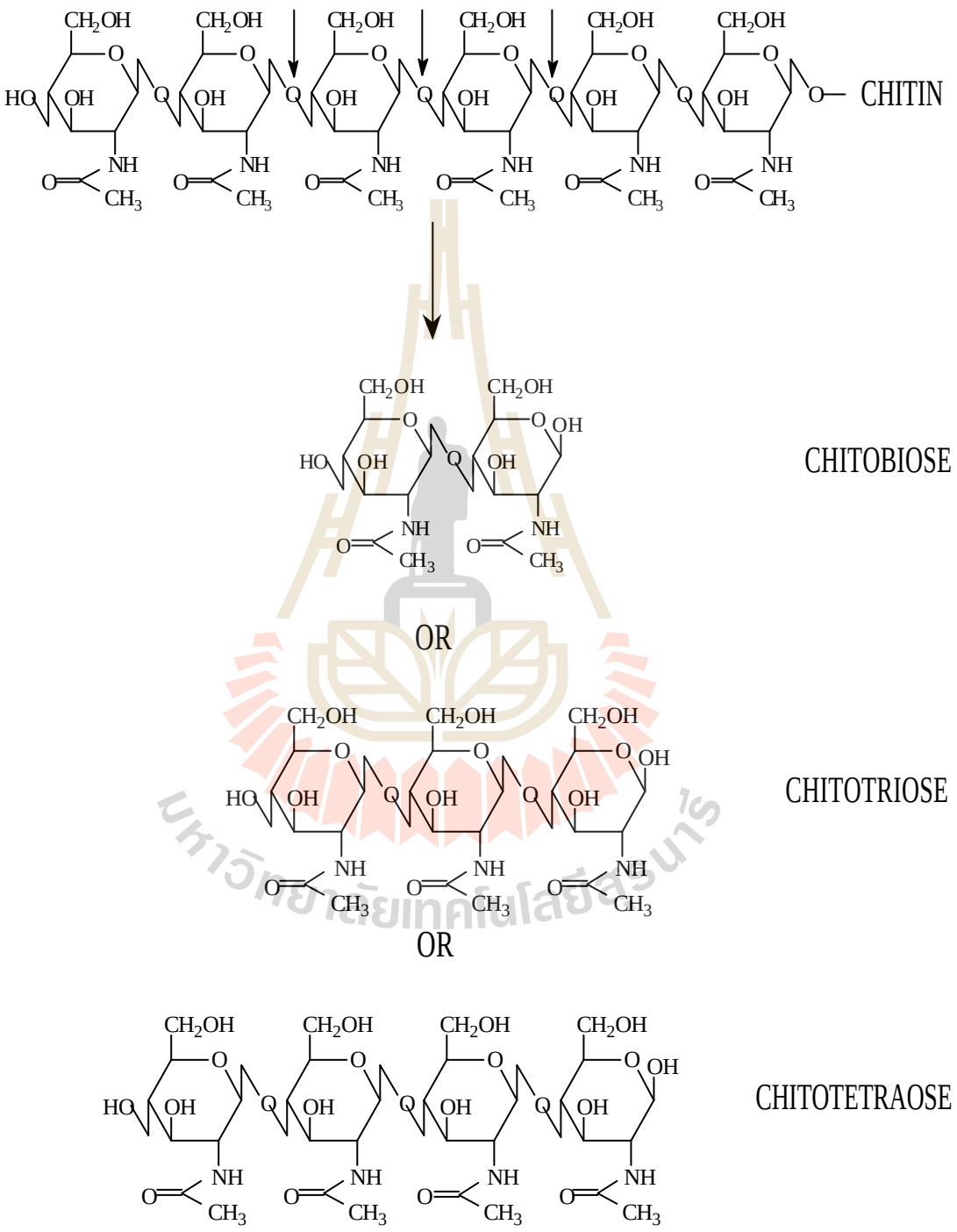

Figure 1.5 Endochitinase hydrolysis at the *b*-1,4-glucosidic bond of chitin to produce the chitobiose, chitotriose and chitotetraose.

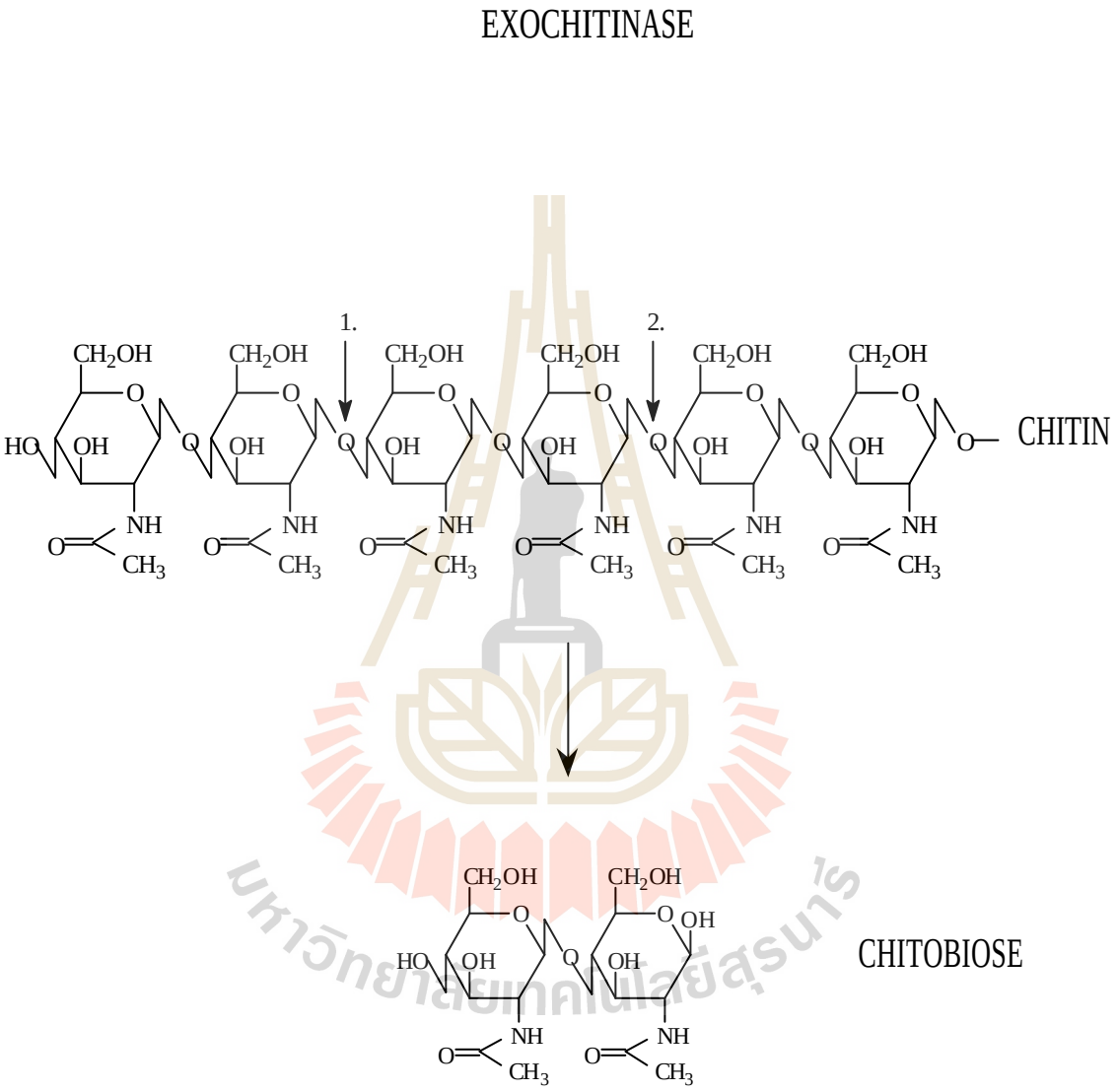

Figure 1.6 Exochitinase hydrolysis at the *b*-1,4-glucosidic of chitin to produce the chitobiose production.

1.3 Classification of chitinases based on amino acid sequence

On the basis of amino acid sequences, chitinases have been divided into six classes (Figure 1.7) (Colinge *et al*., 1993; Menis *et al*., 1994; Shishi *et al*., 1990), which fall into two unrelated families, families 18 and 19 of the glycosyl hydrolases (Henrissat and Bairoch, 1993; Menis *et al*., 1994). Family 18 chitinases have also been classified as classes III, V, and VI which have been found in a wide range of species, including bacteria, fungi, plants, insects, mammals, and viruses (Ayres *et al*., 1994; Hakala *et al*., 1993; Henrissat, 1990; Kramer *et al*., 1993). Within this family, the prokaryotic chitinases show only two short sequence motifs that are homologous to the eukaryotic enzymes. Family 19 chitinases have been found mainly in plants so far. Chitinases in family 19 include classes I, II, IV.

Class I chitinases have both acidic and basic isoforms which are structurally homologous and are primarily localized in the central vacuole (Neuhaus *et al*., 1991). They contain a cysteine-rich domain of about 40 amino acids at their N-terminal, similar to the rubber lectin hevein, and a highly conserved catalytic domain. The hevein domain is thought to serve as a chitin-binding site. There is a variable glycine- and proline-rich spacer region between the hevein and the catalytic domains. They have a leucine-rich or valine-rich signal peptide (Figure 1.7). Class I chitinases have been divided into two subclasses. Class Ia chitinases have basic isoelectric point and the carboxy-terminal extension at C-terminal. Class Ib chitinases have acidic isoelectric point. Examples of class I chitinases have been found in *Arabidopsis*, barley, bean, pea, peanut, tobacco, and tomato (Herget *et al*., 1990; Samac *et al*., 1990; Shishi *et al*., 1990; Swegle *et al*., 1989; Vad, 1991).

Class II chitinases lack the hevein domain (cysteine-rich domain) (Collinge *et al*., 1993). However, the amino acid sequences of the catalytic domains of class II chitinases are similar to those of class I chitinases (Figure 1.7). The primary structure of class I and class II chitinases are 60-65% identical, while there is more than 70% identity within the classes. Class II chitinases are usually found in the extracellular fluid of leaves and in the culture medium of cell suspensions, suggesting that they are localized in the apoplastic compartment, consistent with a major function in defense (Shinshi *et al*., 1990). Class II chitinases have been divided into two subclasses. Class IIa chitinases have basic isoelectric point and a deletion in the catalytic domain. Class IIb chitinases have acidic isoelectric point (Flach *et al*., 1992). Examples of class II chitinases have

been found in *Arabidopsis*, barley, and PR-P and PR-Q protein from tobacco (Samac *et al*., 1990; Leah *et al*., 1991; Linthorst *et al*., 1990).

Class III chitinases are structurally unrelated to classes I, II, and IV (Figure 1.7). They can be acidic and basic isoelectric points (Beintema, 1994). Class III chitinases are located in the extracellular and intercellular fluids. They have high sequence identity with chitinases from yeast and zygomycetes. The primary structures of class III chitinases within yeast and zygomycetes are 45-74% identical (Henrissat, 1990). Examples of class III chitinases have been found in cucumber, sugar-beet and tobacco (Lawton *et al*., 1992; Metraux *et al*., 1989; Mikkelsen *et al*., 1992).

Class IV chitinases contain a chitin binding domain and a conserved main structure that resembles that of class I enzymes, but are significantly smaller due to deletion of four regions (one in the chitin-binding domain, 2 in catalytic domain and one at the end, as shown in Figure 1.7). Class IV chitinases are 35-50% identical with the class I and class II chitinases, and more than 60% among themselves (Beintema, 1994). Examples have been found in bean, rape and sugar-beet (Margis-Pinheiro *et al*., 1991; Mikkelsen *et al*., 1992; Rasmussen *et al*., 1992).

Class V chitinases are structurally unrelated to classes I, II, and IV (Figure 1.7). They have a duplicated N-terminal lectin domain with an amino acid sequence which is similar to the stinging nettle lectin precursor (Morgavi *et al*., 1994). They share some similarity with Class III bacterial exochtinase (Melchers *et al*., 1994). Examples have been found in *Untica dioica*, and Nettle (Lemer and Raikhel, 1992).

Classes VI chitinases have no sequence similarity to classes I, II, and IV chitinases (Figure 1.7), but have significant amino acid sequence similarity to Class III bacterial exochitinase (Menis *et al*., 1994). Examples have been found in beet and tobacco (Melchers *et al*., 1994; Mikkelsen *et al*., 1992).

The amino acid composition (residues/mole) and molecular weight of chitinase has been used to classify chitinases to a known class by comparing these properties to those of known classes of chitinases. The amino acid composition has been used to compare between purified protein and deduced cloned chitinases (Wang and Chang, 1997). The amino acid compositions of class I chitinases have a high content of Asp, Gly, Cys, and Pro such as in tobacco and carrot (*Zhang, et al*., 1996). Plant class I chitinases have 15-17 cysteine residues/ mol, class II of 5-8 cysteine residues/ mol, and class III of 1-6 cysteine residues/ mol (Yamagami *et al*., 1998),

as shown in table 1.1. Class I chitinases also have high amounts of Arg, Ilu, Gly, and Ser as the major amino acid. Class II chitinases have Ala and Thr as the most abundant amino acids. Class III chitinases have Glu, Leu, Lys, and Val as the most abundant amino acids (Wang and Chang, 1997). The amino acid composition has been used to determine whether the enzyme isoforms have on the average high or low isoelectric point (Molano *et al*., 1979).

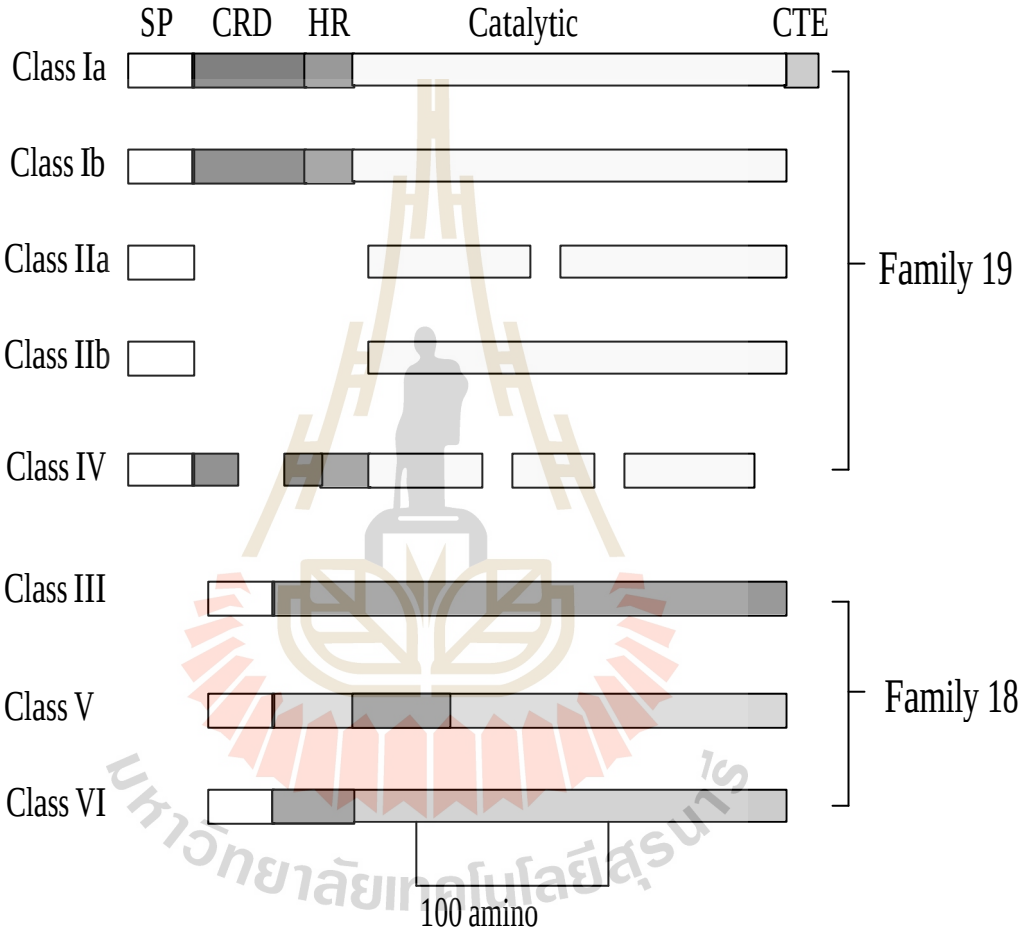

Figure 1.7 Classification of chitinases based on amino acid (Colinge *et al*., 1993; Menis *et al*., 1994; Shishi *et al*., 1990; Verburg *et al*., 1993).

Table 1.1 Comparison of amino acid composition (numbers/protein) of plant class I endochitinases, class II chitinases, class III chitinases, and class IV chitinases

Amino acid	Potato Leaves Class I	Tobacco Class I	Rice Class II	Rye seed Class II	Gladiolus bulb Class III	Pokeweed leaf Class III	Sugar beet Class IV	Corn Class IV
Asp	40	36.02	29	25	39	36	22	30
Thr	12	15.62	20	14	19	12	13	9
Ser	23	26.15	18	14	27	23	28	11
Glu	18	20.08	19	12	20	25	18	20
Pro	22	23.27	10	14	9	12	10	11
Gly	44	47.50	31	29	18	40	28	46
Ala	21	22.31	35	35	17	16	30	28
Cys	17	18.17	7	7	1	6	16	14
Val	9	8.61	13	10	22	13	16	12
Met	3	4.14	4	2	-	2	4	3
Ile	15	15.94	12	10	20	13	13	9
Leu	14	14.98	14	11	16	21	11	6
Tyr	11	11.79	13	12	13	10	12	13
Phe	15	14.98	13	13	21	15	19	14
His	4	4.14	2	6	4	3	4	5
Lys	8	13.48	5	8	12	13	3	8
Arg	18	19.13	12	15	12	7	15	10
Trp	8	8.60	4	6	4	7	3	4
Total	302	324.91	261	243	274	274	265	253
MW	32,400	34,000	27,643	26,098	30,714	29,473	28,365	26,492

(Gaynor, 1988; Huynh, *et al*., 1992; Kim et al., 1998; Mikkelsen *et al*., 1992; Shishi *et al*., 1990; Yamagami and Funatsu, 1993, 1995; Yamagami et al., 1997)

1.4 Sources of chitinases

1.4.1 Animal chitinases

1.4.1.1 Fish chitinases

Chitinases have been described in the digestive tract of some fish feeding on Dover sole or on krill. A chitinase was purified from the stomach of red sea bream. The molecular weight was about 46,000 and pI of 8.3. The optimum temperature and pH were 50°C and 5.5, respectively. The activity was strongly inhibited by Hg^{2+}, Fe^{2+}, and Sn^{2+}. The chitinase was synthesized within the egg and could be induced during the larval period by the consumption of exogenous foods. Japanese eel was also found to have chitinase in the stomach. However, the digestive tract of eel also contained chitin-decomposing bacteria (Flach *et al*., 1992).

1.4.1.2 Insect chitinases

Most insect chitinases were found in the alimentary canal, integument, haemolymph, and moulting fluid. Several insect chitinases are glycoproteins (Funke *et al*., 1989). *Bombyx mori* chitinase is synthesized as an inactive precursor which is activated by limited proteolysis (Koga *et al*., 1989). The isoelectric points of insect chitinases range between 4 and 5, the optimum pH between 4.5-6.0 and K_m values with chitin between 0.2 mg/ml and 5 mg/ml (Kramer and Koga, 1986).

1.4.1.3 Marine invertebrate chitinases

Chitinases have been characterized in marine invertebrates, molluscs and crustaceans, such as oysters, prawns, lobsters, and krill (Wright and Smucker, 1986: Kono *et al*., 1990; Lynn, 1990; Spindler and Buchholz, 1988). *Euphasia superba* and *Meganyctiphanes norvegica* chitinases have been demonstrated. The pH optimum is 5.0 and temperature optimum is between 40°C and 50°C. Enzymes activities in the lower temperature range were still high, suggesting a functional adaptation to the low temperature in seawater.

1.4.2 Bacterial chitinases

Bacteria have a large role in chitin degradation but not all species are able to hydrolyze chitin. Bacterial have been shown to produce extracelluar chitinases. Bacteria which can produce chitinases include gram positive bacteria of the genus *Arthrobacter, Bacillus, Clostridium, Nocardia,* and *Steptomyces* and the gram negative *Aeromonas, Chromobacterium,*

Photobacterium, *Psuedomonas*, *Serratia*, and *Vibrio*. The distribution of chitinase was studied in 29 *Bacillus* species and 10 species were found to produce endochitinase, including *B.alvei*, *B.chitinospours*, *B.licheniformis*, and *B.pulvifaciens*, while 15 species produced exochitinase, including *Bacillus circulans* and *Serratia marcescens* (Cody, 1989). When an exochitinase from *Serratia marcescens* was expressed in *E.coli*, it was secreted into the periplasm (Cabib, 1988). This chitinase has a high specific activity. Because of its stability, wider pH optimun and linear kinetics over a wide range than many other chitinases, the *S. marcescens* chitinase has been recommended for analytical use. For these reasons, it is also a good candidate for the large scale degradation of chitin for industrial purposes.

1.4.3 Fungal chitinases

Fungi can produce enzymes that degrade chitin. For example, an endochitnase and a *ь*-*N*-acetylglucosaminidase have been found in *Aspergillus nidulans* (Bernasconi *et al*., 1986), an exochitinase in *Mucor rouxii* (Pedraza-Reyes and Lopez-Romero, 1989), and *Vericillium albo-atrum* cultures produce a constitutive chitinase which has been implicated in the *in vitro* and *in vivo* lysis of fungal mycelium (Pegg, 1988). Chitinases seem to play a physiological role related to the mycoparasitic activity displayed by several species of fungi, such as the genus *Trichoderma*. Some species of *Trichoderma* have been used as biological control agents against fungal pathogen. The degradation and further assimilation of phytopathogenic fungi, namely mycoparatism, has been proposed as the major mechanism accounting for the antagonistic activity against phytopathogenic fungi displayed by *Trichoderma* (Siwayaprahm, 1997).

1.4.4 Protozoan chitinases

Malaria parasites (ookinetes) have been reported to digest the peritrophic membrane in the mosquito midgut during penetration (Huber *et al*., 1991). A chitinase from the parasite was detected which might be involved in the digestion of the peritrophic membrane.

1.4.5 Plant chitinases

Plant chitinases are monomeric proteins between 25 and 40 kDa that play an important role in a general disease resistance mechanism (Leah *et al*., 1991; Schlumbaum *et al*., 1986). Multiple forms of chitinase have been found in numerous plants (Boller *et al*., 1983). In all plants that have

been analyzed, chitinase activity was strongly and coordinately induced upon infection or by other types of stress, such as treatment with fungal elicitors, ethylene, other hormones, chemicals or heavy metals (Boller *et al*., 1983). It has also been demonstrated that transgenic plants that overexpress chitinases have increased resistance to fungal attack (Collinge *et al*., 1993). Chitinases can be soluble in the apoplastic space or cellular, probably sequestered in lyzosomal vaculoes, membrane-bound or wall-bound (Chagolla *et al*., 1987). Upon cellular fractionation, they have been found in both microsomal and supernatant fractions (Humphreys, and Gooday, 1984).

1.4.5.1 Constitutive and Induced plant chitinases

Chitinases are sometimes observed constitutively in plants. In *Heavea brasiliensis*, the latex contains large amounts of chitinase. Chitinases can also be produced during specific steps of plant development, such as chitinase was expressed in tobacco and *Arabidopsis* explants during flower formation, in apical leaves, and roots (Flach, 1992; Samac and Shah, 1991), in pea pods (Mauch *et al*., 1988) and in cereal grain (Jacobsen *et al*., 1990). Chitinase activity increased markedly at the onset of ripening in grape berries and continued to increase throughout the sugar accumulation phase of berry development (Robinson *et al*., 1997). Three chitinases isolated from barley grain also accumulate extracellularly in embryogenic cell suspension cultures of barley (Kragh *et al*., 1991).

Plants do not contain an immune system and thus are vulnerable to pathogens. In order to protect themselves from pathogens, plant have evolved a number of defense responses that are elicited during their life cycle in response to developmental signals and pathogen attack. Plant chitinases have been speculated to play a crucial role in plant defense against fungal pathogens because of their ability to digest chitin, a major constituent of the cell walls of a number of fungal pathogens. (Huynh *et al*., 1992). Roberts and Selitrennikoff (1988) purified chitinases from three grains, barley, maize and wheat and all three grain chitinases inhibited hyphal extension of *Trichoderma reesei* and *Phycomyces blakesleeanus*.

1.4.5.2 Infection by microorganisms, wounding and fungal elicitors

Hedrick *et al*. (1988) showed that chitinase synthesis was stimulated in bean cell suspension cultures treated with fungal cell wall elicitors and in hypocotyles after fungal infection. Elicitors caused a very rapid activation of chitinase transcription with 10-fold stimulation after 5 minutes. Chitinase transcripts were also greatly accumulated in wounded and

infected hypocotyls. Roby *et al*. (1992) also studied the relationships between oligosaccharide size and elicitor efficiency: for the colorimetric assay, the hexamer of chitin was the most efficient elicitor, whereas the heptamer was the most convenient one for the radiochemical assay.

Some chitinases can be induced by virus infection, for example in tobacco leaves infected with TMV (tobacco mosaic virus) (Trudel *et al*., 1989). Legrand *et al*. (1987) also found two additional chitinases in cucumber, also induced by TMV infection.

1.4.5.3 Phytohormone and chemical induction

Chitinases can be induced by ethylene treatment. Abeles *et al*. (1970) and Boller *et al*. (1983) showed that chitinase activity in bean seedings increased 30-fold after exposure to exogenous ethylene. In carrot cell cultures, four chitinases were induced by ethylene (Kurosaki *et al*., 1989). Chitinase accumulation in tobacco *in vitro* cultures is also regulated by addition of auxin and cytokinin to the culture medium; chitinase was also regulated during development in the intact plant. (Grosset *et al*., 1990; Sinshi *et al*., 1987).

Chitinase activity can be induced by treatments with salicylic acid or mercuric chloride. But, as for most other induction means, the effects of chemical treatments are not specific because other defence mechanisms are simultaneously induced (Nasser *et al*., 1990; Parent and Asselin, 1984).

1.4.5.4 Transgenic plants

The reports concerning transgenic plants with elevated expression levels of chitinase have been published (Broglie *et al*., 1991; Jones *et al*., 1988; Linthorst *et al*., 1990; Neuhaus *et al*., 1991), and other reports have been presented at international meetings. Transgenic tobacco and rape plants containing a bean chitinase gene with a constitutive promoter have been shown to exhibit higher basal levels of chitinase and concomitant increased resistance to *Rhizoctonia solani* when compared with control plants (Broglie *et al*., 1991). In another report (Neuhaus *et al*., 1991) tobacco plants transformed with a basic chitinase from tobacco, under regulation of a constitutive promoter, accumulated up to 120-fold more active chitinase than non-transformed plants. Oilseed rape (*Brassica napus* var. *oleifera*) containing a tomato chitinase gene with a constitutive promoter exhibited an increased tolerance to disease as compared with the nontransgenic parental plants (Grison *et al*., 1996). Japonica rice, Nipponbare and Koshihikari transformed with chitinase from rice (*Oryza sativa* L.) with a constitution promoter exhibited a 12-fold increased tolerance to rice blast pathogen (*Magnaporth grisea* race 007.0) over control (Nishizawa *et al*., 1999).

1.5 Chitinase purification

Chitinase can be purified from a total homogenate, from the intercellular fluid or from latex of plants. Affinity chromatography, using chitin or regenerated colloidal chitin, is the most specific method. It was used successfully for the purification of the chitinases from barley leaf intercellular fluid (Kragh *et al*., 1990), soybean (Wadsworth *et al*., 1984), bean, (Boller *et al*., 1983), tobacco (Shinshi *et al*., 1987), and wheat (Ride and Barber, 1990). This method cannot always be used, as problems in the binding or in the release of chitinases may occur (Flach *et al*., 1992).

Many chitinases have a very high or very low isoelectric points. This characteristic has often been used to purify chitinase. Bernasconi *et al*. (1986) described a one-step purification of *Rubus* chitinase from culture medium, using ion exchange chromatography. However, this step is generally inadequate for obtaining a pure protein. Additional steps such as hydrophobic interaction chromatography (Legrand *et al*., 1987), gel filtration on P-100 (Bernasconi *et al*., 1987) or Sephacryl S-200 gels (Roby and Esquerre-Tugaye, 1987), or chromatofocusing (Hooft *et al*., 1987) have been used. To increase the efficiency of separation, all steps can be performed by HPLC or FPLC (Meins *et al*., 1989). Table 1.2 shows examples of the purification and some properties of several chitinases.

1.6 Chitinase genes (class I and class II chitinases)

Brogile *et al*. (1986) performed Southern blot analysis of bean genomic DNA using a cDNA clone encoding a class I chitinase, and found the chitinase is encoded by a small multigene family consisting of about four members. At least two ethylene-regulated genes are expressed, as shown by sequence analysis of additional cDNA clones. Similar observations were made using cDNA clones from infected bean and rice (Hedrick *et al*., 1988; Nishizawa and Hibi, 1991). In barley, endochitinase genes were present as multiple copies on chromosome (Swegle *et a1*., 1989). In tobacco (Hooft *et al*., 1987), hybridization of cDNA probes to genomic blots indicated that the acidic and basic chitinases are each encoded by two to four genes in the amphidiploid genome of Samsun NN tobacco. A similar complexity was found for the genes encoding other pathogenesis-related proteins of bean (Ryder *et al*., 1987). In peanut, Herget *et al*. (1990) showed an elicitor-specific induction of chitinase genes (chit 1-4); chit 1 gene was activated by yeast

extract, chit 2 gene by fungal wall components; chit 3 gene was constitutively expressed and chit 4 gene induced by each tested stimulus. The structure of a tobacco class I chitinase gene was described by Shinshi *et al*. (1990). The gene contained two introns. The authors suggested that the sequences encoding the cysteine-rich domain was introduced into the ancestral gene for class I enzymes by a transposition event. In the class I chitinase gene from *Arabidopsis*, the second intron is missing and the chitinase is encoded by a single copy gene. There is no intron in rice (Zhu and Lamb, 1991) and potato (Gaynor and Unkenholz, 1989) chitinase genes.

Basic chitinase in dicotyledonous are synthesized as pre-enzymes with a signal peptide for vacuolar targeting (Mauch and Staehelin, 1989). The hydropathy plot of the deduced amino acid sequence of rice (class I basic chitinase) revealed a stretch of hydrophobic amino acids at the N-terminus, representing a typical signal peptide including a splicing motif (Perlman and Halvorson, 1983). This was consistent with a vacuolar location for basic chitinase in monocotyledons. With a tobacco chitinase gene, it has been found that some sequences for targeting the protein to the vacuole are located at the C-terminus (Shinshi et al., 1988). No homology was observed between the C-terminal of tobacco chitinase and the rice class I basic chitinase.

Table 1.2 Properties of some purified chitinases

Source of chitinase	Methods of purification	MW (kDa)	pH optimum	Temperature optimum (°C)	pI
Plant					
- Sugar beet leaves (Mikkelsen *et al*., 1992)	- Heat treament 50°C 20 min - 90% ammonium sulfate - Q Sepharose FF - Chitin column - Chromatofocusing - FPLC, Mono P				
- Basic I		32	-	-	8.1
- Basic II		26	-	-	9.1
- Acidic I		31	-	-	3.0
- Acidic II		35	-	-	4.2
- *Cucurbita* sp. Pumpkin (Esaka *et al*., 1990)	- Sepharose 6B column	29.8	-	-	-
-*Gladiolus gandavensis* (Yamagami *et al*., 1997)	- CM cellulose column - Butyl Toyopearl 650 M - Sephadex G-75 - Mono S	30 30	5.0 5.0	- -	6.0 7.5
-Japanese radish seeds (Kondo *et al*., 1997)	- 0-70% ammonium sulfate - CM Sephadex C-50 - Sephadex G-100	30.4 25.5	6.0 -	55-65 -	- -
- Lycopersion esculentum (Pegg, 1988)	- DEAE cellulose column - ammonium sulfate - Hydroxyapatite - Bio Gel P-100 column	31	5.1	-	-

Table 1.2 (continued)

Source of chitinase	Methods of purification	MW (kDa)	pH optimum	Temperature optimum (°C)	pI
- Tobacco leaves (*Nicotiana tabacum*) (Iseli *et.al*., 1993)	- Ammonium sulphate 65% - Chitin affinity column - Superrose 12-HR 10/30 - CM-Trisacryl				
- Chitnase I		34	6.5	55-60	-
- Chitinase II		32	7.0	50-55	-
- *Phaseolus vulgaris* leaves (Boller *et al*., 1983)	- Heat treatment - 0-60% ammonium sulfate - Regenerated chitin column	32.5	6.5	-	9.4
- *Pisum sativum* Pea (Mauch *et al*., 1988)	- 0-90% ammonium sulfate	33.1	-	-	-
	- Trisacryl DEAE	36.2	-	-	-
	- Chromatofocusing	32.0	-	-	-
- Wheat germ (Molano *et al*., 1979)	- Acetic acid pH 4.5 precipitate -Chitin affinity column - Sephadex G-50	30	6	30	7.5
- Zea mays Maize (Nasser *et al*., 1988)	- 0.80% ammonium sulfate	25	-	-	-
	- Sephadex G-500	25	-	-	-
	- Electroelted 12% PAGE	29	-	-	-
	- DEAE cellulose	34.5	-	-	-

Table 1.2 (continued)

Source of chitinase	Methods of purification	MW (kDa)	pH optimum	Temperature optimum (°C)	pI
Fungal					
- *Allium porrum* (Vergauwen *et al*., 1998)	- Chitin column - Mono Q column				
- Chitinase B		34	5.0	40	4.1
- Chitinase C		35	5.0	40	6.6
- Chitinase D		35	-	50	6.3
- Chitinase D_r		35	6.0	60	6.3
- Chitinase E		33	-	60	7.5
- Chitinase F		33	6.0	50	8.0
- *Metarhizium anisopliae* (Pinto et al., 1997)	- 0-85% ammonium sulfate - DEAE Sephacel	30	4.5-5.0	40-45	-
- *Tricoderma hazianum* (Ulhoa and Peberdy, 1992)	- 0-75% ammonium sulfate - Q Sepharose - Sephadex G-100 - Phenyl Separose CL-4B	40	4.0-4.5	40	-
- *Rhizpus oligosporus* (Yanai *et al*., 1992)	- 0-90% ammonium sulfate - Regenerated chitin column				
- Chitinase I	-Sephadex G-75	50	4.0	-	-
- Chitinase II	- DEAE Toyopearl	52	3.5	-	-
- *Penicillium oxalicum* (Rodriguez *et al*., 1995)	- 0-70% ammonium sulfate - Anion and cation exchange - Sephacry S-200	54.9	5.0	3.5	4.5

Table 1.2 (continued)

Source of chitinase	Methods of purification	MW (kDa)	pH optimum	Temperature Optimum (°C)	pI
- *Mucor rouxii* (Pedraza-Reyes and Iopez-Romero, 1989)	- 0-60% ammonium sulfate - Bio gel P-100 column - DEAE Bio Gel A				
- Chitinase I		30.7	6.5	30	-
- Chitinase II		24.2	6.5	30	-
Insect					
- *Stomoxys calcitrans* stable fly (Chen *et al*., 1982)	- 30-50% ammonium sulfate - Sephadex IEF - Sephadex G-100 - PAGE	63	6-8	-	3.7
Bacterial					
- *Bacillus* SP. strain MH-1 (Sakai *et al*., 1998)					
- Chitinase L	- Chitin column	71	6.5	75	5.3
- Chitinase M	- Chromatofocusing	62	5.5	65	4.8
- Chitinase S		53	5.5	75	4.7
- *Bacillus* sp. BG-11 (Bhushan and Hoondal, 1998)	- 50-80% ammonium sulfate - Chitin column - Sephadex G-100	-	7.5-9.0	45-55	-
- *Bacillus stearothermophilus* Ch-4 (Sakai *et al*., 1994)	- 0-80% ammonium sulfate - DEAE cellulose - Butyl Toyopearl - Sephadex G-100 - Mono Q	74	6.5	75	-

Table 1.2 (continued)

Source of chitinase	Methods of purification	MW (kDa)	pH optimum	Temperature Optimum (°C)	pI
- *Bacillus circulans* WL-12 (Watanabe *et al*., 1990)	- 0-40% ammonium sulfate - Chitin affinity column - Sephadex G-100				
- Chitinase A1		74	-	-	4.7
- Chitinase A2		69	-	-	4.8
- Chitinase B1		38	-	-	6.6
- Chitinase B2		38	-	-	5.9
- Chitinase C		39	-	-	8.6
- Chitinase D		52	-	-	5.2
- *Bacillus licheniformis* X-7U (Takayanagi *et al*.,1991)	- Butyl Toyopearl 650 M - Q Sepharose - Sephacryl S-200				
- Chitinase I		89	6.0	-	-
- Chitinase II		76	6.0	-	-
- Chitinase III		66	5.0	-	-
- Chitinase IV		59	5.0	-	-
- *Enterobacter* sp. G-1 (Park *et al*., 1997)	- 0-75% ammonium sulfate - DEAE Sephadex A-50	60	7.0	40	6.6
- Chitinase A	- Sephadex G-100				
- *Serratia marcescens* (Roberts and Cabib, 1982)	- Chitin affinity column				
- Chitinase A		52	4-7	-	-
- Chitinase B		58	4-7	-	-

Table 1.2 (continued)

Source of chitinase	Methods of purification	MW (kDa)	pH optimum	Temperature optimum (°C)	PI
- *Pseudomonas aeruginose* K-187 (Wang and Chang, 1997)	- 0-80% ammonium sulfate - DEAE Sepharose Cl-6B - 0-80% ammonium sulfate - Econo Pac Q column				
- Chitinase I		30	8.0	50	5.2
- Chitinase II		32	7.0	40	4.8
- *Steptomyces* sp. (Skujins *et al*., 1970)	- 0-70% ammonium sulfate - DEAE column - Hydroxylapatite - Bio Gel P-150	29	-	-	-
- *Steptomyces erythraeus* (Hara *et al*., 1989)	- DEAE cellulose - Bio Gel G-60 - DEAE Sephadex	30	5	-	3.7
- *Steptomyces albidoflavus* (Broadway *et al*., 1995)	- 0-95 % Ammonium sulfate - DEAE column				
- Chitianse I		59.0	5.1	-	-
- Chitinase II		45.0	5.3	-	-
- Chitinase III		38.5	5.7	-	-
- Chitinase IV		27.0	5.8	-	-
- Chitinase V		25.5	5.9	-	-
- *Vibrio* sp. (Ohtakara *et al*., 1979)	-0-70% ammonium sulfate - DEAE Sephadex A-25 - Hydroxyapatite column - Sephadex G-100	48	5.0	-	4.9

1.7 Assay methods of chitinase enzyme

Chitinase activity has been assayed by a variety of procedures, including the monitoring of changes in the molecular size of substrate and the determination of oligosaccharides, aglycones or *N*-acetyl-glucosamine liberated in the reaction.

1.7.1 Viscosimetric assay

Viscosity measurements for chitinase activity monitor the changes in the molecular size of substrate (Jeuniaux, 1966). Insoluble compounds such as colloidal chitin are used as substrates in these assay procedures (Tracey, 1955). This method is a more sensitive and effective procedure to detect a slight activity. However, it is somewhat troublesome and too time consuming to determine the chitinase activity of numerous samples. The assay of chitinase activity by the viscosimetric method was first carried out using the solution of chitosan acetate. Carboxymethylchitin has been reported to be a water soluble substrate suitable for the assay of chitinase activity. However, both compounds have the disadvantage that the viscosity was markedly affected by ionic strength and pH. On the contrary, glycol chitin does not have such disadvantage and is a useful substrate for the viscosimetric assay.

A unit of chitinase activity is defined as the amount of the enzyme which is required to attain a half life time of substrate in 30 min at 30°C and optimum pH. There is a linear relationship between chitinase activity and amount of enzyme. Thus, the viscosimetric unit for chitinase can be obtained by dividing 30 min by half life time (Ohtakara, 1988).

1.7.2 Turbidimetric (Nephelometric) assay

Turbidimetric measurements of the turbidity variation of a colloidal chitin suspension during chitiniolysis has also been used. This method is rapid and accurate, but is suitable only for the estimation of relatively high activities, in limpid and poorly colored solutions (Jeuniaux, 1966).

Ten nephelometric units correspond to a 50% turbidity decrease of a 6 ml colloidal chitin suspension (0.3 g/ml) within 2 hours at 37.5°C and pH 5.3. The amount (c) of chitinase, expressed in nephelometric units, is thus given by the formula.

$$c = \frac{10}{0.3}\log\frac{T_0}{T_2}$$; T_0: turbidity at start time, T_2: turbidity at 2 hours

1.7.3 Tritiated chitin hydrolysis assay (Radiometric assay)

The radiochemical assay uses [acetyl-^{3}H] chitin as a substrate. The assay, based on the formation of soluble oligosaccharides from [acetyl-^{3}H] chitin, is the most sensitive, because of the possibility of using substrate of very high specific activity. It is suitable for both endochitinases and exochitinases as it obviates the need for an auxiliary *b*–*N*-acetylhexosaminidase, and is extremely simple to carry out (Cabib, 1988).

A unit of chitinase activity by this method is defined as that amount of enzyme which catalyzes the release of 1 μmol of soluble product (calculated as *N*-acetylglucosamine, GlcAc) in 1 min at 30°C (Cabib, 1988).

1.7.4 Colorimetric assay

The most widely used colorimetric assay for plant chitinases has been an exochitinase assay, based on the determination of monomeric *N*-acetylglucosamine released from colloidal chitin. However, plant chitinases generally are endochitinases and produce chitoligosaccharides as principal products (Boller *et al*., 1983). Therefore, measurements of plant chitinases with the exochitinase assay should be viewed with caution. For accurate determination, it is essential to measure the chitooligosaccharides produced in the assay. This can be accomplished by the enzymatic hydrolysis of the reaction products to monomeric GlcNAc prior to the colorimetric measurement. The monomeric GlcNAc is then determined with dimethylaminobenzadehyde (DMAB) which replaces the acetyl group and absorbs at 585 nm.

Chitobiosidase and endochitinase were assayed by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-*b*-D-*N*,*N*¢-diacetylchitobiose and *p*-nitrophenyl-*b*-D-*N*,*N*¢,*N*¢¢-triacetyl-chitotriose. These substrates function as trimeric and tetrameric subatrates, respectively, with the *p*-nitrophenyl group serving as one monomer (Roberts and Selitrennikoff, 1988). Plates were read at 410 nm in a microtiter plate reader. Activity was expressed as nkatals (nmoles of nitrophenol releases per second) or U (μmoles of nitrophenol release per min).

1.7.5 Activity stained gels

Chitinase activity in electrophoretic gels can be detected by using a glycol chitin gel as substrate and staining the gel with Calcofluor White M2R which is fluorescent and highly specific for glycol chitin (Maeda and Ishida, 1967). The area of glycol chitin that was hydrolyzed appears as a dark band in ultraviolet on the white background. 4-methylumbelliferone-

chitooligosaccharides substrate can use to detect the product 4-methyl-umbelliferone (4-MU) that fluorescent on the gel under the ultraviolet (Molano *et al*.; Cabib, 1988).

1.7.6 Chitin agar plate

In the chitin agar plate method, the chitinase activity is measured as the zone of clearing around the wells where chitinase is added as it hydrolyzes the colloidal chitin substrate in the media plate to small oligosaccharides (Roberts and Selitrennikoff, 1988).

1.8 Substrate specificity of chitinases

Chitinases from different sources have widely different substrate specificity, hydrolyzing different chitin derivatives. Alternative substrates for plant chitinases are bacterial peptidoglycan, the soluble chitin derivative glycol chitin and in some cases chitosan (deacetylated chitin) (Bernasconi *et al*., 1987: Boller *et al*., 1983; Molano *et al*., 1979). Various commercial chitins are available, but the particle size is generally too large to permit sensitive assays, thus colloidal chitin preparations are currently the most commonly used experimental substrates.

1.8.1 Enzymatic Hydrolysis of colloidal chitin, purified chitin, glycol-chitin, chitosan

Some chitinases can hydrolyze colloidal chitin, glycol-chitin and glycol-chitosan but they have different specific activities toward the different substrates. For example, *Trichoderma harianum* chitinase (CHIT33) was able to hydrolyze colloidal chitin, glycol-chitin and glycol-chitosan, but specific activity of hydrolysis of colloidal chitin was 6.55 fold that of glycol-chitin and 1440 fold that of glycol-chitosan (De La Cruz *et al*., 1992). The hydrolysis products of the enzymatic reaction from colloidal chitin were a mixture of $(GlcNAc)_4$, $(GlcNAc)_3$, $(GlcNAc)_2$, and GlcNAc in which $(GlcNAc)_4$ was the main product, indicating an endo-type action of the chitinase.

Glycol chitin is used in assays which need a soluble substrate (Wood and Kellogg, 1988; Ohtakara, 1988), however it does not work for all chitinases. The wheat germ chitinase is specific for chitin and will not attack even glycol chitin (Molano *et al*., 1979). The pattern of product formation by the wheat germ enzyme, as analyzed by paper chromatography, indicates that the enzyme acts as an endochitinase. The smallest substrate that the enzyme can attack is

a trisaccharide that is a marginal substrate. Even after prolonged incubation, only traces of diacetylchitobiose and acetylglucosamine were observed.

Terwisscha van Scheltinga *et al*. (1995) determined the difference in stereochemistry between chitinases of prokaryotic and eukaryotic organisms from chitin hydrolysis by hevamine, a plant class III chitinase from *Hevea brasiliensis*. Hevamine, like the bacterial chitinases, acts by retention of configuration, despite the absence of stabilizing carboxylate in the active site. Hevamine does not hydrolyze chitin fragments shorter than chitopentose and chitopentose is cleaved mostly into chitotetraose and GlcNAc. The profile of chitopentose, incubated for 5 min with hevamine, clearly shows that the relative amount of the *b*–anomer of the formed chitotetraose is much higher that the *a*-anomer, whereas in equilibrium state the amount of α-anomer is about 1.5 times that of the *b*–anomer. Therefore, hevamine cleaves chitin with retention of the configuration at C1, like the family 18 bacterial chitinases (Armand *et al*., 1994).

Iseli *et al*. (1996) compared the hydrolysis mechanism of a cucumber class III chitinase (family 18) and a bean class I chitinase (family 19). Cucumber chitinase is another class III chitinase like hevamine. Plant class III chitinases of family 18 use the same mechanism and have a very similar three-dimension structure (Armand *et al*., 1994; Perrakis *et al*., 1994). The class I chitinase was found to operate with inversion of the anomeric configuration and the results are in agreement with the recent observation in yam chitinase (family 19) also performs an inversion of the anomeric configuration (Fukamizo *et al*., 1995). It is therefore likely that all family 19 chitinases share the same active site structure, catalytic machinery and stereochemical outcome.

Kragh *et al*. (1990) determined the hydrolysis production of barley leaf (*Hordeum vulgare* L.) chitinase from [^{3}H]chitin. It was found that GlcNAc respresented 6% of the radioactivity detected after 24 h incubation, whereas chitobiose, chitotriose, chitotetraose and higher oligomers accounted for 39, 15, 14 and 25%, respectively. The low amount of GlcNAc and relatively high amounts of chitotriose and chitotetraose implies that barley chitinase is an endochitinase, with little exo-*N*-acetylglucosaminidase activity.

Aiba (1994) determined the production of *Streptomyces griseus* chitinase hydrolysis of chitosan. The yields of the oligomers produced appeared to follow *N*-acetylation. The yields were relatively low when chitosan with degree of *N*-acetylation (degree of *N*-acetylation, da) of 5% was used as a substrate. Higher yields were obtained from chitosans with more than 10% da.

The major products were trimer, tetramer and pentamer of 31%, 28%, and 23%, respectively. The yield of monomer and hexamer and were 6% and 10%, respectively.

Boller *et al*. (1983) determined the hydrolysis products of chitinase from bean leaves (*Phaseolus vulgaris*) on [^{3}H]chitin. The percents of soluble products at 30 min were GlcNAc, chitobiose, chitotriose, chitotetraose, and higher oligosaccharide of 3, 31, 33, 30, and 3% respectively. The main soluble products formed after prolonged incubation for 24 h with purified endochitinase were chitobiose, chitotriose, and some chitotetraose at 56, 31, and 5%, respectively. Even after 24 h, only 7% of the product was in the form of the monosaccharide.

1.8.2 Enzymatic hydrolysis of ***p***-nitrophenyl-***N***-acetyl-D-glucosamine (pNP-GlcNAc)

Chromogenic measurements for chitinase activity monitor the changes in the molecular size of substrate. This method is highly specific because of using different forms of synthetic substrate for the assay of chitinase activity. For this method use *p*-nitrophenyl-chitooligosaccharides as substrate to produce *p*-nitrophenol (pNP). It is convenint, rapid, highly specific, and can use for quantitative and qualitative analysis, but the substrate is expensive and of no physiological relevance (Roberts and Selitrennikoff, 1988; Trosmo and Harman, 1992).

Harman *et al*. (1993) assayed the chitinolytic endochitinase and chitobiosidase of the fungus, *Trichoderma harzianum*, by measuring the release of nitrophenol from *p*-nitrophenyl-*N,N*¢¢diacetylchitobiose and *p*-nitrophenyl-*N,N*¢,*N*¢¢-triacetylchitotriose. The specific activity of the purified endochitinase and chitobiosidase were calculated to be 2.2 and 127 nkat, respectively. The large difference in specific activity of the endochitinase on *p*-nitrophenyl-*N,N*¢,N¢¢-triacetylchitotriose versus chitobiosidase on nitrophenyl-*N,N*¢¢-diacetylchitobiose was probably due to the fact that nitrophenyl-*N,N*¢, *N*¢¢-triacetylchitotriose is a poor substrate for endochitinase.

1.8.3 Enzymatic hydrolysis of 4-methylumbelliferyl-***N***-acetylglucosaminide (4-MU-GlcNAc), 4-methylumbelliferyl- ***N,N***¢-diacetylchitobioside [4- MU-$(GlcNAc)_2$] and 4-methylumbelliferyl- ***N,N***¢,N¢¢-triacetylchitotrioside [4-MU-$(GlcNAc)_3$], and 4-methylumbelliferyl- ***N,N***¢,N¢¢,N¢¢¢-tetraacetylchitotetraoside [4-MU-$(GlcNAc)_4$]

Enzyme assays that use fluorogenic substrates with 4-methylumbelliferone as aglycone are very sensitive and allow selection of low levels of chitinolytic activity (O'Brien and Cowell, 1987; McCreath and Gooday, 1992). They have been compared favorably with other methods for

measuring chitinase (Hood, 1991; O'Brien and Cowell, 1987). Fluorogenic assays measure hydrolysis of substrate which changes 4-methylumbelliferyl-chitooligosaccharides to 4-methylumbelliferone (4-MU), which is fluorescent. This product can be used to detect chitinase activity on gels under ultraviolet light (Molano *et al*., 1977; Cabib, 1988).

4-MU substrates have been proposed as a tool to distinguish between endo- and exo-chitinase activities, 4 MU-GlcNAc for the determination of *N*-acetyl-β-D-glucosaminidase activity, 4-MU-$(GlcNAc)_2$ for the determination of exochitinase, and 4-MU-$(GlcNAc)_3$, 4-MU-$(GlcNAc)_4$ for determination of endochitinase (Robbins *et al*., 1988). Using this method a crude protozoan enzyme exhibited predominantly an 'exo' mode of action for chitinase activity (chitobiosidase activity), producing 4-methylumbelliferone from 4-MU-$(GlcNAc)_2$ 2.4 times faster than from 4-MU-$(GlcNAc)_3$. The crude chitinase from *Streptomyces antibioticus* (chitinase-49 and chitinase-63), on the other hand, showed an 'endo' types of action.

1.8.4 Hydrolysis of Natural substrates

No endogenous substrates in plants have been identified until recently. When boiled homogenates of bean plants were treated with bean endochitinase, no GlcNAc-containing fragments were detected indicating that chitin-like substrates are not present in the plant (Boller *et al*., 1983). However, recent immunochemical studies using gold-labelled wheat germ agglutinin and bacterial chitinases revealed abundant GlcNAc residues in secondary walls of plants (Benhamou and Asselin, 1989). The data suggested that the GlcNAc residues may be present in the form of glycolipids whereas GlcNAc polymers analogous to chitin are absent. In this context, it is noteworthy that rhizobia (*Rhizobium*, *Bradyrhizobium* and *Azorhizobium*) secrete lipo-chitooligosaccharides (nod factors). These signal molecules are tetramers and pentamers of GlcNAc in a beta-1,4 linkage with an acyl group at the non reducing residue (Lerouge *et al*., 1990; Roche *et al*., 1991; Spaink *et al*., 1991). These nod factors are a new class of signal molecules that, at very low concentrations (10^{-7}-10^{-10} M), trigger root hair deformation, nodule primordium formation for establishing specific symbiolic interations with leguminous plants (Lerouge *et al*., 1990; Roche *et al*., 1991; Spaink *et al*., 1991; Truchet *et al*., 1991) and elicit infection-related processes (Van Brusel *et al*., 1992). It has been proposed that plant chitinases might interfere by hydrolyzing these bacterial lipo-oligosaccharides (Roche *et al*., 1991; Staehelin *et al*., 1992). Endogenous pea and bean chitinases have no influence on the pea or

bean nod factors, but soybean chitinase can inactivate the soybean, pea and bean factors. It has been postulated that rhizobial nod factors resemble comparable glycolipid compounds of plant origin (Fisher and Long, 1992). If the endogenous glycolipids are indeed substrates for chitinases, then the products might act as plant signal molecules (Fisher and Long, 1992; Spaink, 1992; Truchet *et al*., 1991). Spaink *et al* (1992) observed molecules which migrate similarly to rhizobial lipo-oligosaccharides in TLC by following chitinase treatment of butanol extracts from *Lathyrus* flowers.

Table 1.3 Some characterization and kinetic properties of purified chitinases

Source	MW (kDa)	Substrate used	Activity (U/ml)	Specific activity (U/mg per protein)	K_M	Vmax (mmol /min/mg)	Reaction Products
Plant							
Bean leaf (*Phaseolus vulgaris*) (ethylene treated) (Boller *et al*., 1983)	32.5	[^{3}H]-chitin	72	144	0.007 mM	0.12	$(GlcNAc)_2$ $(GlcNAc)_3$ $(GlcNAc)_4$
Barley (*Hordeum vulgare* L.) (Hollis *et al*., 1997)	26	$(GlcNAc)_4$ 4-MU-$(GlcNAc)_3$	- -	- -	0.003 0.033 mM	$12X10^{-3}$ $12\ X10^{-6}$	- -
Bulbs of Gladious (*Gladiolus gandavensis*) (Yamagami *et al*., 1997)	30 32	Glycol-chitin	51.12 28.35	159.7 135.1	- -	- -	$(GlcNAc)_2$ $(GlcNAc)_3$ $(GlcNAc)_4$
Carrot (*Daucus carota*) (Zhang *et al*.,1996)	39.5	Glycol-chitin	4.0	166.7	-	-	-
Latex (*Hevea brasiliensis*) (Martin, 1991)	25.5 26.0 27.5	[^{3}H]-chitin	37 91 33	- 19 6.9	- - -	- - -	- - -
Pea (*Pisum sativum*) (Mauch *et al*., 1988)	33 36	[^{3}H]-chitin	12 34	17.7 12.7	- -	- -	- -

Table 1.3 (continued)

Source	MW (kDa)	Substrate Used	Activity (U/ml)	Specific activity (U/mg per protein)	K_M	Vmax (mmol /min/mg)	Reaction Products
Sorghum seeds (Krishnaveni *et al*., 1999)	24 28 33	CM-chitin-RBV	- - -	7.42 6.05 2.0	- - -	- - -	- - -
Tobacco (class I) (*Nicotiana tabacum*) (Iseli *et al*., 1993)	34	[^{3}H]-chitin	-	1.3 μkat /mg	23 mM	3.5	-
Tomato (*Lycopersicon esculentum*) (Pegg, 1988)	31	Colloidal chitin	22	12.22	10.46 mg dry wt chitin/ml	97.8 μg GlcNAc/h /ml	$(GlcNAc)_2$ higher oligo-saccharide
Wheat germ (Moana *et al*., 1979)	30	[^{3}H]-chitin	7.29	14.6	2 mM	-	$(GlcNAc)_2$ $(GlcNAc)_3$ $(GlcNAc)_4$
Yam tuber (*Dioscorea opposita*) (Arakane *et al*., 2000)	33.5	Glycol-chitin	-	-	0.639 mg/dry wt chitin/ml	-	-
Insect Hornworm (Manduca sexta) (Huang *et al*., 2000)	75	[^{3}H]-Glycol-chitin	-	-	0.6 mM	-	-

Table 1.3 (Continued)

Source	MW (kDa)	Substrate used	Activity (U/ml)	Specific activity (U/mg per protein)	K_M	Vmax (mmol /min/mg)	Reaction Products
Blowfly (*Lucilia cuprina*) (Londershausen *et al*., 1996)	83	[^{3}H]-chitin	-	-	0.043 mM	133.23	-
Stable fly (*Stomoxis calcitrans*) (Chen *et al*., 1982)	48	[^{3}H]-chitin	-	-	33 mM	-	$(GlcNAc)_2$ some GlcNAc
Spider (*Cupiennius salei*) (Mommsen, 1980)	48	[^{3}H]-chitin	-	-	-	-	$(GlcNAc)_2$ some $(GlcNAc)_3$
Bacterial							
Bacillus circulans (Watanabe, 1993)	70	4-MU-$(GlcAc)_3$	-	-	3.6 mM	-	-
Saccharomyces cerevisiae (Correa *et al*., 1982)	-	[^{3}H]-chitin	-	-	3.9 mM	-	$(GlcNAc)_2$
Streptomyces antibioticus (Jeuniaux, 1966)	30	[^{3}H]-chitin	625	420	0.5 mM	-	$(GlcNAc)_2$

Table 1.3 (Continued)

Source	MW (kDa)	Substrate Used	Activity (U/ml)	Specific activity (U/mg per protein)	K_M	Vmax (mmol /min /mg)	Reaction Products
Streptomyces Erythraeus (Hara *et al.*, 1989)	30	Glycol-chitin	-	27.6	-	-	$(GlcNAc)_2$
Streptomyces griseus (Jeuniaux, 1966)	-	[^{3}H]-chitin	-	-	5 mM	-	$(GlcNAc)_2$
Serratia macrescens (Cabib, 1988)	58	[^{3}H]-chitin	1.84	1.33	4.4 mM	-	$(GlcNAc)_2$ some $(GlcNAc)_3$
fungal							
Fusarium chlamydosporum (Mathivanan *et al.*, 1998)	40	Colloidal chitin	180	200	-	-	-
Trichoderma harzianum (DE LA CRUZ *et al.*, 1992)	37 42	Colloidal-chitin	- -	2.78 6.54	0.5 1.0 mg dry wt chitin/ ml	- -	mainly $(GlcNAc)_4$ some $(GlcNAc)_3$ $(GlcNAc)_2$ GlcNAc
Verticillium albo-atrum (Pegg, 1988)	64	Colloidal chitin	16	43.2	97 μg dry wt chitin/ml	-	$(GlcNAc)_2$

1.9 The catalytic mechanism of family 19 chitinases

The most important aspect of chitinase catalysis is the stereochemistry of the catalyzed reaction. Acid-catalyzed glycosidic hydrolysis may proceed to yield a hydrolysis product with either retention or inversion of anomeric configuration (at C1′) relative to the starting conformation and this will indicate whether one chemical transition state or two are involved in the pathway. All of the family 19 chitinases reported to date yield the *α*-anomer the hydrolysis product of the inverting mechanism (Fukamizo *et al*., 1995).

The X-ray crystal structure of a family 19 plant endochitinase from barley (*Hordeum vulare* L.) seeds (Hart *et al*., 1995) reveals two well-separated acidic residues in the active site and inverting mechanism requires these two largely separated acidic residues within the active site. The hydrolysis products for two family 19 chitinases show inversion of the anomeric configuration (Fukamizo *et al*., 1995; Iseli *et al*., 1996). These results led to the conclusion a single displacement mechanism is used for family 19 chitinases (Figure 1.8). The single displacement mechanism requires one acidic residue to act as a general acid and the other as a general base, activating water for a concerted nucleophilic attack at C1′. The single displacement mechanism proposed for family 19 chitinases necessarily involves an intermediate with considerable oxocarbenium ion character. The basic mechanism involves the following features: an acid catalyst donates a proton to the β-1,4-glycosidic oxygen linking the sugar residues in the substrate; the other acid residue acts as a general base, to activate the water molecule for nucleophilic attack; and a conformation change occurs within the active site to bring the oxocarbenium ion intermediate to product with inversion of the anomeric configuration.

Brameld and Goddard (1998) have examined the binding of hexaGlcNAc substrate and two potential hydrolysis intermediates (an oxazoline ion and an oxocarbonium ion) in the family 19 barley chitinase by computational methods. They found the hexaGlcNAc substrate most likely binds with all sugars in a chair conformation, unlike the family 18 chitinase, which causes substrate distortion. Glu 67 is in a position to protonate the anomeric oxygen linking sugar residues D and E whereas Asn 199 serves to hydrogen bond with the C2′ *N*-acetyl group of sugar D, thus preventing the formation of an oxazoline ion intermediate (Figure 1.9). In addition, Glu 89 is part of a flexible loop region allowing a conformational change to occur within the active site to bring the oxocarbenium ion intermediate and Glu 89 closer by 4-5 Å. A hydrolysis

product with inversion of the anomeric configuration occurs because of nucleophilic attack by a water molecule, which is coordinated by Glu 89 and Ser 120 to bond in the α-conformation. So family 19 chitinases use a single displacement mechanism to hydrolyze their substrates (Figure 1.8).

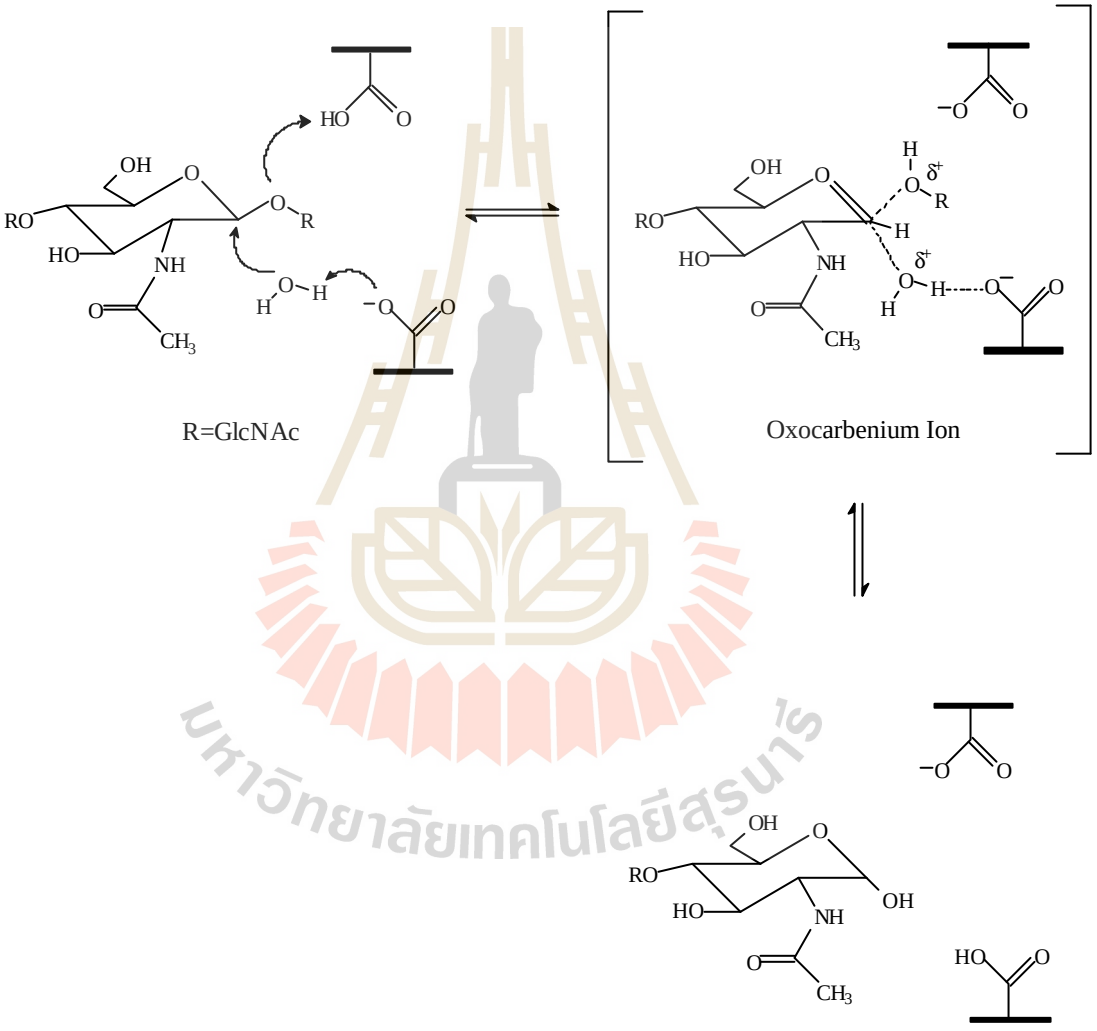

Figure 1.8 Pathway of the single displacement hydrolysis mechanism proposed for the inverting family 19 chitinases. (Brameld and Goddard, 1998)

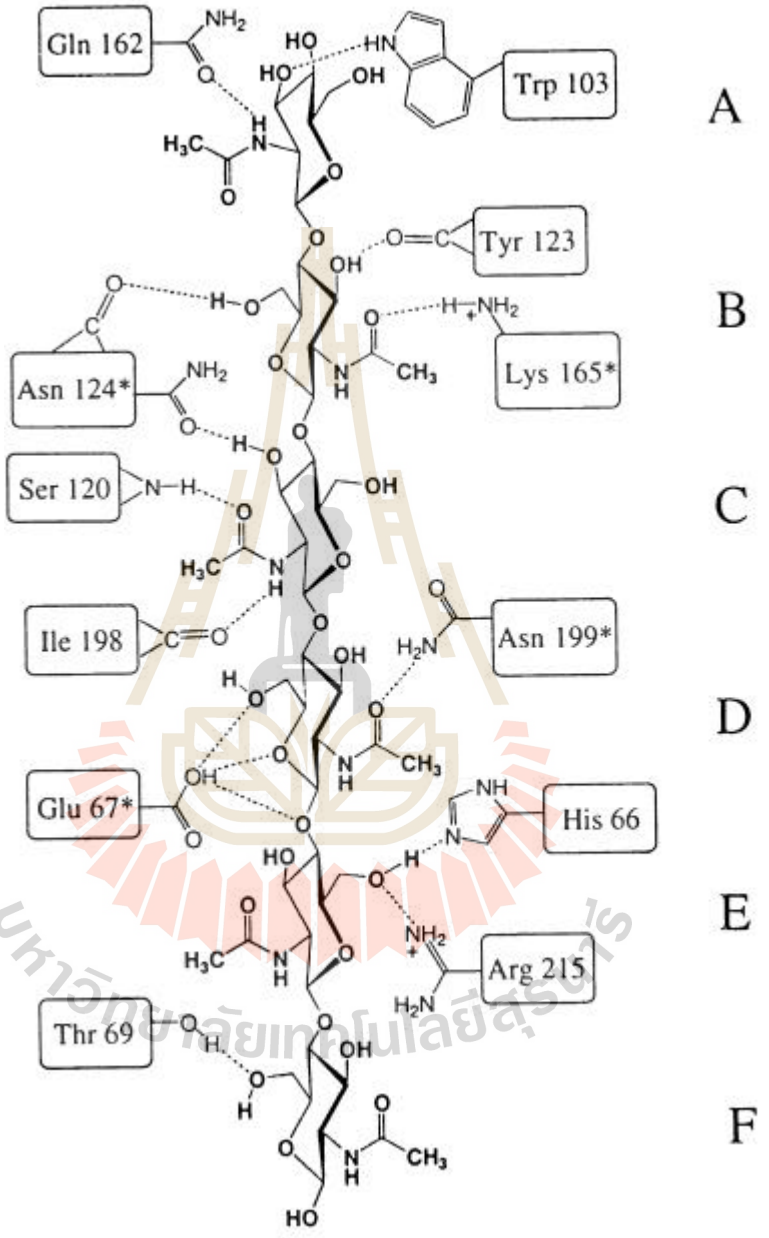

Figure 1.9 A schematic of the hydrogen bonds observed for a hexaGlcNAc substrate bound to barley chitinase. * Residues strongly conserved in family chitinases.

1.10 Antimicrobial Activity

Wang and Chang (1997) determined the antibacterial properties of chitinases I and II from *Pseudomonas aeruginosa* K-187 grown in a shrimp and crab shell powder medium. The reaction mixtures containing 1.5 ml of the cell suspension of the substrate and 1.5 ml of the purified enzyme (2 mg/ml) was used as the lytic enzyme for measurement of growth inhibition at 37°C for 30 min, and the enzyme activity was determined spectrophotometrically by measuring the decrease in optical density at 660 nm. The bacteria cell-lytic activities of the type I and II chitinases against both gram-negative and gram-positive bacteria were examined. The two chitinases showed potent activities toward *S. aureus*, *P. aerugious* M-1001, and *P. aeruginosa* K-187.

The difference in antifungal activities in plant, bacterial, and fungal chitinases have been explored. Plant chitinases can inhibit growth of fungus, but most fungal and bacterial chitinases cannot inhibit fungal growth (Roberts and Selitrennikoff, 1986) (table 1.4).

Krishnaveni *et al.* (1999) have shown that three sorghum seed chitinases inhibited the growth of *Trichoderma viride* and *Rhizoactonia salani* at a concentration of 1μg/5 mm disc and *Fusarium moniliforme* at a concentration of 5 μg/5 mm disc, but these enzymes did not inhibit the growth of *P. irregulare* which lacks chitin in its cell wall.

Iseli *et al.* (1993) have examined cysteine-rich domain (CBD) for its affects on the biochemical and functional characteristic of chitinase. Chimeric genes encoding the basic chitinase A of tobacco (*Nicotiana tabacum*) with and without this domain were constructed and constitutively expressed in transgenic *Nicotiana sylvestris.* The chitinases were subsequently isolated and purified to homogeneity from the transgenic plants. Both chitinases were capable of inhibiting growth of *Trichoderma viride*, although the form with the CBD was about three times more effective than the one without CBD. Thus, the CBD is not necessary for catalytic activity but improves antifungal activity of the enzyme.

Table 1.4 Comparison of antifungal and enzyme activities from plant, bacteria, and fungus. The minimum amounts of chitinase (μg protein per 5 mm disc) required to produce detectable fungal growth inhibition are shown.

Source	Fungus (mg protein)
A. thaliana (Verburg and Huynh, 1991)	As (8), Fo (8), Gg (8), Pm (8), Ss (8), Tr (0.5)
Barley (Henrik *et al*., 1991)	Fs (1.5), Pb (3), Tr (1.5)
Bean (Boller *et al*., 1988)	Tv (2)
Carrot (Zhang *et al*., 1996)	Ts (5)
Cucumer (Zhang and Punja, 1994)	Ts (4)
Maize (Roberts and Selitrennikoff, 1986)	Pb (3), Tr(1)
Sorghum seeds (Krishnaveni *et al*., 1999)	Fm (5), Pi (NI), Rs (1), Tv (1)
Tobacco (Sela-Buurlage *et al*., 1993)	Fs (5)
Wheat (Roberts and Selitrennikoff, 1986)	Pb (10), Tr (0.3)
Pseudomonas stutzeri (Roberts and Selitrennikoff, 1986)	Pb (>50), Tr (>50)
Serratia marcescens(Roberts and Selitrennikoff, 1986)	Pb (>50), Tr (>50)
Streptomyces griseus (Roberts and Selitrennikoff, 1986)	Pb (>50), Tr (>50)
Fusarium cklamydosporum (Mathivana *et al*., 1998)	Pa (30)
Trichoderma harianum (CHIT 42) (De La Cruz *et al*., 1992)	Bc (10)

As : *Alternaria solani* Bc : *Botrytis cinerea* Fm : *Fusarium moniliforme*
Fo : *Fusarium oxysporum* Fs : *Fusarium sporotrichioides* Pa : *Puccinia arachidis*
Gg : *Gaeumannomyces graminis* Pb : *Phycomyces blakesleeanus* Pi : *Pythium irregulare*
Pm : *Phytophthora megasperma* Rs : *Rhizoactonia salani* Ss : *Sclerotinia sclerotiorum*
Tr : *Trichoderma reesei* Ts : *Trichoderma sp.* Tv : *Trichoderma viride*
NI = not inhibit

1.11 ***Leucaena leucoccephala*** de Wit

Leucaena leucocephala de Wit is in the genus *Leucaena*, family *Leguminosae* and subfamily *Minosoideae*. The genus *Leucaena* includes about 50 species of trees and shrubs that are native to central America and southeast Asia. Humans have used leaves, lugums (pods), and young seeds of at least 4 *Leucaena* species for food (Brewbaker at al., 1970).

Leucaena leucocephala de Wit, the most widespread species of the genus, is considered pantropical. This species is evergreen when moisture is not a limiting factor. It is a drought-tolerant, branchy, abundantly flowering, and aggressive shrub or small tree with finely divided, light green, bipinnate leaves. The leaflets, about 15 pairs, are 1-1.5 cm. long, opposite, asymmetrical and slightly curved. The flowering heads grow in clusters from branch ends. The individual flowers are white, the head turning brown as it matures. Blooming time: summer. The spherical, whitish flower heads are 2.0 to 2.5 cm in diameter and are borne on stalks 2 to 3 cm long at the ends or sides of twigs (Parrotta, 1992). The pods are flat, about 15 cm long, green when immature, turning brown with age. The oval seeds are flat, brown and glossy. It has been widely planted in the tropics for fodder and firewood. It is quickly becoming a nuisance tree, out competing many native species (Daguma *et al*., 1988).

Leucaena leucocephala is easily propagated from seed. Seeds need to be scarified or treated with boiling water (80°C) for 3-4 min, followed by soaking in water at room temperature for up to 12 hours or soaking in concentrated sulfuric acid for 15 to 30 min. Scarification may be followed by inoculation with nitrogen-fixing *Rhizobium* bacteria (mixed with finely ground peat) after coating the seeds with a gum arabic or concentrated sugar solution (Parrotta, 1992). Germination rates are commonly 50-98% for fresh seeds (Daguma *et al*., 1988). Scarified seeds germinate 6-10 days after sowing but unscarified seeds germinate 6-60 days after sowing (Parrotta, 1992).

Paudel and Upasena (2000) have used green leaf manure of *L. leucocephala* for growing rice (*Oryza sativa*) cultivar Suphan Buri 90. The results were revealed that application of green leaf manure nitrogen at 120 kg/ha from *Leucaena* produced yield and agronomical component rice similar to that obtained from nitrogen at 60 kg/ha from urea. The increase in grain yield over control treatment in green leaf manure treated plots was from 19-49%. Thus, *Leucaena* is of potentially economic importance in agriculture aside from its own use as a food or herbal medicine.

1.12 Objectives

The objectives of this thesis include:

1) To determine the amino acid sequence of chitinase enzyme from germinated *Leucaena leucocephala* de Wit by DNA sequence determination and analysis; 2) To determine the catalytic properties of the enzyme purified from germinated seeds and expressed from the cDNA in *E.coli*; 3) To compare the chitinase activity from the chitinase gene expressed in *E.coli* to that of chitinase purified from 4-week-old seedlings; 4) To determine the antifungal activity of the *L. leucocephala* chitinase on 14 fungal strains.

Chapter II
Materials and Methods

2.1 Materials

2.1.1 Plant Material

Leucaena leucocephala (*L. leucocephala*) seeds were collected from trees at Suranaree University of Technology and Lumtaklong Dam, Nakhon Rachasima. For Nested PCR and 3′ RACE, the seeds were sown for 5 days in moist filter paper. For construction of cDNA library and 5′ RACE, the seed covers were cut and the seeds were sown for 2 days in moist filter paper and grown in a growth chamber at 25◦C with a 15-h/day light period for 1 week and they were transferred to long day conditions in the greenhouse for 3 weeks. For purification of chitinase from the plant and amplification of full-length cDNA for expression in *E.coli*, the seeds were soaked for 4 days in water and grown in soil for 3-4 weeks.

2.1.2 PCR primer

Degenerate oligonucleotides were ordered from Gibco BRL, Life Technologies, Grand Island, NY, USA as show in Table 2.1. The primers for 3′ RACE and 5′ RACE were designed for the initial chitinase PCR-amplified cDNA fragment and ordered from the National Science and Technology Development Agency (NSTDA) BIOSERVICES UNIT (BSU, Thailand) and for the final chitinase PCR-amplified cDNA fragment were ordered from the Expedite Nucleic Acid Synthesis System (Workstation), Tsukuba, Japan as shown in Table 2.2. The primers for expression in *E.coli* were designed without prepeptide for pET23d, which were ordered from the NSTDA BSU (Thailand) and for pET32a ordered from Geneset Oligos Ltd. (Singapore) as show in Table 2.3.

2.1.3 Cloning and expression vectors, and host cells

The pGEM-T plasmid vector (shown in Appendix A) systems were purchased from Promega (Madison, WI, USA) and pT7blue T-vector (shown in Appendix A), pUC19 vector

(shown in Appendix A) were purchased from Takara (Tokyo, Japan). They were used to clone PCR products with the recommended protocol. *Escherichia coli* strain DH5α were generally used as host cells for amplification of recombinant plasmids. Plasmid expression vector pET23d (shown in Appendix A), pET32a (shown in Appendix A), BL21 (DE3), and Origami (DE3) *E.coli* were from Novagen (Madison, WI, USA).

Table 2.1 Degenerate oligonucleotide primer sequences derived from conserved peptide sequences for amplification of the initial cDNA fragment of *L. leucocephala* chitinase by Nested PCR.

Primer name	Peptides sequences (restriction site)	Nucleotide sequences
Chit19E191f (outer forward)	E-I/V/L-A-A-F-F/L-A/G-Q-T	5′-GA(A/G)(A/G)TIGCIGCITT(T/C) (T/C)TIG(G/C)ICA(A/G)AC-3′
Chit19H201f (inner forward)	(*Bam*H I)-H-E-T-T-G-G-W	5′-CTGGATCCA(T/C)GA(A/G) ACIA CIGGIGGNTGG-3′
Chit19P301r (inner reverse)	(*Xho* I)-P/A-T-M-W-F-W-M/F/I-A	5′-AACTCGAG(G/C)IGTCATCCA (A/G)AACCAIA(A/T)NGC-3′
Chit19A326r (outer reverse)	G-F/Y-G-P-V/L-R-N/G/R-A	5′-CC(A/G)(A/T)AICCIGGIA(C/G/A)IC (T/C)I(T/C)(T/C/G)NGC-3′

Table 2.2 Primers sequences used for 3′ RACE and 5′RACE. Primers QT, KB-1f, and KB-1r were used for 3′RACE and KB-1f, KB-2r, KB-3r, and KB-4r were used for 5′ RACE.

Primer name	Nucleotide sequences
QT	5′-CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTT TTTTTTTT-3′
KB-1f	5′-TCCTTACGCCTGGGGTTACTGC-3′
KB-1r	5′-CAGTGAGCAGAGTGACGAGGACTC-3′
KB-2r	5′-GCACATGGATATTGG GAGCTGGG-3′
KB-3r	5′-CTCCCTCTTGCGCGTGGC-3′
KB-4r	5′-CGTCGTTGCGGTGTTGAGCATC-3′

Table 2.3 Primer for amplification of cDNA for expression. Primer sequences were derived from the full-length nucleotide sequences for amplification of the protein coding sequence without the prepeptide of the *L. leucocephala* chitinase cDNA. Chi-Ex1f (forward) and Chit-Ex1r (reverse) primers were used for pET23d, KBEX-2F (forward) and KBEX-2R (reverse) primers for pET32a.

Primer name	Nucleotide sequences
Chi-Ex1f	5′-CCATGGAGCAATGCGGCAGAC-3′
Chi-Ex1r	5′-CTCGAGGAGGACGTCGATGAG-3′
KBEX-2F	5′-CCCATGGAGCAATGCGGCAGA-3′
KBEX-2R	5′-CCTCGAGGAGGACGTCGATGA-3′

2.1.4 Chemicals and molecular reagents

Acrylamide, bis-*N*,*N*¢-acrylamide, ammonium persulfate, *N*,*N*¢,*N*¢¢,*N*¢¢¢-tetramethyl-ethylenediamine (TEMED), sodium dodecyl sulfate (SDS), Sephadex G-100, cDNA synthesis kit, lysozyme, triton X-100, and PhasGel blue R were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Restriction endonucleases including *Bam*H I, *Nco* I, and *Xho* I were purchased from New England Biolabs (Beverly, MA, USA). Ammonium sulfate, bromophenol blue, Folin reagent, chloroform, copper sulfate, sodium hydroxide, methanol, ethanol, sodium bicarbonate, sodium dihydrogen phosphate, glacial acetic acid, hydrochloric acid, HPLC water, and disodium ethylenediamine tetraacetate (EDTA) were purchased from Carlo ERBA (Rodano, Milano, Italy). Bacteriological peptone, yeast extract, and agar were purchased from DIFCO (Grayson, USA). Coomassie brilliant blue G-250, sodium chloride, imidazole, phenyl-methylsulfonyl fluoride (PMSF), diethyl pyrocarbonate, ethidium bromide (EtBr), and magnesium chloride were purchased from Fluka (Steinheim, Swizerland). Superscript Reverse Transcriptase II, RNase H, *Msp* I, and *Nsp* I were purchased from Gibco-BRL. Ampicillin, sodium tetraborate, and boric acid were purchased from Merck (Damsladt, Germany). YM-10 Amicon filters were purchased from Millipore Corporation, Bedford, USA. Agarose, deoxyribonucleotide (dATP, dCTP, dGTP,dTTP), X-Gal, PolyATtract mRNA isolation systems IV, Taq polymerase, Pfu DNA polymerase, T4 DNA ligase and restriction endonucleases including, *Bam*H I, *Eco*R I, *Hinc* II, *Nco* I, *Pst* I, *Sac* II, *Xho* I were products from Promega.

QIA prep spin Mini prep kit, and Ni-NTA superflow were purchased from QIAGEN. Bovine serum albumin (BSA), isopropyl-*b*-D-thiogaltopyranoside (IPTG), Tris-hydroxymethylene-diamine, polyvinylpolypyrrolidone (PVPP), dimethylaminobenzaldehyde (DMAB), chitin flakes from crab shells, chitosan, purified chitin powder from crab shells, chitosan *b*-mercaptoethanol, chloroform/isoamyl alcohol (24:1), RNase Zap, *p*-nitrophenyl-*N*-acetylglucosamine, *p*-nitrophenyl-*N*,*N*′-diacetylchitobiose, *p*-nitrophenyl-*N*,*N*′,*N*′′-triacetylchitotriose, *N*-Acetyl-*b*-D-glucosamine, *N*,*N*′-diacetylchitobiose, *N*,*N*′,*N*′′-triacetylchitotriose, and *N*,*N*′,*N*′′-tetraacetyl-chitotetraose were purchased from Sigma. Lambda gt11 was purchased from Stratagene. 5′-Full RACE Core Set and P^{32} label isotope were purchased from TaKaRa, Japan. All chemicals used were analytical reagent grade or molecular biology grade. Geneclean II kit was produced from BIO 101, Inc., La Jolla, USA. ABI PRISM Dye terminator cycle sequencing kit was produced from Perkin Elmer Applied Biosystems Inc., Branchburg, NJ, USA. Other chemicals and solvents used but not listed were analytical or molecular biology grade and were purchased from a variety of suppliers.

Glycol chitin was synthesized according to the method of Trudel and Asselin (1988) from glycol chitosan.

Fourteen type strains of fungi for test chitinase activity were from the Plant Pathology and Microbiology Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok, Thailand and Department of Biology Faculty of Science, Chiang Mai University, Chiang Mai, Thailand. There were *Anthacnose collectotrichum*, *Cerospora* sp., *Cladosporium* sp., *Collectotrichum* sp. 1 and 2, *Drechslera* sp., *Fusarium* sp, *Fusarium monoliforme*, *Fusarium oxysporum*, *Pestalestiopsos* sp. 1, 2, 3, and 4, and *Sclerotium* sp.

2.2 Methods

2.2.1 Cloning and sequencing of the initial chitinase fragment

2.2.1.1 Oligonucleotide primer design

To perform PCR reactions, two sets of oligonucleotide primers were designed from amino acid sequences in plant chitinase family 19 from Swiss-Prot database by using the computer programs of Feng and Doolittle (1996) to build multiple sequence alignments of known chitinase sequences from which conserved sequences were identified. Conserved protein sequences were translated to yield possible nucleotide sequences. Degeneracy was calculated

as the total number of sequences possible from the combination of based each protein and sequences with lowest degeneracy were used to make Nested [Chit19H201f (inner forward), Chit19P301r (inner reverse), Chit19E191f (outer forward), and Chit19A326r (outer reverse)] primers for PCR as shown in Table 2.1.

2.2.1.2 Total RNA Isolation

Isolation of total RNA from cells and tissues were done using TRIZOL Reagent (GIBCO-BRL, Life Technologies, Grand Island, NY) as developed by Chomczynski and Sacchi (1987). One hundred micrograms of germinated *L. leucocephala* seeds were homogenized using a cold mortar and pestle in liquid N_2 and transferred into a new sterile microtube containing 1 ml of Trizol reagent. The homogenized sample was incubated for 5 min at room temperature, followed by adding 0.2 ml of chloroform. The mixture was mixed vigorously by hand for 15 sec, incubated at room temperature for 2-3 min, and then centrifuged at 12,000 x *g* for 1 min at 4◦C. The colorless upper aqueous phase containing RNA was transferred into a fresh tube. The RNA was precipitated from the aqueous phase by mixing with 0.5 ml of isopropyl alcohol, incubating at room temperature for 10 min, and pelleting by centrifugation at 12,000 x *g* for 10 min at 4◦C. The pelleted RNA was washed with 75% (v/v) ethanol (in DEPC-treated water) and dried briefly at 37◦C. Finally the dried RNA pellet was resuspended in 20 μl of DEPC-treated water. The RNA solution was immediately used to synthesize first strand cDNA, as described in next step, or kept at –70◦C. The concentration of the RNA solution was determined by measuring the UV absorbance at 260 nm of a 1 μl aliquot, diluted to 1 ml in distilled water. One absorbance unit is equivalent to 40 μg/ml RNA. The purity of RNA was indicated with absorbance of A_{260}/A_{280} ratio between 1.80-2.00.

2.2.1.3 First-strand cDNA synthesis

The extracted total RNA was used as the template for the synthesis of first-strand cDNA using Superscript Reverse Transcriptase II (GIBCO-BRL). The reaction mixture was composed of 5 μg total RNA, 1 μl Oligo $(dT)_{17}$ (500 μg/ml) and sterile distilled water up to 12 μl. The mixture was heated at 70◦C for 10 min and quick chilled on ice. The contents of the tube were collected by brief centrifugation and 4 μl of 5X First Strand Buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl and 15 mM $MgCl_2$) , 2 μl of 0.1 M DTT, 1μl of 10 mM dNTP mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH) were added. The contents of the tube were mixed gently and incubated at 42◦C for 2 min. Then, 1 μl (200 units) of SUPERSCRIPT II was

added and mixed by pipetting gently up and down. The mixture was incubated at 42◦C for 50 min, and the reaction was stopped by heating at 70◦C for 15 min. To remove RNA complementary to the cDNA, 1 μl (2 units) of *E. coli* RNase H was added and incubated at 37◦C for 20 min. The cDNA was used as a template for amplification in PCR.

2.2.1.4 DNA amplification by polymerase chain reaction (PCR)

DNA amplification by polymerase chain reaction (PCR) was performed using 10% of the first-strand cDNA reaction as a template with the same components, but different primers, DNA templates and amplification conditions for amplification of different products. The reaction mixture was composed of 2.5 μl first-strand cDNA, 0.5 μl 10 pmol forward primer, 1 μl 10 pmol reverse primer, 2.5 μl 2 mM dNTPs mixed, 1.5 μl 1.5 mM $MgCl_2$, 2.5 μl 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0 and 1% Triton X-100), 0.25 μl (1U) Taq Polymerase (5 unit/μl) and autoclaved distilled water to make the volume up to 25 μl.

Two sets of Nested PCR programs were used for inner and outer primers as shown in Table 2.4, Table 2.5 respectively.

2.2.1.5 DNA analysis

The amplified PCR products were analyzed on 0.8% agarose gel electrophoresis in 1X TAE buffer (0.04 M Tris-HCl, 0.04 M acetic acid, 0.001 M EDTA (pH 8.0)). Aliquots of DNA samples were mixed 5:1 with 6X loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol). The sample in loading dye and the DNA marker were applied to the gel wells. Electrophoresis was performed at constant 80 V for 1h 30 min using 1X TAE buffer. After electrophoresis the gel was stained with ethidium bromide solution (0.1 μg/ml EtBr) for 10 min and destained with distilled water. The band in the gel was visualized by UV light transillumination at 254 nm.

2.2.1.6 Purification of DNA fragment in polyacrylamide gel

The DNA on agarose gel was extracted by using polyacrylamide gel. A mixture of DNA product was separated on a TBE (0.09 M Tris-HCl, 0.09 M boric acid, 0.002 M EDTA (pH 8.0)) 5% polyacrylamide gel. The DNA bands on the polyacrylamide gel were visualized under UV light, and transferred into 1.5 ml microtubes. The polyacrylamide gel was ground with a teflon pestle and suspended by adding 3 volumes of 0.5 M $NH_4CH_3CO_2$, 10 mM $MgCl_2$, 1 mM EDTA, and 0.1% of SDS, then the solution was mixed by vortex and shaken overnight. The mixture was separated by centrifugation at 10,000 x *g* for 3 min, the solution was transferred

to a new tube and 3 volumes phenol/chloroform was added. Then, the DNA was precipitated by adding 1/10 volume 3 M sodium acetate pH 5.2 and 2 volumes absolute ethanol and incubating at –20°C for 5 min. The DNA mixture was centrifuged at 14,000 x *g* for 10 min at 4°C, the DNA pellet was washed with 1 volume 70% ethanol and the DNA was dissolved with 10 μl of sterile distilled water. The concentration of DNA solution was determined by measuring the absorbance at 260 nm.

Table 2.4 First set of Nested PCR program

Cycle	Step	Temperature (°C)	Time
Prerunning	Denaturation	94	5 min
1-5	denaturation	94	30 s
	annealing	45	1 min
	extension	72	1 min
6-35	denaturation	94	30 s
	annealing	50	1 min
	extension	72	1 min
Postrunning	extension	72	7 min

Table 2.5 Second set of Nested PCR program

Cycle	Step	Temperature (°C)	Time
Prerunning	denaturation	94	5 min
1-5	denaturation	94	30 s
	annealing	45	1 min
	extension	72	1 min
6-35	denaturation	94	30 s
	annealing	52	1 min
	extension	72	1 min
Postrunning	extension	72	7 min

2.2.1.7 Ligation of DNA fragment into vector and transformation of competent cells

Ligation of gel-purified PCR products into pGEM-T vector was done according to the plasmid supplier's recommendation. The reaction mixture (10 μl) was composed of 0.5 μl of pGEM-T vector (50 ng/μl), 7.5 μl of eluted DNA fragment (10-50 ng), 1 μl of 10X ligation buffer, and 1 μl of T_4 DNA ligase. The mixture was incubated at 14°C overnight. A 100 μl aliquot of fresh or frozen competent cells was thawed on ice. The ligation mixtures were added to thawed competent cells, mixed gently by swirling and the tube store on ice for 30 min. The cells were heat shocked at 42°C for 90 sec and the tube rapidly transferred to ice for 2 min. The transformed cells were grown by adding 800 μl of SOC medium (20 g/l trytone, 5g/l yeast extract, 0.01 M NaCl, 0.0025 M KCl, 0.005 M $MgCl_2$, 0.005 M $MgSO_4$, and 0.02 M Glucose) and incubated the cultures at 37°C for 1 h. This culture was spread on LB plate containing 100 μg/ml ampicillin, 0.5mM IPTG, and 40 μg/ml X-Gal (LB/amp/IPTG/X-Gal), and the plate was incubated overnight at 37°C. The recombinant clones were identified as white colonies with non recombinant plasmid colonies staining blue.

2.2.1.8 Preparation of competent cells

Competent DH5α *E. coli* cells were used to prepared using the modified $CaCl_2$ method (Hanahan, 1983). A single colony of *E. coli* was inoculated into 100 ml of LB broth and incubated the culture for 10 h at 30°C at 55-70 rpm shaking until the optical density (OD) at 600 nm was approximately 0.5. The cells were transferred into sterile disposable, ice cold 50 ml polypropylene tube, chilled on ice for 10 min and the cells were recovered by centrifugation at 3,000 x *g* for 10 min at 4°C. The media was decanted from the cell pellets, and each pellet was resuspended in 15 ml of ice cold TB buffer (10 mM PIPES, 20 mM $CaCl_2$, 250 mM KCl, and 100 mM $MgCl_2$) and stored on ice and pelleted by centrifugation in the same condition. The supernatant from the cell pellets was decanted, and the tube was placed in an inverted position for 1 min to allow the last traces of fluid to drain away. Each pellet was resuspended in 2.79 ml of ice cold TB buffer and 0.21 ml of dimethyl sulfoxide for each 50 ml of original culture. Then, 100 μl aliquots of competent cells were transferred to sterile microtubes. These competent cells were transformed immediately or kept at –70°C.

2.2.1.9 Plasmid isolation using QIA prep Spin Miniprep Kit

The QIAprep spin Miniprep protocol was used to purify plasmid for sequencing using the recommended protocol (QIAGEN, 1999). A white colony was grown in LB broth with 100 μg/ml ampicillin at 37◦C overnight. Cells were pelleted from three milliliters of culture by centrifugation at 3,000 x *g* for 10 min. The cell pellets were resuspended in 250 μl of P1 buffer, followed by adding 250 μl of P2 buffer. The mixture was gently mixed by inverting the tube 4-6 times. Then 350 μl of N3 buffer was added and the tube immediately inverted. The DNA solution was separated by centrifugation at 12,000 x *g* for 10 min at 4◦C. A QIAprep spin column was placed in a 2 ml collection tube. The DNA liquid supernatant was applied into the QIAprep column, followed by centrifugation at 12,000 x *g* for 30-60 sec. The plasmid solution was packed into column and it was washed with 0.5 ml of PB buffer then with 0.5 ml PE buffer. Plasmid was eluted from column by adding 50 μl of EB buffer (elution buffer) (10 mM Tris-HCl, pH 8.5) to the center of QIA prep column, leaving it to stand for 1 min and centrifuging at 14,000 x *g* for 1 min. The concentration of plasmid solution was determined by measuring the absorbance at 260 nm. One absorbance unit was assumed to be equivalent to a plasmid concentration of 50 μg/ml. The purity of DNA was indicated by an A260/280 absorbance ratio between 1.60-1.80.

The size of recombinant plasmid insert was determined by digestion at the *Pst* I and *Sac* II sites present in the pGEM-T plasmid. The reaction mixture contained 1 μl purified plasmid, 1 μl *Pst* I (10 unit/μl), 1 μl *Sac* II (10 unit/μl), 1 μl 10X buffer H and 6 μl distilled water. The mixture was incubated at 37◦C overnight, and 0.8% agarose gel electrophoresis was carried out to determine the DNA insert size.

2.2.1.10 DNA sequencing and analysis

The purified DNA sample was sequenced in cycle-sequencing using the ABI PRISM dye labeled terminator kit with the recommended protocol. The reaction mixture was composed of 4 μl of Terminator Ready Reaction Mix, 1 μl purified plasmid DNA (100 ng/μl), 1 μl primer (3.2 pmol/μl) and 4 μl distilled water. Amplification was performed using the Gene Amp PCR system. The program was conducted by rapidly increasing the temperature to 96◦C, followed by 25 cycles of 96◦C for 20 s, 50◦C for 10 s and 72◦C for 4 min. The amplified products were purified by ethanol precipitation as described in the kit manual and loaded onto an ABI 310 DNA automated sequencer. The determined cDNA fragment sequence of *L. leucocephala* chitinase was searched to find confirm its relationship to known chitinases using BLASTX at the Basic Blast

facility of the Genbank database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The DNA sequence was further interpreted, converted to single letter amino acid code, and analyzed using the sequence analysis programs from the Baylor College of Medicine (BCM) Search Launcher Index (http://www.hgsc.bcm.tmc.edu).

2.2.2 DNA amplification by using 3′ RACE method

Total RNA from *L. leucocephala* seedlings were extracted using TRIZOL Reagent as described in method 2.2.1.2. First strand cDNA synthesis was performed with the QT primer. The mixture was prepared by using method 2.2.1.3 with 10 pmol QT primer, a universal primer. After the reaction was finished, the reaction was treated with 1 μl of RNase H to destroy the RNA template by incubating at 37°C for 20 min. For DNA amplification, the reaction mixture was composed of cDNA reaction with 10 pmol KB-1f primer (derived from the chitinase cDNA fragment sequence) and 10 mM KB-1r primer (derived from the QT primer internal sequence) and amplified using the program described in Table 2.6. The PCR product was analyzed by 0.8% agarose gel electrophoresis. The specific PCR product (expected size about 650 bp) was eluted from the polyacrylamide gel as described previously in method 2.2.1.6, cloned into pT7blue T-vector at *Eco*R V site and the clones sequenced by standard methods. The sequence was analyzed by using the computer program GENETYX Mac version 9.0 (Software Development, Shibuya-ku, Tokyo, Japan).

Table 2.6 Set of 3′ RACE PCR program

Cycle	Step	Temperature (°C)	Time
Prerunning	denaturation	94	5 min
1-35	denaturation	94	10 s
	annealing	58	10 s
	extension	72	30 s
Postrunning	extension	72	7 min

2.2.3 Northern bolt analysis

2.2.3.1 RNA isolation by CTAB method

RNA was isolated from shoots and roots of *L. leucocephala* at four weeks of germination using a CTAB isolation method modified from Doyle and Doyle (1987). Shoot and root tissues were ground to a powder in liquid nitrogen using a mortar and pestle. Five grams of frozen powder was added to 16 ml of 2X CTAB RNA isolation buffer (2% CTAB, 0.1 M Tris-HCl pH 9.5, 20mM EDTA, 1.4 M NaCl, 5% (v/v) *b*-mercaptoethanol). The mixture was incubated at 65◦C for 10 min. Samples were extracted twice in an equal volume of chloroform/isoamyl alcohol (24:1) and the phases were separated by centrifugation at 15,000 x *g* for 10 min at room temperature. Following centrifugation, the supernatant was moved to a new tube the same volume of phenol:chloroform was added, the tube was mixed by shaking and then centrifuged at 15,000 x *g* for 10 min at room temperature. One fourth volume of 10 M lithium chloride was added to the supernatant, and RNA was allowed to precipitate at least 2 h at –20◦C. RNA was pelleted by centrifugation at 15,000 x *g* for 10 min at 4◦C. The RNA pellet was dissolved with 500 µl of TE and the sample was centrifuged again at 15,000 x *g* for 10 min at 4◦C, 500 µl of TE saturated phenol pH 9.0 was added to the supernatant, it was mixed and centrifuged at 15,000 x *g* for 10 min at room temperature. The RNA was extracted by adding 500 µl of phenol-chloroform and shaking vigorously, the phases were separated by centrifugation at 15,000 x *g* for 10 min at 4◦C and the aqueous phase moved to a new tube. Then, 500 µl of chloroform:isoamyl (24:1) was added to this tube, it was mixed and centrifuged at 15,000 x *g* for 10 min at room temperature. One fourth volume of 10 M lithium chloride was added to the aqueous phase and it was incubated at –20◦C at least 2 h. The RNA was pelleted by centrifugation at 15,000 x *g* for 15 min at 4◦C, washed with 500 µl of 70% ethanol and resuspended in 15 µl of 0.1% DEPC-treated water.

2.2.3.2 Transfer RNA onto nitrocellulose membrane

Total RNA was isolated from shoots and roots of *L. leucocephala* at four weeks of germination using CTAB method as previously described. Aliquots of five micrograms of each total RNA sample were mixed with 6 µl of 50% DMSO, 1.2 µl of 100 mM phosphate buffer, 2 µl of 6 M glyoxal and distilled water up to 12 µl, denatured at 50◦C for 1 h and placed on ice for 3 min. The RNA was separated by electrophoresis through a 1.2% agarose gel in 0.01M phosphate buffer and capillary transferred to nitrocellulose membrane overnight (Sambrook *et al*.,

1989). The membrane was dried at room temperature for 5 min, RNA was fixed and linked onto membrane with UV light for 2 min using a transilluminator and kept at 4◦C until hybridization.

2.2.3.3 Preparation of probe DNA

2.2.3.3.1 Middle scale plasmid isolation by alkaline lysis method.

MJ 1-1-1 (plasmid containing 3′ RACE product) was isolated in medium scale by the alkaline lysis method (Birnboim and Doly, 1979). A single bacteria colony that contained MJ 1-1-1 plasmid was transferred into 50 ml of LB medium containing the 5 mg of ampicillin and incubated at 37◦C with vigorous shaking overnight. The culture was centrifuged at 3,000 x *g* for 10 min at room temperature and the pellet was resuspended in 1.5 ml of 50 mM Tris-HCl, 0.5 M EDTA pH 8.0, 50 mM glucose. Then 3 ml of 1% SDS/0.2 M NaOH was added, it was cooled on ice 3 min, 2 ml cold 3 M potassium acetate pH 4.8 was added and the tube inverted briefly. The mixture was centrifuged at 14,000 x *g* for 5 min at room temperature, the supernatant transferred to a new tube, 10 ml absolute ethanol added and the mixture centrifuged at 14,000 x *g* for 5 min at 4◦C. The supernatant was removed, and the pellet resuspended in 1 ml 20 mM Tris-EDTA pH 8.0, 10 μl of 1 mg/ml RNase A was added and it was incubated at 37◦C for 15 min. Then, 500 μl 20% polyethylene glycol 6000/2.5 M NaCl solution was added, on ice for 1 h, and mixture was centrifuged at 14,000 x *g* for 5 min at 4◦C. The pellet was washed with 70% ethanol, dried in vacuum and dissolved in 50 μl 20 mM Tris-EDTA pH 8.0. The purity and concentration of DNA solution was evaluated by measuring the A_{260}/A_{280} ratio using a spectrophotometer.

2.2.3.3.2 Radio active labeling of cDNA fragment by the random primer method.

Five microliters (10 μg) of MJ 1-1-1 plasmid was mixed with 5 μl of 10X buffer M, 3 μl of *Bam*H I (15 units/μl), 3 μl of *Xba* I (15 units/μl) and 34 μl of distilled water to adjust the total volume to 50 μl and the reaction incubated at 37◦C for 1 h. The solution was loaded onto 5% polyacrylamide gel with TBE for electrode buffer and electrophoresed using voltage and current limits of 250 V, and 40 mA for 30 min. The band at 650 bp was cut and extracted with Gilbert buffer (Sambrook *et al*., 1989). Two microliters (20 ng) of digested gel-purified 3′ RACE fragment from above, 4.5 μl of distilled water, and 1 μl of random primer were mixed, denatured at 95◦C for 3 min and cooled on ice for 5 min. Then, 1.25 μl of 10X buffer, 1.25 μl of dNTP mixture, 1 μl of α–^{32}P dCTP, and 0.50 μl of *Bca* BEST DNA polymerase

were added and the mixture was incubated at 55◦C for 60 min. To stop the reaction, 1 μl of 0.5 M EDTA was added and incubated at 70◦C for 10 min. The mixture was adjusted to 100 μl with TES buffer (0.15 M NaCl, 10 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0) and centrifuged through a Sephadex G-50 spin column, which was equilibrated with TES buffer, to remove unincorporated nucleotides. The labeled probe was measured using a scintillation counter and kept at –20◦C. Prior to hybridization, the probe was heated at 100◦C for 5 min and immediately put on ice for approximately 10 min.

2.2.3.4 Hybridization and detection

The nitrocellulose membrane prepared as described in section 2.2.2.3 was put into a plastic hybridization bag. About 5 ml of prehybridization buffer (1.25 ml of 20X SSPE, 0.05 ml of 10% SDS, 0.1 ml of 50X Denhardt's solution, and 3.6 ml of distilled water) was transferred onto the plastic bag to soak the membrane. The membrane was prehybridized at 60◦C for 30 min in a hybridization oven, and then 30 μl of labelled DNA probe (4 ng) mixed with 20 μl of salmon sperm DNA was added to the bag. Hybridization was performed at 60◦C for 18 h. After hybridization, the nitrocellulose membrane was washed twice with 50 ml of 2X SSC for 2 min at room temperature, 50 ml of 2X SSC containing 0.1% SDS for 15-30 min at 60◦C and 2X SSC for 5 min at room temperature. The wash solution was aspirated and the membrane was dried at room temperature for 1-2 min. The membrane was placed in the plastic bag and exposed to high sensitivity X-ray film (Hyper film ™-MP, Amersham) inside a cassette at –80◦C overnight before developing the film.

2.2.4 Construction and screening of 4 week old seedling shoot cDNA library

2.2.4.1 Isolation of mRNA

Total RNA was isolated by CTAB method (as described in 2.2.3.1). Poly(A)$^+$ mRNA was isolated using polyA tract mRNA isolation system III (Promega Madison, WI). One milligram of total RNA was incubated at 65◦C for 10 min and 3 μl of the biotinylated-Oligo (dT) and 13 μl of 20X SSC were added, mixed and incubated at room temperature for 10 min. SA-PMP was added to the tube and resuspended the tube was captured in a magnetic stand for 30 sec, the supernatant removed, and the SA-PMP washed with 0.3 ml of 0.05X SSC 3 times. Then, 0.1 ml of 0.5X SSC, was poured into the washed SA-PMPs and incubated at room temperature for 10 min. The tube was captured in a magnetic stand for 30 sec, the supernatant

removed and the pellet washed with 0.3 ml of 0.1X SSC 4 times. Finally, 0.1 ml of water was added to the SA-PMP, it was captured on the magnetic stand for 30 sec and the aqueous mRNA was transferred to a sterile tube and kept at –80◦C.

2.2.4.2 Precipitated mRNA by using isopropanal

Two hundred and fifty milligrams of mRNA were mixed with 25 μl of 3 M sodium-acetate and 250 μl of isopropanal and incubated at –20◦C overnight. After that, the mixture was centrifuged at 15,000 x g for 10 min and the supernatant removed. The pellet was washed with 500 μl of 70% ethanol and centrifuged at 15,000 x *g* for 10 min, then the supernatant was removed. The pellet was dried under vacuum for 5 min and DEPC-water added to give a final mRNA concentration of 1 μg/μl.

2.2.4.3 Construction of λgt11 cDNA library

2.2.4.3.1 Poly A^+ mRNA denaturing and the first strand reaction

The cDNA was prepared from 5 μg of poly$(A)^+$mRNA with a cDNA synthesis kit (Pharmacia) by adding DEPC water up to 20 μl, incubation at 65◦C for 10 min, chilling on ice for 5 min, then adding 10 μl of 1st-strand reaction mixes (Cloned Murine Reverse Transcriptase, RNAguard, RNase- and DNase-Free BSA, Oligo$(dT)_{12-18}$ primer, dATP, dCTP, dGTP, dTTP), 1 μl of [α-32P] dCTP (5 μCi/μl), 1 μl of DTT solution and incubated at 37◦C for 1 h.

2.2.4.3.2 Monitoring

Three microliters of 1st-strand reaction mixes was transferred to a new tube, 17 μl of 0.5 M EDTA (pH 8.0) and 80 μl of ssDNA (salmon sperm DNA) was added and then, 2 μl was spotted onto a glass filter, air dried and counted with a scintillation counter (T1 value). Then 1 ml of 10% TCA was mixed with 98 μl of 1st-strand reaction, incubated on ice for 10 min, filtered and the filtrate washed with 10 ml of 10% trichloroacetic acid (TCA), 2 ml of 100% ethyl alcohol in 1 ml of filter sample, air dried and counted (P1 value)

2.2.4.3.3 The second strand reaction

The second strand reaction mixture composed of 5 μl of [α-32P] dCTP, 70 μl of 2nd-strand reaction mixes (*E. coli*, RNase H and *E .coli* DNA polymerase I in aqueous buffer containing dNTPs), and 30 μl of 1st-strand reaction. The mixture reaction was agitated, spun down, and incubated at 12◦C for 1 h. and 22◦C for 1 h., then 1 μl of Klenow DNA polymerase fragment was added and incubated at 37◦C for 30 min. Then, 50 μl of DEPC-H_2O (to give a final

volume of 150 μl), 150 μl of 4 M ammonium acetate, and 300 μl of phenol/chloroform were added to the reaction mixture, which was mixed, centrifuged at 12,000 x *g* for 3 min at room temperature and the supernatant transferred to a new tube. The supernatant was precipitated with 700 μl of 100% of ethyl alcohol. The pellet was separated by centrifuged at 12,000 x *g* for 10 min at 4◦C, dried under vacuum and dissolved in 37 μl of DEPC-H_2O. The 2nd-strand reaction was monitored as described in the method 2.2.4.3.2 with replacement of T1 with T2 and P1 with P2.

The yield of cDNA was calculated as follows:

$$\text{First-strand cDNA} = \frac{P1}{T1} X\ 1.75$$

$$\text{Second-strand cDNA} = \frac{\left[P2-\left(\frac{P1}{0.31}\right)\right]}{\left[T2-\left(\frac{P1}{0.31}\right)\right]} X5.57$$

2.2.4.3.4 Ligation of ***Eco***R I/***Not*** I adaptors, phosphorylation and purification

Thirty seven microliters of double stranded cDNA were mixed with 5 μl of 10X ligation buffer, 5 μl of adapter solution, 3 μl of T_4 DNA ligase and incubated at 8◦C overnight. The ligation solution was incubated at 65◦C for 10 min and cooled down. Fifty microliters the ligation mixture was mixed with 6 μl of 10X ligation buffer, 10 μl of ATP solution, 43 μl of DEPC-water and 1 μl of T4 poly nucleotide kinase, and incubated at 37◦C for 30 min and 65◦C for 10 min. The reaction was extracted with 110 μl of phenol/chloroform. The supernatant was separated by centrifuged at 12,000 x *g* for 5 min at room temperature and 100 μl of supernatant was transferred to a spin column and eluted by centrifuged at 12,000 x *g* for 5 min at room temperature. Then, 100 μl of phenol/chloroform was added to the 100 μl of cDNA solution, mixed and centrifuged at 12,000 x *g* for 5 min at room temperature. The supernatant was precipitated with 10 μl of sodium acetate pH 5.2 and 500 μl of 100% ethyl alcohol. The pellet was separated by centrifuging at 12,000 x *g* for 5 min at 4◦C and incubating at –80◦C for 1 h. The pellet was washed with 500 μl of 70% ethyl alcohol and centrifuged at 12,000 x *g* for 5 min at 4◦C, the supernatant removed, and the pellet dried and dissolved in 10.5 μl of DEPC-water. Then, 0.5 μl of cDNA was added with 4.5 μl of DEPC-water. Three microliters was counted with a scintillation (P3 value) and two microliters of cDNA was analyzed by agarose gel elctrophoresis.

The gel was washed twice with 7% TCA for 10 min, stained 0.1 μg/ml ethidium bromide to visualize the maker and autoradiographied.

2.2.4.3.5 Vector ligation and packing

The molar ratio of vector and cDNA was adjusted with the calculation below.

$$\text{mole of } \lambda gt11 = \text{mole of cDNA}$$

$$\frac{44 \text{ kb}}{1000 \text{ng}} = \frac{1.1 \text{ kb}}{X \text{ng}}$$

$$X = 25 \text{ ng}$$

or $$\frac{0.3}{10.5} xP3{:}X = \frac{3}{100} xP2{:}0.44$$

Two microliters of cDNA solution (25 ng) was mixed with 1 μl of λgt11, 0.5 μl of ATP solution, 0.5 μl of ligation buffer, and 1 μl of T4 DNA ligase and incubated at 4◦C overnight. Five microliters of the cDNA library was added to the packing extract (stored at -80◦C) immediately when it began to thaw and the tube was incubated at 22◦C for 2 h. The reaction mixture was mixed with 500 μl of SM buffer (0.1 M NaCl, 0.01 M $MgSO_4$, 1 M Tris-HCl pH7.0, 2% gelatin), and 20 μl of chloroform, spun down and stored cDNA library at 4◦C.

2.2.4.4 Titering

Two microliters of cDNA library was diluted 100 fold by addition of 198 μl of SM buffer. Two micro liters of the 100 fold dilution of the cDNA library was diluted to 1,000 fold by adding 18 μl of SM buffer. After that aliquots of 10, 5, and 2 μl of each dilution of cDNA library were mixed with 200 μl of Y1090r$^-$ competent cells, incubated at 37◦C for 15 min and mixed with 8 μl of 100 mM IPTG, 8 μl of 10% X-gal, 3 ml of melted top agarose (LB, 25 mg/ml ampicillin, 0.01 mM $MgSO_4$, 0.8% agarose) and 50 μl of 25 mg/ml ampicillin. The mixture was poured on top of a LB ampicillin plate and incubated at 42◦C for 12 h. The number of phage plaques was then counted.

2.2.4.5 Preparation of probe for screening the cDNA library

The two fragments of 3′ RACE product produced by digestion with *Msp* I and *Nsp* I were prepared as separate probes, 331 bp (5′ terminus of 3′ RACE) and 242 bp (3′ terminus of 3′ RACE) using the same method used to prepare probes for northern blotting (Section 2.2.3.3).

2.2.4.6 First round screening of the cDNA library and collection of plaques

A single colony of *E.coli* (Y1090r-$^{-}$) was inoculated into 500 ml of LB medium containing 0.2% maltose, 10.0 mM $MgSO_4$. The culture was incubated with shaking at 37◦C for 5 h until the OD_{600} was approximately 1.0, then 2 ml of this culture was mixed with 350 μl of a 1,000X dilution of λ phage solution. The mixture was incubated without shaking at 37◦C for 15 min to allow phage particles to be adsorbed to the host cells. To propagate the λ phage, 45 ml of 0.8% top agarose containing 10 mM $MgSO_4$, 25 μg/ml ampicillin was added to the mixture and immediately poured onto a LB big plate (20x40 cm). After the agarose hardened, the plate was inverted and incubated for 8 h at 42◦C until cleared plaques appeared. The LB plates containing cleared plaques were incubated at 4◦C for 1 h before making a replica plate. Phage particles, the plaques on the LB plate were transferred to a 10x20 cm nitrocellulose membrane by applying the reactive side of the membrane on top of the agar. The phage particles were allowed to adsorb to the membrane for 5 min. Then, the membrane was gently peeled off and dried for 5 min at room temperature. The phage particles were lysed by treating the membrane with denaturing solution containing 0.2 M NaOH and 1.5 M NaCl on filter paper for 5 min and letting the membrane dry for 5 min at room temperature, followed by treating the membrane twice with neutralizing solution containing 1M Tris pH 7.4 and 1.5 M NaCl. The DNA was immobilized by baking the treated membrane in a hot air oven at 80◦C for 2 h.

Nitrocellulose membrane prepared as described above were folded in a plastic hybridization bag. About 10 ml of hybridization solution was transferred into the plastic bag to soak the membrane. The membrane was prehybridized at 60◦C for 6 h and then labelled DNA probe (chitinase fragment), prepared as previously described in section 2.2.4.5 was added to the plastic bag. Hybridization was performed at 60◦C for 18 h. After hybridization, the nitrocellulose membrane was washed twice with 2X SSC for 5 min at room temperature and twice with 2X SSC containing 1% SDS at 60◦C for 15 min. The wash solution was aspirated and the membrane was dried at room temperature for 5 min. The membrane was covered with saran wrap and exposed to high sensitivity X-ray film (Hyper film-MP, Amersham). The membrane and film were placed inside a metal cassette and incubated at –80◦C for 4 h before developing.

Plaques that gave very high hybridization signal were collected. Top agarose plugs containing the positive plaques were picked from the plate by using a pasteur pipette and transferred to 1 ml of sterile SM buffer in a microtube. The tube was then vigorously mixed by

vortexing. About 3-4 drops of chloroform was added. The tube was kept for 1 h at room temperature, followed by centrifugation at 14,000 x *g* for 5 min at 4◦C to separate the supernatant from the pellet before keeping the supernatant at 4◦C.

2.2.4.7 The second and the third round screening of the cDNA library.

Two microliters of 1,000X dilution λ phage from the first round screening was mixed with 200 μl of *E.coli* (Y1090r-) prepared as described in method 2.2.4.4. The mixture was incubated with shaking at 37◦C for 15 min to allow phage particles to be adsorbed to the host cells. To propagate the λ phage, 3 mL of 0.8% top agarose containing 10 mM $MgSO_4$ and 25 μg/ml ampicillin was added to the mixture and immediately poured onto a 10 cm circular LB plate. After the agarose hardened, the plate was inverted and incubated at 42◦C for 8 h until clear plaques appeared. The plaques were transferred to 3x7 cm nitrocellulose membrane by applying the nitrocellulose membrane on top of the agar. Following these plaques lifts, hybridization, detection by autoradiography and collection of the positive plaque was done as previously described. A third round of screening was conducted in the same manner to allow isolation of individual clones.

2.2.5 Isolation of plaque DNA

After the third screening, plaques that gave high hybridizing signal were collected. Top agarose containing a single positive plaque was picked from plate using pasteur pipette and transferred to 200 μl of SM buffer in a sterile microtube. Chloroform was added to a final concentration of 10% (v/v). The tube was then vigorously vortex and centrifuged at 14,000 x *g* for 10 min at 4◦C to separate the supernatant, before keeping at 4◦C.

Fifteen clones from positive plaques were selected and designated as 1, 2, 3, 4, 5, 6, 7, 8,9, 10, A, C, D, E, and J. Each of these selected plaques was transferred into host cell as described in section 2.2.4.7. After clear plaques were appeared, 5 ml of SM buffer was added to each plate and shaken for 1 h. at room temperature, and the supernatants were completely collected using pasteur pipettes and put into sterile 15 ml tubes. One hundred microliters of chloroform was added to the supernatants. The tubes were vigorously vortexed and spun down at 14,000 x g for 10 min at 4◦C. The supernatants containing phage particles, were transferred into new tubes and keep at 4◦C. To amplify the phage particles, 4 μl of the phage solution was mixed with 200 μl of bacterial suspension ($Y1090r^-$) and incubated at 37◦C for 15 min. The mixture was then transferred to

20 ml of LB medium containing 25 μg/ml ampicillin and shaking continued at 37◦C for 6 h until cell debris appeared.

To complete cell lysis, 200 μl of chloroform was added and vortexed. Cell debris in the lysate was removed by centrifugation at 5,000 x *g* for 5 min at 4◦C and then the supernatant was transferred into a new tube. The solution was mixed with an equal volume of 20% (w/v) polyethylene glycol (PEG800) and 10 ml of 2.5 M NaCl, and incubated at 0◦C on ice for 1 h. Phages particles were recovered by centrifugation of the mixture at 3,000 x *g* for 20 min at room temperature. The supernatant was discarded and the precipitate was allowed to dry at room temperature. The phage particles were resuspended in 1 ml of TE buffer pH 8.0. In order to the digest host's DNA and RNA, *DNase* I and *RNase* A were added to a final concentration of 10 μg/ml each, the mixture was incubated at 37◦C for 1 h followed by addition of 200 μl of chloroform and centrifugation at 12,000 x *g* for 10 min at 4 ◦C. The supernatant was transferred into new tube and 32 μl of 10% SDS, 40 μl of EDTA and 2 μl of 10 mg/ml of proteinase K added, followed by an incubation at 65◦C for 30 min. Recombinant DNA was extracted by the phenol/chlorofom method. The DNA pellet was precipitated with 500 μl of isopropanol, rinsed 500 μl of 70% ethanol and dried under vacuum. The DNA pellet was dissolved in 50 μl of TE and stored at 4◦C until used.

2.2.6 Subcloning of 1 DNA insert into pUC19 and characterization

Phage DNA insert size from the previous step was determined by digest with *Eco*R I enzyme. The restriction mixture was composed of 10 μl of DNA (50 ng), 3 μl of *Eco*R I (12 U/μl), 3 μl of buffer H and 14 μl of distilled water. The solution was incubated at 37◦C overnight. DNA was loaded onto a 5% polyacrylamide gel. The restriction fragment was ligated into pUC19 vector. The reaction mixture (10 μl) was composed of 1 μl *Eco*R I digested pUC19 vector (50 μg/μl), 1 μl 10X ligation buffer, 1 μl T_4 DNA ligase, 7 μl eluted DNA fragment (200 ng). The mixture solution was incubated at 14◦C overnight and then, transformed into competent DH5α cells, followed by plasmid isolation as previously described (Section 2.2.1.7). To determined the DNA sequence, the recombinant plasmid was sequenced using a ABI 310 automated DNA sequencer and ABI PRISM dye label terminator kit as previously described. The sequence was translated to amino acid sequence using the computer program GENETYX

Mac version 9.0 and the amino acid sequence aligned with other chitinase sequences using the NCBI BLAST search.

2.2.7 Completion of the chitinase cDNA sequence by 5′ RACE and sequencing

The oligonucleotide primers for 5′ RACE, shown in Table 2.2, were designed from sequence of the cDNA library clones. Total RNA from *L. leucocephala* seedlings was extracted using TRIZOL Reagent as described in section 2.2.1.2. First strand cDNA synthesis was performed with a KB-2r primer, derived from the internal cDNA chitinase fragment sequence. The mixture was prepared as described in section 2.2.1.3 with 10 pmol KB-2r. After the reaction was finished, the reaction was treated with 1 μl of RNase H to destroy the RNA template by incubating at 37◦C for 20 min. The single stranded cDNAs were purified by ethanol precipitate and ligated with T_4 RNA ligase. For DNA amplification, the reaction mixture was contained 1 μl of 10 fold diluted cDNA, 10 pmol KB-1f primer and KB-3r primer and the other components described in section 2.2.1.4, and was amplified using the program described in Table 2.7.

After the first PCR experiment was finished. A second amplification was performed using 1 μl of a 500-fold dilution of the first PCR product with 10 pmol KB-1f primer and KB-4r primer according to the program described in Table 2.7. The product was analyze by 0.8% agarose gel electrophoresis, as previously described. The specific PCR product (expected size about 200 bp) was gel-purified using a polyacrylamide gel as described previously in section 2.2.1.6. The gel purified product was cloned into pT7blue T-vector at *Eco*R V site according to the plasmid supplier's recommendation. The reaction mixture (10 μl) was composed of 1 μl pT7blue T-vector (50 μg/μl), 1 μl 10X ligation buffer, 1 μl T_4 DNA ligase, 7 μl eluted DNA fragment (200 ng). The mixture solution was incubated at 14◦C overnight and then, transformed into competent DH5α cells, followed by plasmid isolation as previously described in section 2.2.1.10. The recombinant plasmid insert was determined by digestion at the *EcoR* I and *Pst* I sites present in the pT7blue T-vector. The reaction mixture contained 1 μl (1 μg/μl) purified plasmid, 0.5 μl *EcoR* I (15 units/μl), 0.5 μl *Pst* I (15 units/μl), 1 μl 10X buffer H, and 7 μl distilled water. The mixture was incubated at 37◦C overnight and 0.8% agarose gel electrophoresis was carried out to determine the DNA insert size. The purified DNA sample was sequenced as previously described in section 2.2.1.12.

Table 2.7 Set of 5′ RACE PCR program

Cycle	Step	Temperature (°C)	Time
Prerunning	denaturation	94	5 min
1-30	denaturation	94	30 s
	annealing	60	30 s
	extension	72	1 min
Postrunning	extension	72	7 min

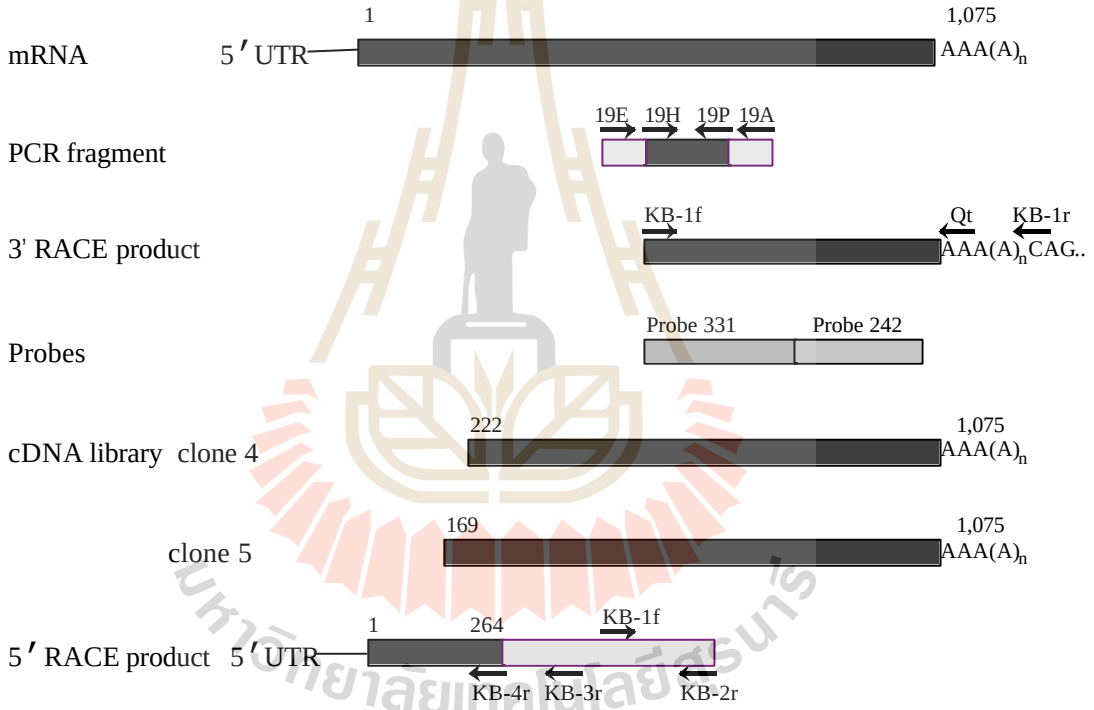

Figure 2.1 Diagram of cloning strategies for leucaena chitinase showing the cDNA sequence length and positions of primers used for cloning. Simple numbers indicate the position relative to the first nucleotide of the protein coding sequence. Other symbols are as follows:

19E: Chit19E191f (position 391-417)
19H: Chit19H201f (position 418-441)
19P:Chit19P301r (position 785-708)
19A: Chit19A326r (position 778-800)
KB-1f (position 459-478)
KB-2r (position 521-542)
KB-3r (position 376-392)
KB-4r (position 264-285)
Probe 331 (position 459-789)
Probe242 (position 790-1031)

2.2. 8 Expression in ***E.coli***

2.2.8.1 RNA extraction and PCR amplification.

Total RNA was extracted from four week-old seedling shoots and roots by Trizol reagent (the same method as section 2.2.1.2). Oligonucleotide primers for DNA expression were designed from *L. leucocephala* class I chitinase without prepeptide. Single-stranded cDNAs were synthesized by reverse transcription, using poly T primer and Superscript II reverse transcriptase (Gibco BRL) and amplified in a 25 μl PCR reactions contain 1 μl 50 pmoles template, 1 μl 50 pmoles forward primer, 1 μl 50 pmoles reverse primer (Table 2.3), 2.5 μl 2 mM dNTPs mixed, 1.5 μl 1.5 mM $MgCl_2$, 2.5 μl 10X *Pfu* buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0 and 1% Triton X-100), 0.5 μl *Pfu* DNA polymerase (1 unit/μl), and autoclaved distilled water to make the volume up to 25 μl according to the program described in Table 2.8. The expected size PCR product was purified by 5% polyacrylamide gel electrophoresis. The pUC19 was digested with *Hinc* II to give blunt ends. The restriction mixture was composed of 1 μl pUC19 vector (1 μg), 1 μl 10X buffer B, 0.5 μl *Hinc* II (10 units/μl), 1 μl 1% BSA, and 6.5 μl of distilled water. The purified PCR product was cloned into the *Hinc* II blunt-end site of the pUC19 vector by standard methods (Maniatis *et al*., 1982). The reaction mixture (10 μl) was composed of 6 μl eluted DNA fragment (600 ng), 1 μl pUC 19 vector (50 ng) 1 μl 10X ligation uffer, 1 μl of T_4 DNA ligase, and 1 μl distilled water. The mixture was incubated at 14◦C overnight and then, transformed into competent DH5α cells, followed by plasmid isolation as previously described in section 2.2.1.10. The recombinant plasmid insert was determined by digestion at *Nco* I and *Xho* I sites present in the linker-primers. The reaction mixture contained 1 μl (1 μg/μl) purified plasmid, 0.5 μl *Nco* I (10 units/μl), 0.5 μl *Xho* I (15 units/μl), 1 μl 10X buffer K, and 7μl distilled water. The mixture was incubated at 37◦C overnight and 0.8% agarose gel electrophoresis was carried out to determine the DNA insert size. The purified plasmid DNA sample was sequenced as previously described in section 2.2.1.12.

2.2.8.2 Construction of pET 23d and pET32a leucaena chitinase gene.

pET23d and pET32a plasmids were digested with *Nco* I and *Xho* I. The restriction mixture was composed of 1 μl pET23d or pET32a vector (1 μg), 1 μl 10X buffer K, 0.5 μl *Nco* I (10 units/μl), 0.5 μl *Xho* I (15 units/μl), and 7 μl distilled water. The mixture was incubated at 37◦C overnight and the expected size was purified by 5% polyacrylamide gel electrophoresis. The purified DNA sample from section 2.2.8.1 was cloned into the *Nco* I and *Xho* I site of the

pET23d and pET32a vector with by standard methods (Maniatis *et al*., 1982). The reaction mixture was composed as described in section 2.2.8.1. The solution was incubated at 14◦C overnight and then, transformed into competent DH5α cells, followed by plasmid isolation as previously described in section 2.2.1.10. The recombinant plasmid insert was determined by digestion at *Nco* I and *Xho* I sites present in the linker-primers as described above. The mixture was incubated at 37◦C overnight and 0.8% agarose gel electrophoresis was carried out to determine the DNA insert size. The correct assembly of the resulting plasmid and the two sequence of the amplified DNA were confirmed by automated DNA sequencing.

The plasmids retransformed into *E.coli* strain BL21 (DE3) and Origami (DE3) by the following methods. For pET23d insert plasmid, used the same method in section 2.2.1.7 with 1μl (1 μg/μl) plasmid. For pET32a insert plasmid, 1 μl (1 μg/μl) plasmid was added to 50 μl thawed Origami (DE3) competent cells, mixed gently by swirling and the tube store on ice for 5 min. The cells were heat shocked at 42◦C for 30 sec and the tube rapidly transferred to ice for 2 min. The transformed cells were grown by adding 800 μl of SOC medium and plated directly on an LB plate containing 50 μg/ml ampicillin, which was then incubated overnight at 37◦C. The recombinant clones were confirmed by PCR amplification using expression primer, T7 promoter and T7 terminus which present in pET23d and pET32a.

Table 2.8 Set of PCR program for expression

Cycle	Step	Temperature (◦C)	Time
Prerunning	denaturation	95	5 min
1-35	denaturation	95	30 s
	annealing	50	1 min
	extension	72	2 min
Postrunning	extension	72	7 min

2.2.8.3 Expression of leucaena chitinase in BL21 (DE3) and Origami (DE3) ***E.coli***

Optimum conditions for protein production in BL21 (DE3) and Origami (DE3) *E. coli* containing the expression plasmid pET23d and pET32a, respectively, were determined.

To do this, a colony was grown in 3 ml LB broth with ampicillin (50 μg/ml) at 37◦C overnight. Cells were pelleted by centrifugation at 3,000 x g for 10 min. The cells pellets were resuspended in 3 ml fresh LB broth with ampicillin (50 μg/ml) and grown at 30◦C for 8 h to A_{600}= 0.6 and induced with 0.05, 0.1, 0.3, 0.5, 0.7, and 0.9 mM isopropyl *b*–D-thiogalactopyranoside (IPTG) for 0, 2, 5, 8, 12, 18, and 24 h of pET-23d and grown for 0, 4, 8, 12, 16, 20, and 24 h of induction at 15 ◦C and 30 ◦C. Cells were harvested by centrifugation at 5,000 x g for 10 min at 4◦C, washed two times with 5 ml 50 mM sodium dihydrogen phosphate pH 8.0 with centrifugation at 5,000 x g for 10 min at 4◦C, and stored at –70◦C overnight.

2.2.8.4 Extraction of chitinase from BL 21 (DE3) and Origami (DE3) ***E.coli***

After growth and induction as described above, the cells were resuspended in 20 mM TE pH 8.0, 100 μg/ml lysozyme, 1% triton X-100, 1 mM phenylmethylsulfonylfluoride (PMSF) to a concentration factor of 50x (volume buffer X 50 = volume culture X OD_{600}) and lysed by sonication on ice at highest output (100 W) for 10 s, 5 times (David and Thomas, 1989) using probe sonicator (Ultrasonic Processor GE 100, Treadlitei, Woodstock, USA). Soluble and insoluble proteins were separated by centrifugation at 14,000 x *g* for 30 min. Protein concentrations were determined by Lowry assays. Samples for SDS/PAGE were heated at 100◦C for 5 min in the 1% mercaptoethanol of 0.5 M Tris/HCl, pH 6.8. SDS/PAGE was performed using 12-15% polyacrylamide gels. The gels were stained with Coomassie brilliant blue R 250 (0.1% Coomassie brilliant blue R 250 dissolved in 40% methanol, 10% acetic acid, 50% water) for 30 min and destained with destaining solution (40% methanol, 10% acetic acid, 50% water) for 1 h until protein bands appeared clearly.

2.2.8.5 Protein determination by Lowry method

Protein concentration was estimated by the method of Lowry using bovine serum albumin as standard (20-100 μg BSA). The diluted sample (1 ml) was mixed with 1 ml freshly prepared alkali copper solution made by mixing 1% copper sulfate, 2% potassium-sodium tartate and 2% sodium carbonate in the ratio of 1:1:100 by volume, respectively. The reaction was left to stand for 10 min at room temperature before adding 0.5 ml Folin reagent. The mixture was vigorously mixed and allowed to stand at room temperature for 30 min. The absorbance at 750 nm was measured using a Genway UV-VIS spectrophotometer (Feisted, Dunmow, Essex, UK).

2.2.8.6 Preparation of Colloidal chitin

Colloidal chitin was prepared according to the modified method of Shimahara and Takiguchi (1988). Chitin flakes (10 g) were added into 200 ml of concentrated hydrochloric acid on ice. The suspension was vigorously stirred for 2 h on ice and kept overnight at 4◦C. The suspension was filtered through cheesecloth and the filtrate was poured into 600 ml of 50% ethanol on ice with stirring. After 1 h., the suspension was filtered with suction through Whatman No. 1 filter paper. The residue was washed with water until the washing become neutral. The acid-free residue was diluted with buffer to the final concentration of 1% dry weight volume, and resuspended with vigorous stirring to prepare the so-called colloidal chitin solution. This colloidal chitin solution was stored in a dark place below 5◦C.

2.2.8.7 Determination of chitinase activity by Colorimetric assay

Plant chitinase activity was determined by a Colorimetric assay (Boller and Mauch, 1988) measuring *N*-acetylglucosamine from enzymatic hydrolysis of the principle products of plant chitinase using colloidal chitin as substrate (ES). For assay, the protein was diluted with 0.1 M sodium acetate buffer pH 5.0 to yield a volume of 200 μl of 2 mg/ml. The reaction was started by the addition of the protein to 800 μl of 1% colloidal chitin. After 10 min of incubation at 40◦C, the reaction was centrifuged at 4◦C for 10 min at 5,000 x *g*. Five hundred microliters of supernatant was mixed with 100 μl of 0.8 M sodium tetraborate and boiled for 3 min, cooled on ice and mixed with 3 ml of 1% of *p*-dimethylaminobenzadehyde in 87.5% glacial acetic acid, 12.5% conc HCl. The absorbance of supernatant was measured photometrically at 585 nm after incubation at 37◦C for 20 min.

For each enzyme preparation measured, an enzyme blank (EB), and a substrate blank (SB) were carried through the procedure. The enzyme blank contained 0.8 ml water instead of the colloidal chitin. The substrate blank contained 0.2 ml of 100 mM sodium acetate buffer, pH 5.0, instead of the enzyme preparation. The amount of *N*-acetylglucosamine equivalents released by the enzyme is calculated as followed :

$$A_{585}(ES)-A_{585}(EB)-A_{585}(SB)$$

Definition of Unit. One unit of chitinase was defined as the amount that catalyzes the release of soluble chitooligosaccharides containing 1 μmol of GlcNAc in 1 min. The initial slope of the standard curve is used to calculate the unit. The standard curve used 0-0.20 μmol of GlcNAc.

2.2.8.8 Purification of recombinant protein by Ni-NTA superflow and Sephadex G-100 chromatography

Four milliliters of soluble extraction of bacteria expressing chitinase-fusion protein from pET32a was added to a milliliter of Ni-NTA superflow column equilibrated with 10 ml (10 volumes) of lysis buffer (500 mM, sodium chloride 50 mM sodium dihydrogen phosphate pH 8.0). The column was washed with 20 ml (20 volumes) of lysis buffer, wash buffer 1 (10 mM imidazole in lysis buffer pH 8.0), and wash buffer 2 (50 mM imidazole in lysis buffer pH 8.0). After that, the recombinant protein was eluted with 5 ml (5 volumes) of elution buffer (250 mM imidazole in lysis buffer pH 8.0). The fractions with chitinase activity were pooled and concentrated 4 fold with 10 kDa cutoff ultrafiltration membrane (YM-10, Amicon). The concentrated enzyme solution was loaded onto a Sephadex G-100 gel filtration column (swollen in distilled water for 72 h), which was eluted with 20 mM Tris-HCl pH 7.5 at a flow rate of 0.5 ml/min. The fractions with chitinase activity were pooled and concentrated four fold as before.

2.2.8.9 Cleavage of recombinant protein

Three hundred micrograms of recombinant protein was cleaved with 2 unit of enterokinase in 30 μl of 200 mM Tris-HCl, 500 mM sodium chloride, 20 mM calcium chloride overnight at 23◦C. The reaction was added to 100 μl of Ni-NTA superflow and incubated at 4◦C for 30 min with vigorous shaking. The reaction was centrifuged at 4◦C for 10 second at 15,000 x *g* and the supernatant, which contained purified chitinase, was collected and evaluated by SDS PAGE and by hydrolysis of colloidal chitin.

2.2.9 Extraction and purification of chitinase from ***L. leucocephala*** seedlings

2.2.9.1 Extraction of chitinase from ***L. leucocephala*** seedlings

L. leucocephala seeds were germinated an clean potting soil and grown in a growth chamber at 25◦C with a 15-h/day light period for 1 week and they were transferred to long day conditions in the greenhouse for 3 weeks. Forty grams of these seedlings were homogenized using a cold mortar and pestle in liquid nitrogen and the homogenate was transferred to a centrifuge tube in 90 milliliters ice-cold 0.1 M sodium acetate buffer, pH 5.0, containing 1 mM phenylmethylsulfonyl fluoride (PMSF), to inhibit proteolysis and 5% (w/v) polyvinylpoly-pyrrolidone (PVPP) to absorb polyphenolic substances. The homogenate was centrifuged

at 13,800 x *g* for 30 min at 4◦C and the supernatant (crude enzyme) was used for assays and further purification.

2.2.9.2 Ammonium sulfate precipitation

The crude enzyme was precipitated from 90 ml homogenate by using 39.24 g (70% w/v) of ammonium sulfate by slow addition of solid ammonium sulfate with gentle mixing and left at 4◦C overnight. The protein precipitate was obtained by centrifugation at 13,800 x *g* for 45 min at 4◦C. The pellet was dissolved in 5 ml of 0.1 M sodium acetate buffer pH 5.0, mixed with 1 ml sodium bicarbonate, pH 8.4 and the pH adjusted to 8.4 with sodium hydroxide. The enzyme mixture was dialyzed twice against 1 l 0.02 M sodium of bicarbonate, pH 8.4 at 4◦C overnight.

2.2.9.3 Chitin affinity Chromatography

The chitin affinity step was based on the method described by Molano *et al* (1979). The dialyzed solution was added to 3.5 g colloidal chitin, saturated with 25 ml 0.02 M sodium bicarbonate pH 8.4, and allowed to bind 30 min. The mixture was centrifuged at 27,000 x *g* at 4◦C for 45 min. The pellet was resuspended in 160 ml 0.02 M sodium bicarbonate pH 8.4, then 160 ml 0.02 M sodium acetate pH 5.5, with centrifugation at 27,000 x *g* at 4◦C for 45 min after each step. In the final step, the pellet was resuspended with 80 ml 0.2 M acetic acid pH 3.0 and centrifuged at 27,000 x *g* at 4◦C for 45 min. The supernatant containing the purified chitinase and concentrated 4 fold with 10 kDa cutoff ultrafiltration membrane (YM-10, Amicon). The concentrated enzyme solution was dialyzed against with 1 l 0.1 M sodium acetate buffer pH 5.0 for two changes.

2.2.10 Determination of the subunit molecular weight of chitinase by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Denaturing polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970). The SDS-polyacrylamide gel was prepared from a stock solution of polyacrylamide consisting of 30% (w/v) acrylamide and 0.8% (w/v) of *N,N*¢-methylene-tris-acrylamide. The separating gel contained 12% acrylamide in 1% SDS and 0.1 M Tris-glycine buffer pH 8.3 while the stacking gel contained 5% acrylamide in 1% SDS and 0.5 M Tris-HCl pH 6.8. The gel was chemically polymerized by the addition of 0.05% of TEMED and 0.5% of ammonium persulfate. Four parts of sample were mixed with one part of sample buffer containing

0.06 M Tris-HCl buffer pH 6.8, 40% (v/v) glycerol, 2% of SDS, and 0.5% (w/v) of bromophenol blue. The mixture was boiled at 100◦C for 5 min then applied onto the gel. Electrophoresis was carried out under a constant current of 50 mA to separate protein for about 2 h until the tracking dye reached the bottom of the gel, at which time the electrophoresis was stopped. The gel was stained Coomassie brilliant blue R 250 for 30 min, then destained with destaining solution until protein bands appeared clearly as previously described in section 2.2.8.4.

The molecular masses of protein bands were estimated by comparison to the low molecular mass markers, phosphorylase b (rabbit muscle, 97.4 kDa), bovine serum albumin (66.0 kDa), ovalbumin (hen egg white, 45.0 kDa), bovine carbonic anhydrase (31.0 kDa), typsin inhibitor (soybean, 21.5 kDa), and bovine α-lactalbumin (14.0 kDa).

2.2.11 Determination of pI of the chitinase

Isoelectric focusing polyacrylamide gel was performed in PhastGel IEF pH 3-9 kit (Amersham Pharmacia Biotech) on Pharmacia phast gel IEF system as described in the Amersham Pharmacia Biotech manual (Copyright 1995). The method for isoelectric focusing contain three steps: a prefocusing step, a sample application step, and a focusing step. Prefocusing, in which the pH gradient is formed, was done at 2000 V, 2.5 mA, 3.5 W, 15◦C, and 75 Vh. Then, the purified chitinase was applied to the polyacrylamide gel (gradient pH 3-9) and the power set at 200 V, 2.5 mA, 3.5 W, 15◦C, and 15 Vh. The applicators were raised at the beginning of step 3 and the proteins migrated to their isoelectric points at 2000V, 2.5mA, 3.5W, 15◦C, and 410 Vh. A mixture of pI calibration standards (pI 4.45-9.60; Bio-RAD), composed of phycocynin (3 bands, 4.45, 4.65, 4.75), β-lactalbumin B (5.10), bovine carbonic anhydrase (6.00), human carbonic anhydrase (6.50), equine myogolobin (7.00), human hemoglobin A (7.10), human hemoglobin C (7.50), lentil lectin (7.80, 8.00, 8.20) and cytochrome c (9.60), was loaded in one lane in the electrophoretic run.

For protein staining, the gel on the gel support film was placed into staining solution (0.1% $CuSO_4$, 0.02% PhastGel Blue R, 60% double distilled water, 30% methanol, and 10% acetic acid) for 5 min and was placed into destaining solution (60% double distilled water, 30% methanol, and10% acetic acid) for 5 min.

2.2.12 Determination of pH optimum of the chitinase

The purified enzyme was used to study the effect of pH on the enzyme activity. The chitinase activity was measured at various pH by the colorimetric method described in section 2.2.8.5, using 1% colloidal chitin as a substrate. The enzyme was incubated for 10 min at 40◦C in 0.1 M acetate buffer at pH 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0; 0.1 M phosphate buffer at pH 5.5, 6.0, 6.5, 7.0, and 7.5; 0.1 M Tris-HCl at pH 7.0, 7.5, 8.0, 8.5, and 9.0.

2.2.13 Determination of temperature optimum of chitinase

The purified enzyme was used to study the effect of temperature on the chitinase activity. The enzyme was incubated for 10 min in 0.1 M sodium acetate buffer pH 4.5 at 30, 35, 40, 45, 50, 55, 60, 65, and 70◦C. The reaction was followed by measuring chitinase activity with colorimetric method as described in 2.2.8.5, using 1% colloidal chitin as a substrate.

2.2.14 Determination of substrate specificity

The purified enzyme was used to study substrate specificity for chitinase activity. Two hundred microliters enzyme was incubated in buffer at 40◦C for 10 min, using 800 μl of each substrate: colloidal chitin, purified chitin, swollen chitin, glycol chitin, and chitosan. The reaction was followed by colorimetric method as described in 2.2.8.7.

2.2.15 Determination of enzymatic assay

The endochitinase assay with the purified chitinase was performed in 0.1 M acetate buffer, pH 4.5, according to Tronsmo and Harman (1993) using *p*-nitrophenyl-*N*-acetyl-*b*-D-glucosaminide, *p*-nitrophenyl-*N,N¢*-diacetylchitobiose, and *p*-nitrophenyl-*N,N¢,N¢¢*-triacetyl-chitotriose as substrate. One hundred microliters of 10 mM solution of each substrate and 850 milliliters of 0.1 M sodium acetate buffer pH 4.5 were mixed with 0.05 ml of purified enzyme, incubated at 40◦C for 15 min and 2 ml of 2 M sodium carbonate was added. The absorbances of the solutions were measured at 400 nm and the concentrations calculated by comparison to 0-0.20 μmol *p*-nitrophenol standard curve. The standard curve was used to calculate the unit.

2.2.16 Determination of chitinase reaction products by HPLC

Colloidal chitin was incubated with purified enzyme for periods 15 min, 30 min, 1 h, 2 h, 4h, 8h, 16 h, and 24 h in 0.1 M sodium acetate buffer pH 4.5 under the standard assay conditions. Substrate blanks were included in parallel. The reactions were stopped by boiling for 3 min. After centrifugation at 5000 x *g* for 10 min, the soluble products were filtered through a 0.45 μm filter. Twenty microliters filtered supernatant was separated by HPLC Series 1100 (Hewlett Packard, Waldbronn, Germany) on a CARBOSep CHO-411 oligosaccharide column (Transgenomic, Omaha, NE USA), which contain a 0.78 x 30-cm bed packed with an trangenomic cation exchange resin in the Na^+ ionic form, and CARBOSep CHO611 guard column. It is specifically designed for the separation of polysaccharide such as those found in corn syrups and related food products on a single chromatographic profile. The column conditions were performed at 70◦C and eluted with water at a flow rate 0.4 ml/min. The products were detected on the basis of their absorbance at 210 nm and identified by comparison to $(GlcNAc)_n$ standards from n = 1-4.

2.2.17 Determination of kinetic values

The kinetic properties of chitinase were performed using colloidal chitin, *p*-nitrophenyl-*N,N¢,N¢¢*-triacetylchitotriose, and *N,N¢,N¢¢,N¢¢¢*-tetraacetylchitotetraose as substrate. The colloidal chitin was suspended in 0.1 M sodium actetate buffer, pH 4.5, ranging in concentration from 0 to 20 mg dry weight chitin/ml. The activity was determined by detecting the product using colorimetric assay as previously described in section 2.2.8.7. For *p*-nitrophenyl-*N,N¢,N¢¢*-triacetylchitotriose was dissolved in 0.1 M acetate buffer pH 4.5, ranging in concentration from 0-0.25 mM. The reaction mixture containing 450 μl substrate, 50 μl enzyme was incubated at 50◦C for 5 min and stop reaction by 1 ml of Na_2CO_3. The absorbance of supernatant was measured at 400 nm and identified by comparison to a 0-0.20 μmol *p*-nitrophenol standard curve. The standard curve was used to calculate the unit.

For chitotetraose, 100 μl enzyme was incubated in 500 μl acetate buffer pH 4.5 containing 300 μg/ml chitobiose, at 40◦C for 10 min and the reaction was stopped by boiling for 3 min. The soluble products were isolated by centrifuging at 10,000 x *g* for 10 min and filtering through a 0.45 μm filter. Twenty microliters filtered supernatant was separated by HPLC as described in the

section 2.2.16 and identified by comparison to a 0-1.0 μM GlcNAc standard curve. The standard curve was used to calculate the unit.

Initial rate velocities were fit to the hyperbolic rate equation and Lineweaver-Burk double reciprocal plots to determine K_m and V_{max} values.

2.2.18 Determination of antifungal activity from purified chitinase

Fourteen fungi were tested to determine the antifungal activity of the chitinase using the hyphal extension-inhibition assay (Robert and Seliterennikoff 1988). A slant of fungal conidia was placed in the center of potato dextrose agar (PDA) plate and was growth at 30°C for 2-3 days. After that, twenty micrograms of control (the recombinant protein without chitinase), and recombinant protein solution at concentration of 0.5, 1.0, and 2.0 μg were added to 0.5 mm filter paper discs and the discs placed at equal distance from the fungal slant and each other on the plate. Resulting hyphae grew outward from the central disc as a circle unless an effective concentration of inhibitor was contacted in a perimeter disc. In the later case, a crescent of growth inhibition was observed around the disc for 2-3 days.

Chapter III
Results and Discussion

3.1 Cloning and sequencing of chitinase fragment

Degenerate primers were designed based on the most highly conserved regions of shared amino acid identity among 36 glycosyl hydrolase family 19 plants chitinase sequences from the Swiss-Port database by multiple sequence alignment as shown in Figure 3.1. Two forward primers (chit19E191f, outer and chit19H210f, inner) and two reverse primers (chit19P301r, inner and chit19A326r, outer) were used in Nested PCR. A single product was obtained from the outer primer set of approximately 400 bp (Figure 3.2 A lane 2, 3) and a single product of approximately 300 bp was produced by reamplification of this product with the inner primer set (lane 4, 5). The products were purified and extracted from 8% polyacrylamide gel electrophoresis (Figure 3.2 B) and cloned into pGEM-T plasmid vector.

The cloned amplified fragment plasmid inserts were first confirmed by restriction digest with *Pst* I and *Sac* II and agarose gel electrophoresis (Figure 3.3). The size of the recombinant plasmids are No. 1 about 3,200 bp (lane 2) which gave the insert size about 200 bp (lane 3) and No. 2 about 3,300 bp (lane 4) which gave the expected insert size about 300 bp (lane 5). The result of expected size leucaena chitinase cDNA fragment sequence was translated to amino acid sequence (Figure 3.4).

Comparison of the deduced amino acid sequence with the GENBANK database indicated it was homologous to a number of plant chitinase with the closest match being chick pea (*Cicer arietinum*, AC: CAA101891) class I chitinase with 83% identity (Figure 3.5). The amino acid sequence contained many residues that were identical to conserved amino acids found in other plant chitinase sequences. This indicated that this cDNA fragment was from a chitinase homologue mRNA as expected.

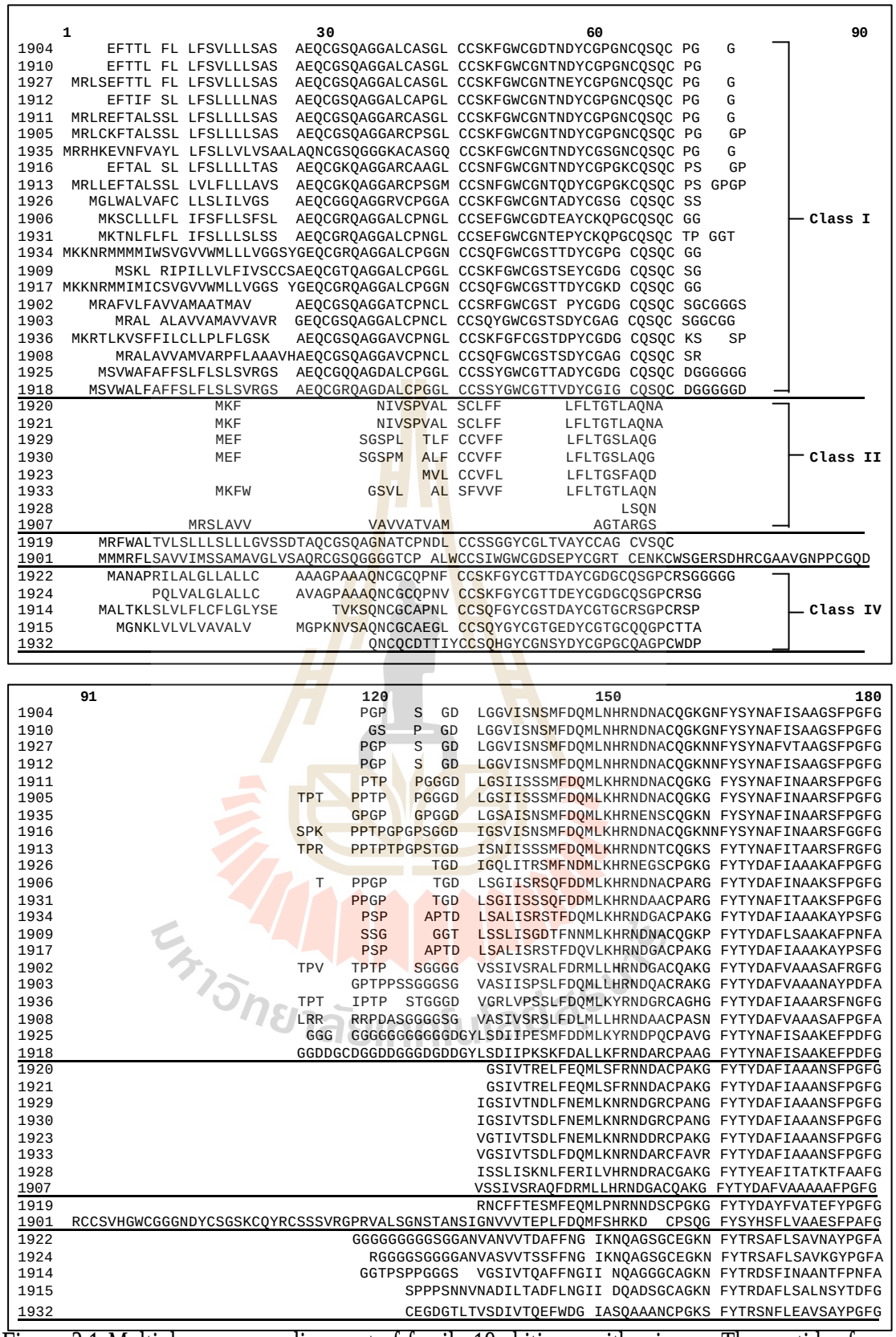

Figure 3.1 Multiple sequence alignment of family 19 chitinase with primers. The peptides from which primers were derived. Underlines demark classes (Feng and Doolittle, 1996, propack protein sequence "alignment program").

Figure 3.1 (Continued)

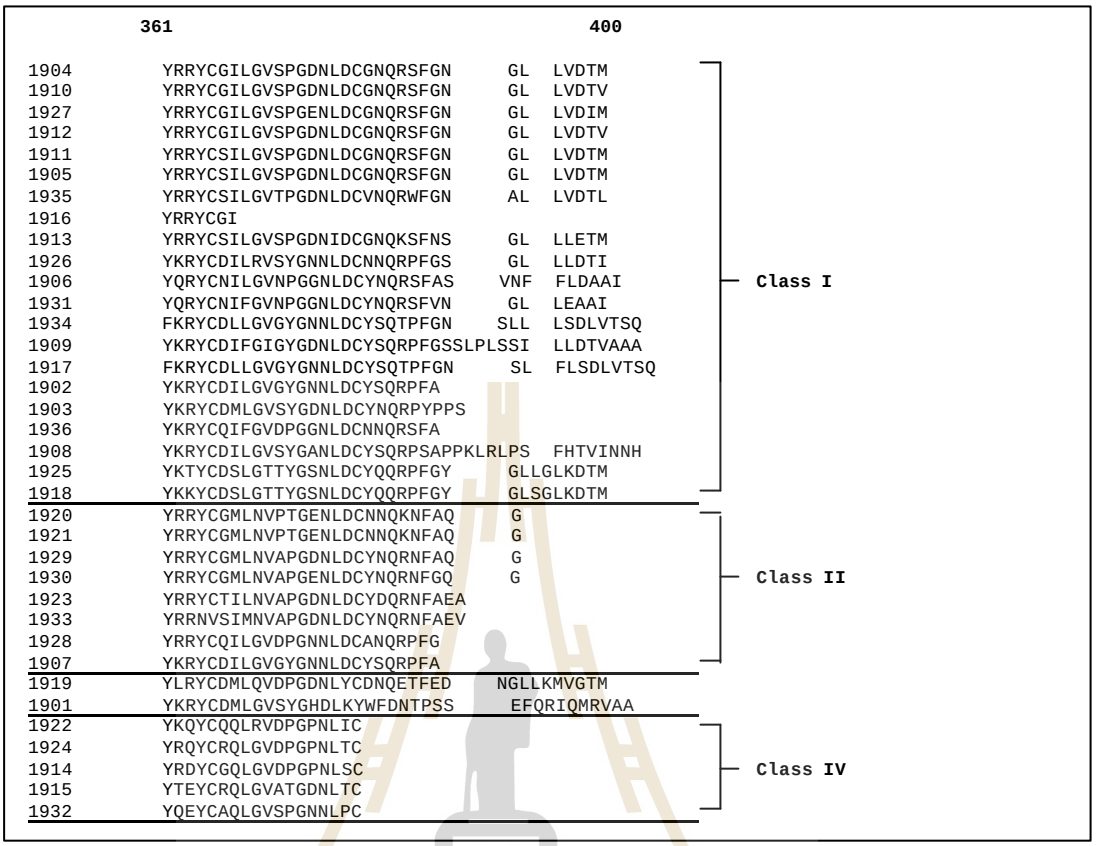

Figure 3.1 (Continued)

1904 Endochitinase 1 precursor, *Solanum tuberosum*, (AC: P52403), potato

1910 Endochitinase 2 precursor, *Solanum tuberosum*, AC: P52404, potato

1912 Endochitinase 3 precursor, *Solanum tuberosum*, AC: P52405, potato

1927 Basic 30 KD endochitinase precursor, *Lycopersicon esculentum*, (AC: Q05538), tomato

1911 Endochitinase B precursor, *Nicotiana tabacum*, (AC: P24091), common tobacco

1905 Endochitinase A precursor, *Nicotiana tabacum*, (AC: P08252), common tobacco

1935 Endochitinase precursor, *Solanum tuberosum*, (AC: P05315), potato

1913 Endochitinase 3 precursor, *Nicotiana tabacum*, (AC: P29059), common tobacco

1916 Endochitinase 4 precursor, *Solanum tuberosum*, AC: P52406, potato

1926 Basic Endochitinase precursor, *Vitis vinifera*, (AC: P51613), grape

1906 Endochitinase CH25 precursor, *Brassica napus*, (AC: Q09023), rape

1931 Basic endochitinase precursor, *Arabidopsis thaliana*, (AC: P19171), mouse-ear cress

1934 Endochitinase precursor, *Phaseolus vulgaris*, (AC: P06215), kidney bean, French bean

1917 Endochitinase CH5B precursor, *Phaseolus vulgaris*, (AC: P36361), kidney bean, French bean

1909 Endochitinase A2 precursor, *Pisum sativum*, (AC: P21226), garden pea

1902 26 KD Endochitinase 1 precursor, *Hordeum vulgare*, (AC: P11955), barley

1936 Endochitinase precursor, *Pisum sativum*, (AC: P36907), garden pea

1903 Basic Endochitinase 1 precursor, Oryza sativa, (AC: P24626), rice

1908 Basic Endochitinase 2 precursor, Oryza sativa, (AC: P25765), rice

1928 Basic endochitinase precursor, *Lycopersicon esculentum*, (AC: Q05537), tomato

1907 26 KD Endochitinase 2 precursor, *Hordeum vulgare*, (AC: P23951), barley

1925 Acidic endochitinase Win6.2B precursor, *Poplus trichocarpa*, (AC: P29031), western balsam poplar

1918 Acidic endochitinase Win6 precursor, *Poplus trichocarpa*, (AC: P16579), western balsam poplar

1919 Endochitinase Win8 precursor, *Poplus trichocarpa*, (AC: P16061), western balsam poplar

1920 Acidic endochitinase PCHT28 precursor, *Lycopersicon chilense*, (AC: Q40114), solanum chilense

1921 Acidic 26 KD endochitinase precursor, *Lycopersicon esculentum*, (AC: Q05539), tomato

1929 Acidic endochitinase P precursor, *Nicotiana tabacum*, (AC: P17513), common tobacco

1930 Acidic endochitinase Q precursor, *Nicotiana tabacum*, (AC: P17514), common tobacco

1923 Acidic 27 KD endochitinase precursor, *Lycopersicon esculentum*, (AC: Q05540), tomato

1933 Acidic endochitinase precursor, *Petunia hybrida*, (AC: P29021), petunia

1901 Lectin endochitinase precursor, *Urtica dioica* (AC: P11218), great nettle

1922 Endochitinase A precursor, *Zea mays* (AC: P29022), maize

1924 Endochitinase B precursor, *Zea mays* (AC: P29023), maize

1914 Basic endochitinase CHB4 precursor, *Brassica napus* (AC: Q06209), rape

1915 Endochitinase PR4 precursor, *Phaseolus vulgaris*, (AC: P27054), kidney bean, French bean

1932 Acidic endochitinase precursor , *Dioscorea japonica* (AC: P80052)

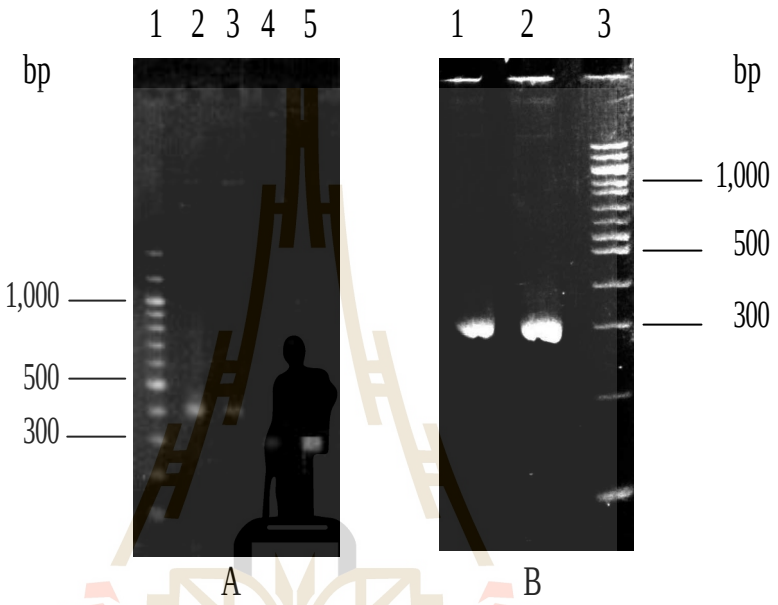

Figure 3.2 A, 1% agarose gel electrophoresis of the first and the second PCR amplification using the outer set (chit19E191f, chit19A326r) and inner set (chit19H210f, chit19P301r) of chitinase primers;

Lane 1: 100 bp DNA marker

Lane 2, 3: product of the first PCR with outer primer set

Lane 4, 5: product of the second PCR with inner primer set

B, 8% polyacrylamide gel of the second PCR amplification using chitinase primers;

Lane 1, 2: product of the second PCR with inner primer set

Lane 3: 100 bp DNA marker

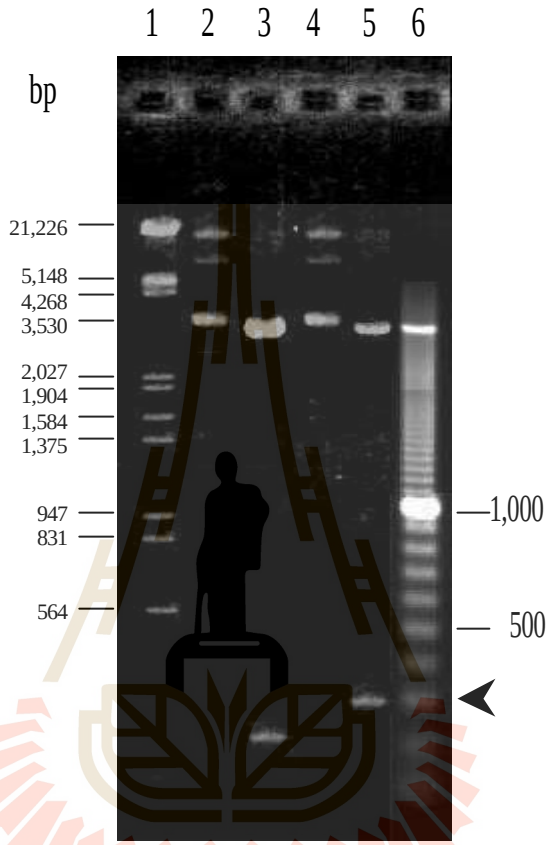

Figure 3.3 1% agarose gel electrophoresis of restriction analysis of recombinant plasmids containing chitinase cDNA fragment.

Lane 1: Lambda/*Eco*R I + *Hind* III marker

Lane 2: Undigested recombinant plasmid from white colony No. 1

Lane 3: *Pst* I and *Sac* II digested recombinant plasmid from white colony No. 1

Lane 4: Undigested recombinant plasmid from white colony No. 2

Lane 5: *Pst* I and *Sac* II digested recombinant plasmid from white colony No. 2

Lane 6: 100 bp DNA marker

Figure 3.4 DNA sequence and deduced amino acid sequence of leucaena chitinase from Nested-PCR product.

```
Leucaena    :HETTGGWPSAPDGPYAWGYCFKQERNPPSAYCQPSSEYPC

Cicer       :HETTGGWSSAPDGPYAWGYCFLREQNP-STYCQPSSEYPC
Arabis      :HETTGGWPTAPDGPFAWGYCFKQERDPPSNYCQPSAAWPC
Medicago    :HETTGGWATAPDGPYAWGYCFVREQNPSSTYCQPSSEFPC
Pea:        :HETTGGWPTAPDGPYAWGYCFLREQNP-STYCQASSEFPC
             *******  ***** ******  * ** * *** *   **

Leucaena    :APGKQYYGRGPMQLSWNYNYGQCGRAIGADLLNDPDLVAN

Cicer       :ASGKQYYGRGPIQLSWNYNYGQCGRAIGVDLLNNPDLVAT
Arabis      :ARDKRYYGRGPMQLTWNYNYGQCGRAIGVDLLNNPDLVAN
Medicago    :ASGKQYYGRGPIQISWNYNYGQCGRAIGVDLLNNPDLVAT
Pea         :ASGKQYYGRGPIQISWNYNYGQCGRAIGVDLLNNPDLVAT
             *  * ****** * ************** **** *****

Leucaena    :DATISFKTAFWFWMTA   96 A.A.

Cicer       :DPVISFKTAIWFWMTP   83% ID
Arabis      :DAVIAFKAAIWFWMTA   82% ID
Medicago    :DPVISFKTALWFWMTP   81% ID
Pea         :DPVISFKTALWFWMTP   80% ID
             *  * ** * *****
```

Figure 3.5 Multiple sequence alignment of deduced amino acid sequences of chitinase from leucaena, *Leucaena leucocephala* de Wit PCR clone; Cicer, *Cicer arietinum* (Genbank AC: CAA10189); Arabis, *Arabis glabra* (AC: AAF69778); Medicago, *Medicago truncatula* (AC: CAA71402); and Pea, garden pea, *Pisum sativum* (AC: L37876). Conserved amino acid residues are marked by asterisks.

3.2 DNA amplification by using 3′ RACE method

To determine the 3′ end of the chitinase mRNA, total RNA extracted from germinated seeds and the first strand DNA synthesis was performed using reverse transcriptase with the QT primer. The first strand cDNA was then amplified by PCR using Taq polymerase with KB-1f forward primer and KB-1r reverse primer. The PCR product (Figure 3.6) gave the expected size band of about 650 bp, but also was contaminated with large smeared bands less than 650 bp. The expected band (650 bp) was eluted and cloned into pT7blue T-vector.

The recombinant plasmids were first extracted and the inserts confirmed by restriction digest with *Eco*R I and *Pst* I (Figure 3.7). The recombinant plasmids from clone 1, 4, 8, 9, and 11 gave the pT7 blue plasmid size, 2900 bp, band and the insert size about 650 bp band (Figure 3.7 lanes 2, 5, 9, 10, 12). The other bands contained insert sizes less than 650 bp band. The leucaena chitinase cDNA 3′ RACE fragment was sequenced and the sequence was translated to amino acid sequence using the GENETYX version 9.0 program for Macintosh (Software Development, Shibuya-ku, Tokyo, Japan) as shown in Figure 3.8. Sequencing of the PCR product indicated that it is composed of 663 bp of DNA sequence that translated to 173 amino acids, and included primers, stop codon, and polyA.

The comparison of the deduced amino acid sequence with the GENBANK database indicated it was homologous to a number of plant chitinases, with the closest match being garden pea (*Pisum sativum*, AC: L37876) chitinase with 82% identity (Figure 3.9). The amino acid sequence region contained many amino acid residues that were identical to those conserved in other plant chitinase sequences.

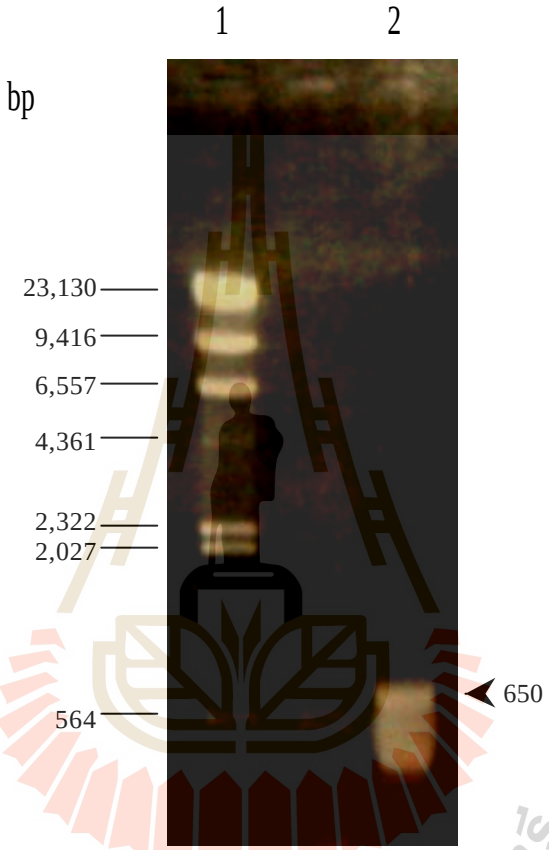

Figure 3.6 0.8% agarose gel electrophoresis of the 3′ RACE PCR product amplified using KB-1f and KB-1r chitinase primers.

Lane 1: Lambda/*Hind* III marker

Lane 2: 3′ RACE product

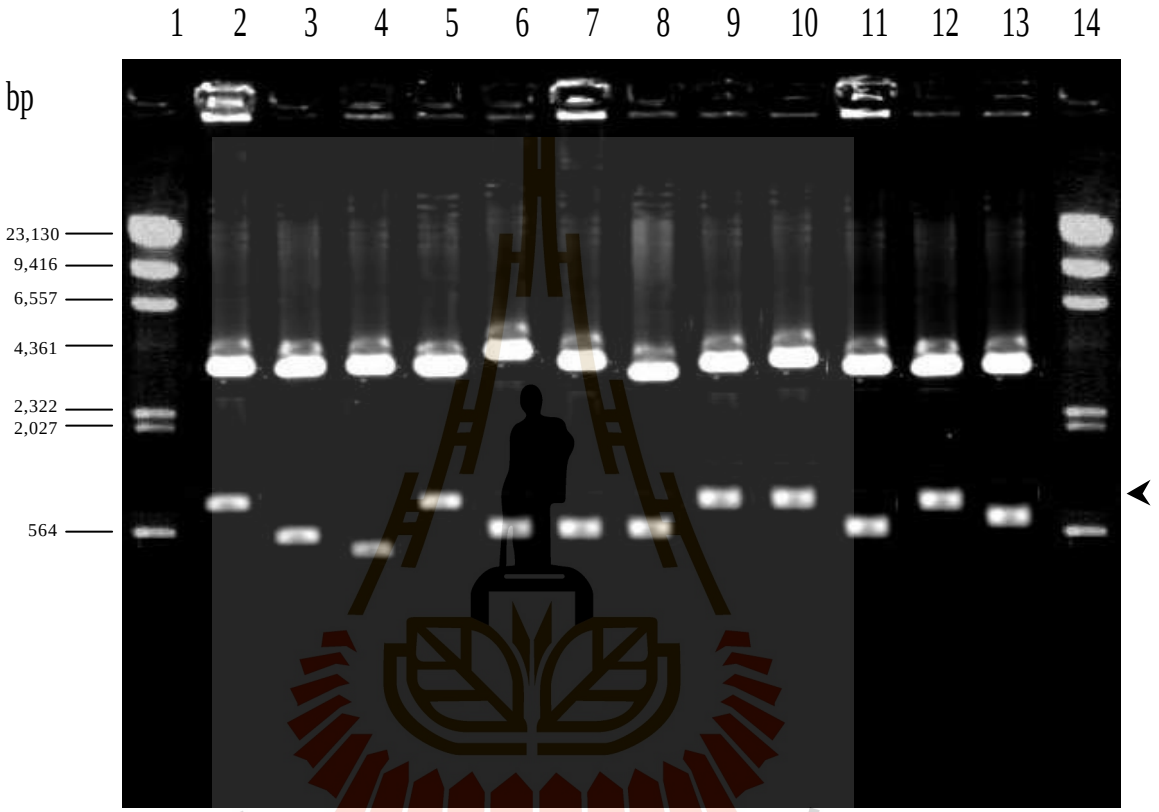

Figure 3.7 0.8% agarose gel electrophoresis of the 3′ RACE insert clone plasmids after restriction digest with *Eco*R I and *Pst* I.

Lane 1,14: Lambda/*Hind* III marker

Lane 2-13: *Eco*R I and *Pst* I digested recombinant plasmids from white colonies No.1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12, from left to right respectively.

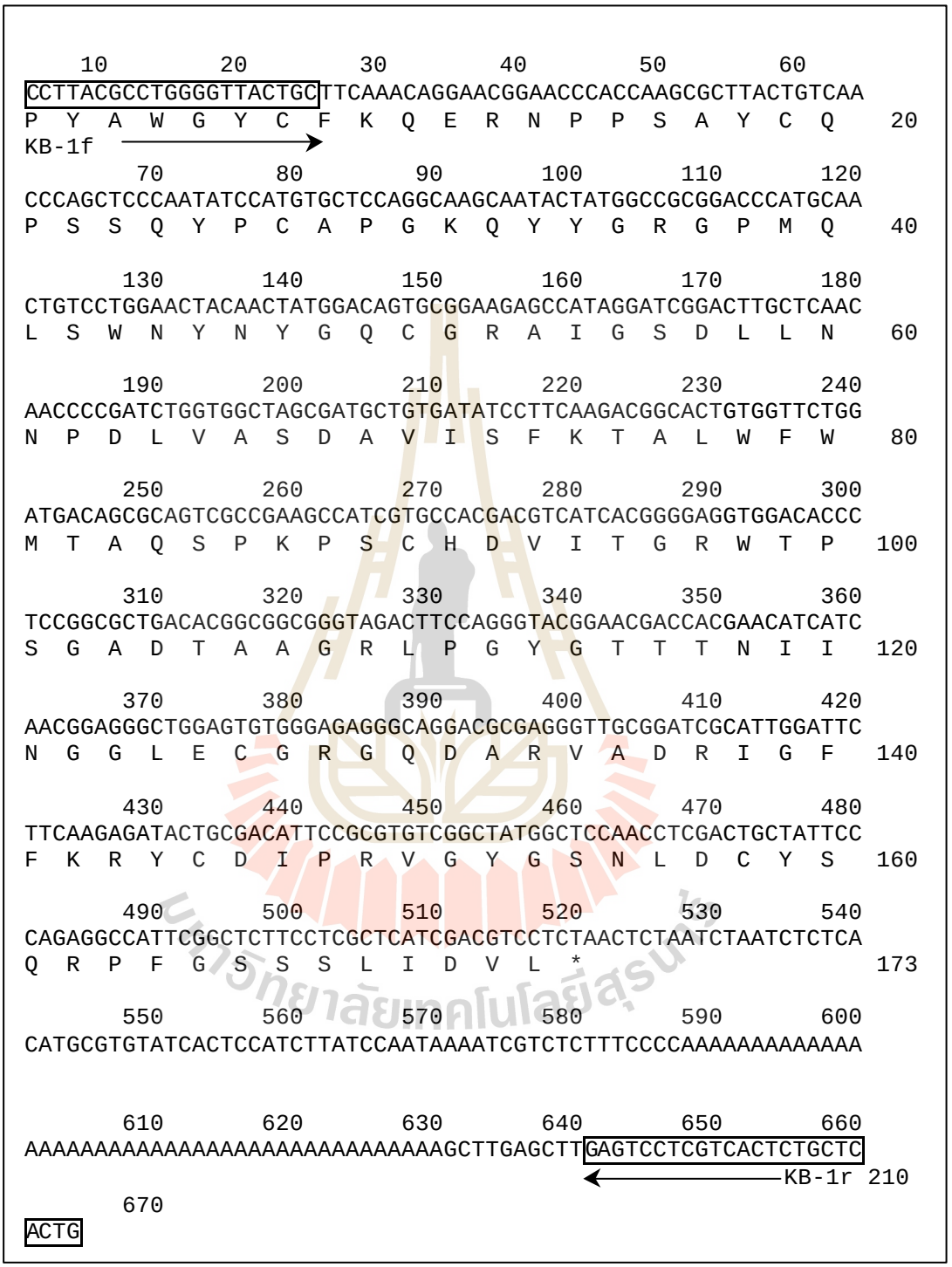

Figure 3.8 DNA sequence and deduce amino acid sequence of chitinase 3′ RACE product. Boxes represent the DNA sequence of PCR primers.

Figure 3.9 Multiple sequence alignment of the deduced amino acid sequences from the *Leucaena leucocephala* de Wit Nested PCR product, Leucaena-Nest, and 3′ RACE product, Leucaena-3′ RACE; *Pisum sativum* (AC: L37876), Pea; kidney bean, *Phaseolus vulgaris* (AC: S43926), Bean; *Arabidopsis thaliana* (AC: BAA82818), Arabidopsis; *Brassica napus* (AC: S59953), Rape; and *Hordeum vulgare* (AC: S04131), Barley. Conserved residues are marked by asterisks.

3.3 Northern blot analysis

The 650 bp fragment from the cloned leucaena cDNA 3′ RACE product was used as a probe of *L. leucocephala* RNA from plants at four weeks of germination to determined the RNA transcript size. A weak signal was detected in the total cellular RNA extracted from *L. leucocephala* shoot, but a strong signal was detected from RNA from the whole plant (Figure 3.10), which had hybridization of the 3' RACE product to a specific band of about 1,100 bp.

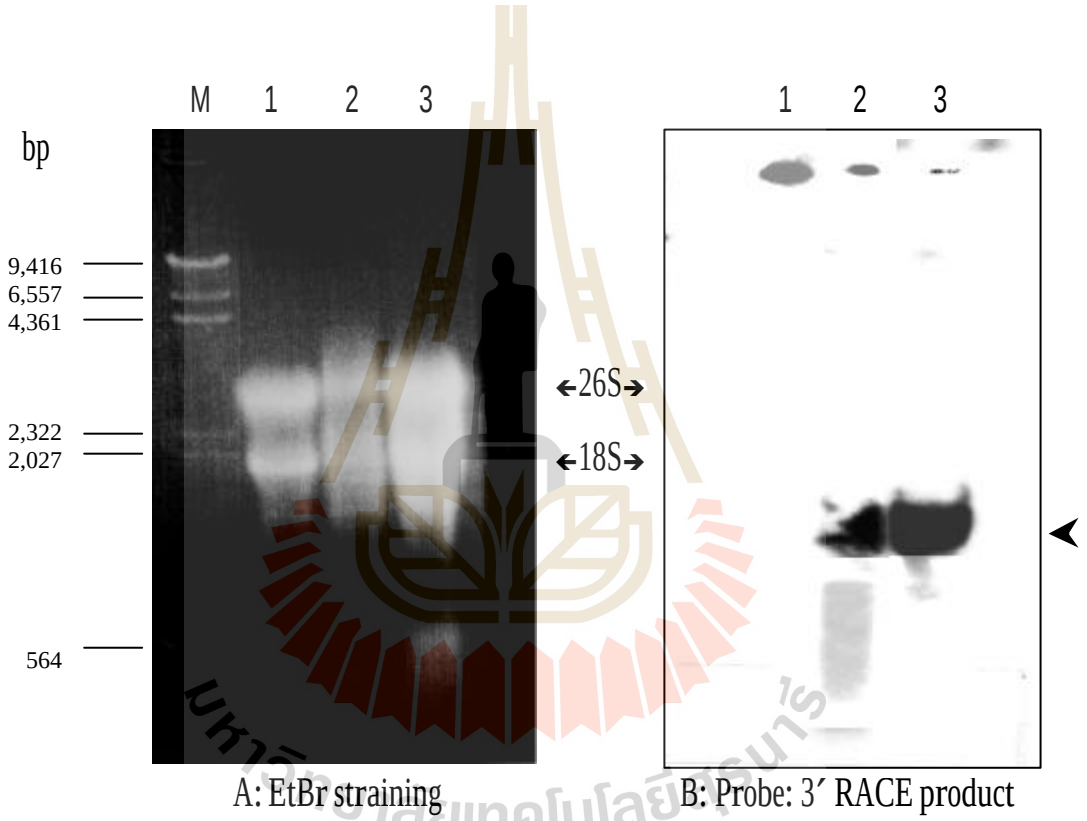

Figure 3.10 Detection of chitinase hybridization by RNA gel-blot analysis. A stained in EtBr and B hybridized to the 3′ RACE cDNA probe labeled with ^{32}P.

Lane M: Lambda/*Hind* III maker

Lane 1: 10 μg total RNA from rice

Lane 2: 10 μg total RNA from *L. leucocephala* shoots seedlings

Lane 3: 10 μg total RNA from *L. leucocephala* whole seedlings

3.4 Screening of the shoot cDNA library and determination of the DNA insert size

A *L. leucocephala* young shoot λgt11 cDNA library was constructed and screened with two separate, non overlapping cDNA fragments (242 bp and 331 bp) derived from the 3' RACE product. After the third screening, plaques that gave high hybridization signals were collected. Totally, fifteen positive clones were isolated and the sequences of these clones were determined. Ten clones were isolated using the 242 bp fragment (3' region of 3' RACE product) and five using the 331 bp fragment (5' region of 3' RACE product) as probes, respectively. Phage DNA insert from all fifteen clones which was digested with *Eco*R I restriction enzyme.

The inserts which used 242 bp fragment as probe, No. 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10, had sizes of 1092, 525, 579, 789, 807, 519, 519, 282, 249, and 420 bp (lane 2 to 11, Figure 3.11), respectively. Using the 331 bp fragment as probe, clones A, C, D, E, and J with insert sizes of 597, 432, 201, 198, and 339 bp, respectively, were isolated (lane 12 to 16, Figure 3.11).

These fragments were subcloned into pUC19 vector at the *Eco*R I site to determine their DNA sequences using M13 forward and M13 reverse primers. The resulting DNA sequences from the leucaena chitinase cDNA library were translated to amino acid sequence and aligned using GENETYX version 9.0 program for Macintosh (Figure 3.12). They were divided into 4 groups. Group 1 had clones 1, 2, 6, 8, and 9, group 2 had clones 3, 4, and 10, group 3 had clones 5, 7, D, and E, group 4 had clones A, C, and J. The longest insert contained a long open reading frame and a stop codon (TAA), but did not contain the initiation codon (ATG) and the 5' untranslated (UTR) region. Almost all of these sequences are chitinase homologue sequences, except clone No. 1, which matched chitinases at the 3' end, but was not similar other chitinase sequences at the 5' end. The chitinase sequence from clone No. 5 is composed of 813 bp of DNA sequence that translated to 271 amino acids in the uninterrupted reading frame (Figure 3.13).

Comparison of the deduced amino acid sequence of clone 5 with the GENBANK database indicated it was homologous to a number of plant chitinases with the closest matches being kidney bean, *Phaseolus Vulgaris* (AC: S43926) with 78% identity (Figure 3.14). The amino acid sequence region contained many amino acid residues that were conserved in other plant chitinase sequences.

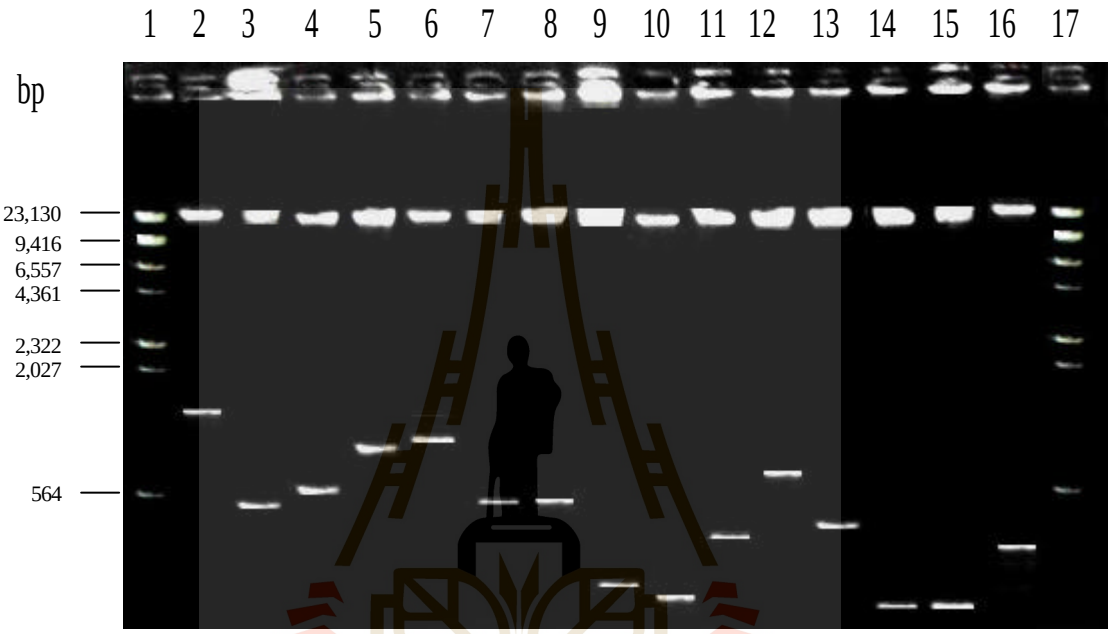

Figure 3.11 0.8% agarose gel electrophoresis of *Eco*R I restriction enzyme digest of recombinant phage λgt11 cDNA containing chitinase cDNA.

Lane 1, 17: λ/*Hind* III marker

Lane 2-11: Phage DNA digested with the *Eco*R I from clones 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10

Lane 12-16: Phage DNA digested with the *Eco*R I from clones A, C, D, E, and J

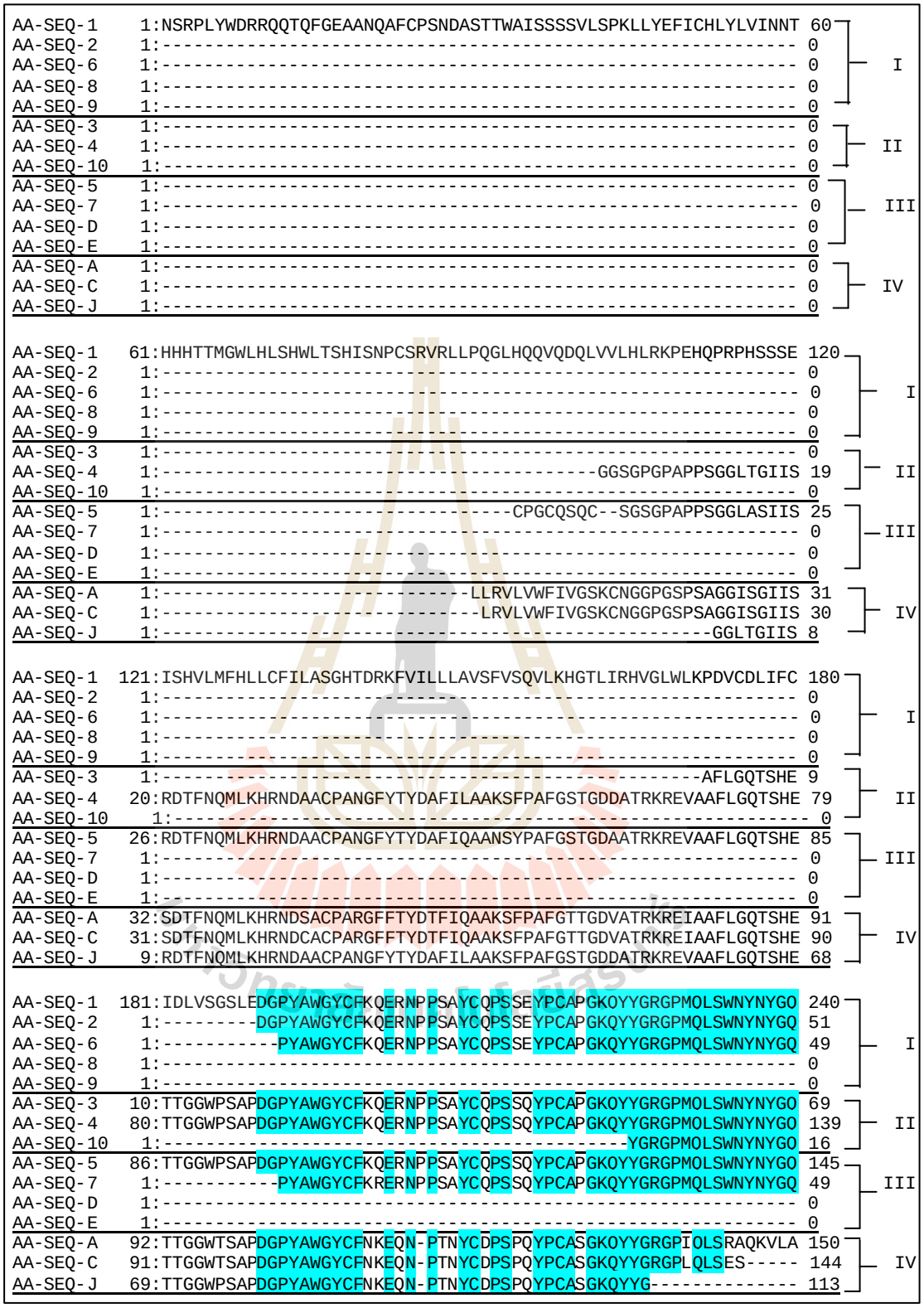

Figure 3.12 Multiple alignment of amino acid sequences from screening the *L. leucocephala* shoot cDNA library. They were separated into 4 groups; group 1, clone 1, 2, 6, 8, and 9; group 2, clones 3, 4, and 10; group 3, clones 5, 7, D, and E; and group 4, clones A, C, and J.

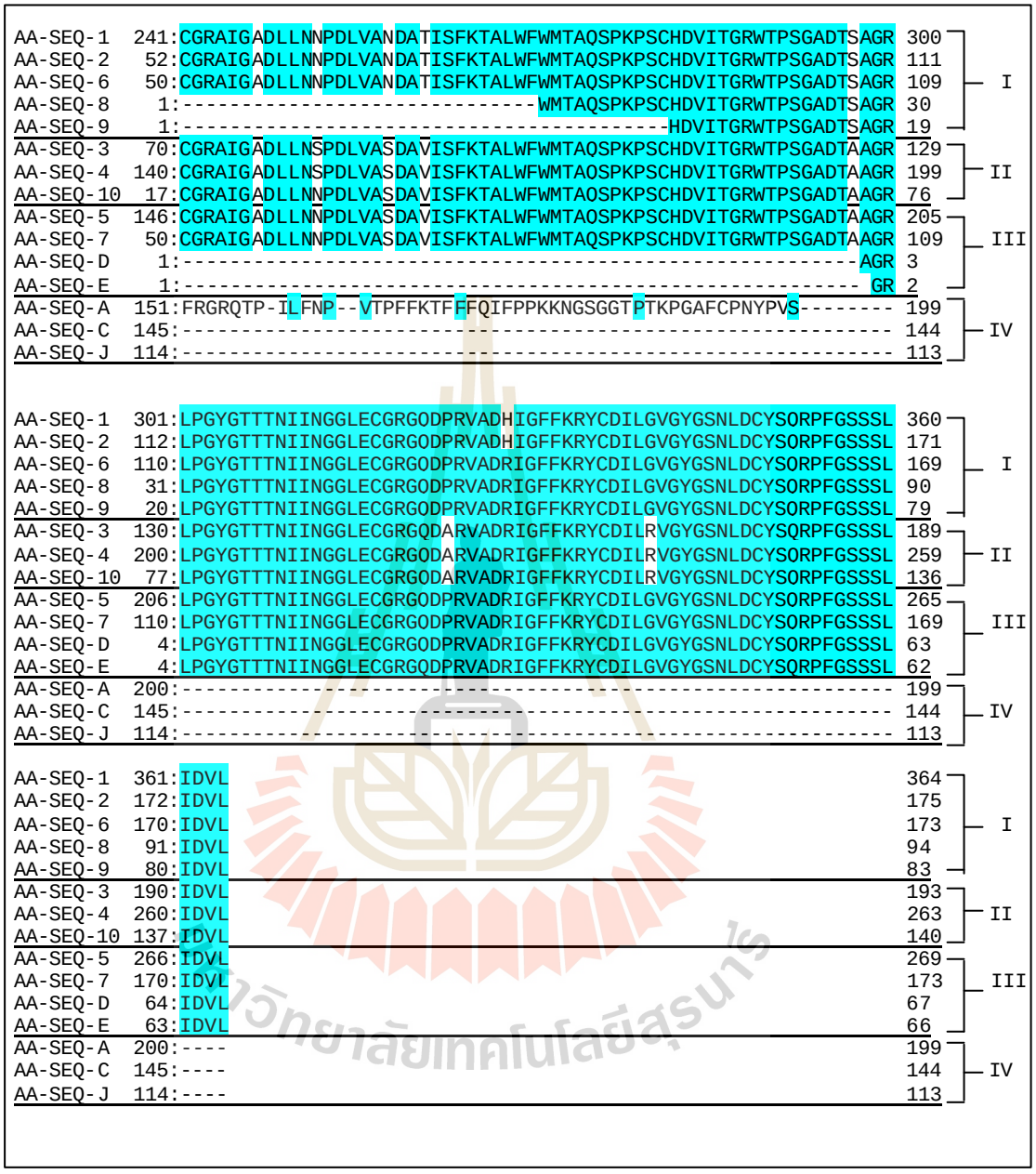

Figure 3.12 (Continued)

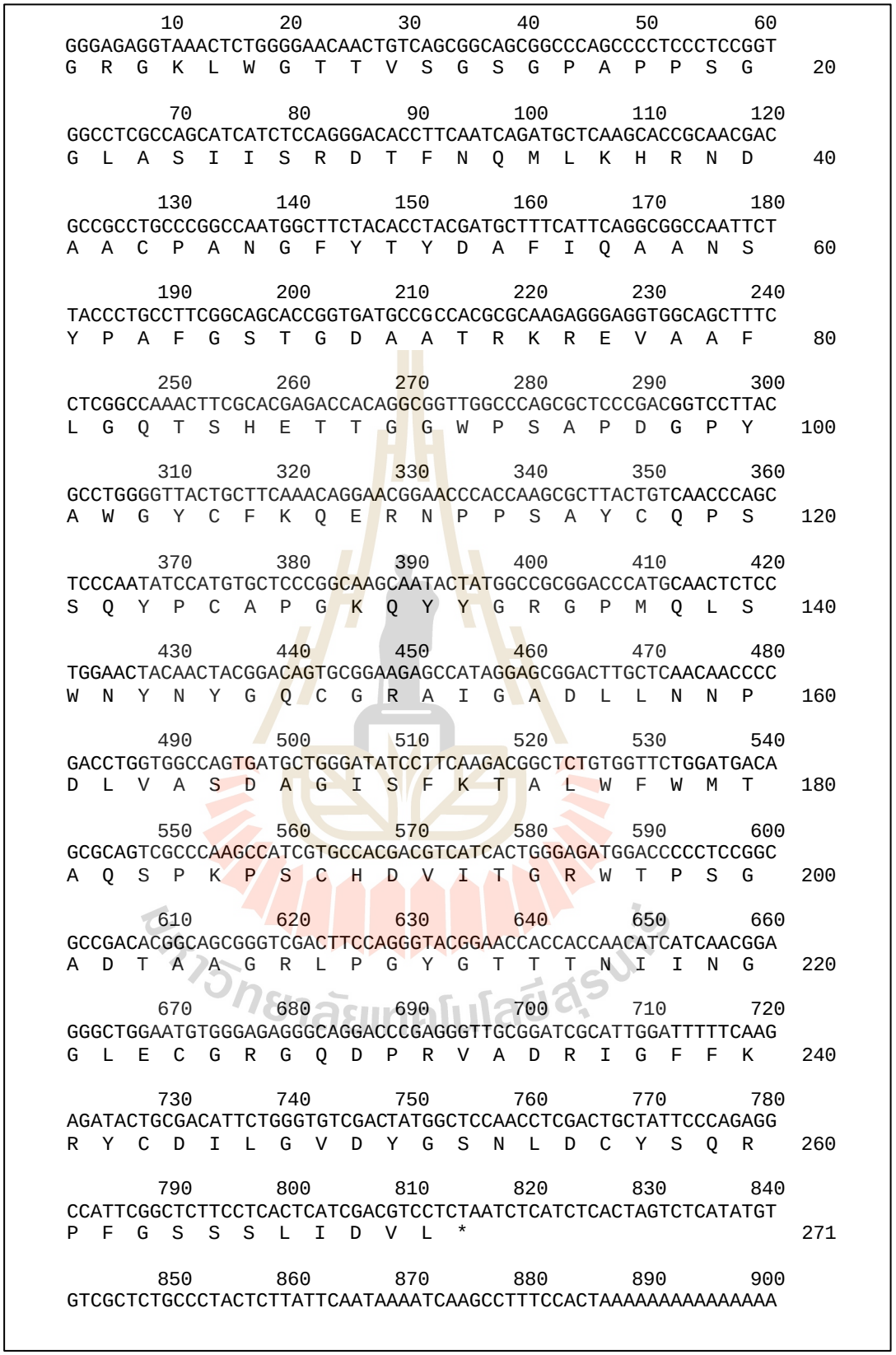

Figure 3.13 DNA sequence and deduced amino acid sequence of leucaena chitinase clone No. 5 from screening of shoot cDNA library.

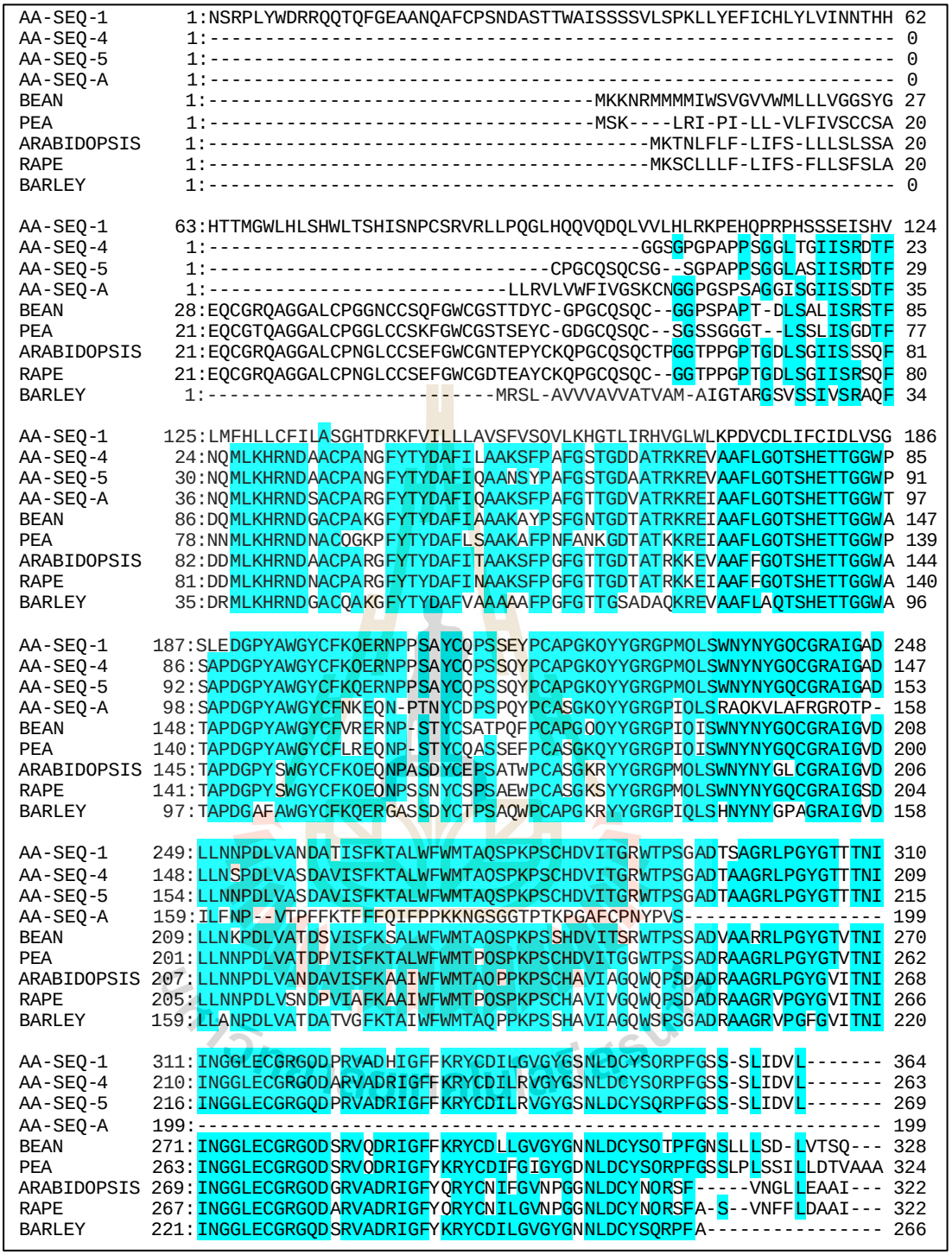

Figure 3.14 Multiple sequence alignment of deduced amino acid sequences from clones 1, 4, 5, and A screening leucaena cDNA library (*Leucaena leucocephala* de Wit); Bean, *Phaseolus vulgaris* (AC: S43926); Pea, *Pisum sativum* (AC: L37876); Arabidopsis, *Arabidopsis thaliana* (AC: AA82818); Rape, *Brassica napus* (AC: S59953), and Barley, *Hordeum vulgare* (AC: S04131). Conserved residues are marked by black boxes.

3.5 Completion of the chitinase cDNA sequence by 5¢ RACE and sequencing

The 5′ RACE was performed using leucaena chitinase-specific primers prepared from the sequence data of the leucaena chitinase cDNA library clone number 5. To determine the 5′ end mRNA chitinase, total RNA was extracted from *L. leucocephala* seedling, reverse transcribed from the KB-2r primer, and then amplified by PCR using Taq polymerase with KB-1f and KB-3r primers. The PCR product was amplified again with KB-1f and KB-4r primers. These PCR products were separated by 0.8% agarose gel electrophoresis. In the second PCR reaction, the expected size band of about 210 bp (lower band in lanes 6, 7, 8) and a major band of approximately 350 bp were produced (Figure 3.15). The specific band (210 bp) was purified by 5% polyacrylamide gel electrophoresis, cloned into pT7blue T-vector. The recombinant plasmids were first extracted and the inserts confirmed by restriction digest with *Eco*R I and *Pst* I (Figure 3.16). The recombinant plasmids from clone 1, 2, 3, 4, 5, 6, 8, and 11 gave the pT7 blue plasmid size 2900 bp, and the insert size about 210 bp (Figure 3.16 lanes 3, 4, 5, 6, 7, 8, 10, 13). The other bands contained insert sizes more and less than 210 bp. The recombinant plasmids were isolated, sequenced, and translated to amino acid sequences as shown in Figure 3.17.

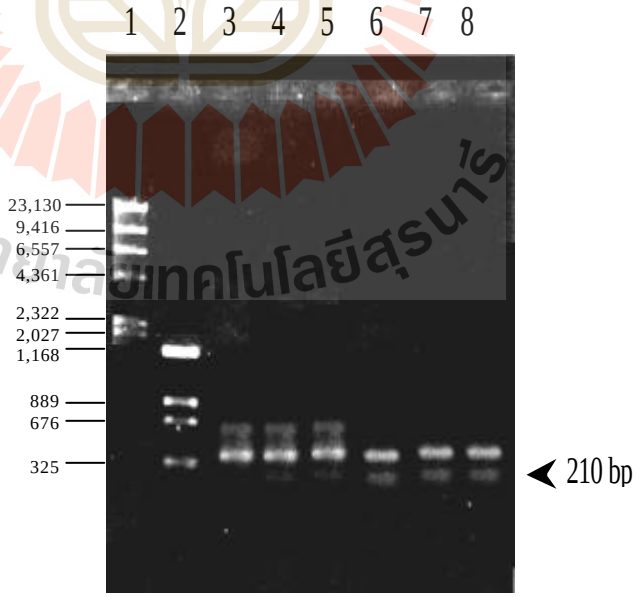

Figure 3.15 0.8% agarose gel electrophoresis of the 5′ RACE product. Lane 1: λ/*Hind* III marker, lane 2: pGEM EX/*Rsa* I marker, lane 3, 4, 5: First PCR product from KB-1f primer and KB-3r primer, and lane 6, 7, 8: Second PCR product from KB-1f primer and KB-4r primer.

The sequence of the 5′ RACE clone was found to match the cDNA library clone. After combining the sequence of cDNA library fragment and the 5′ RACE product, the coding sequence of the cDNA as composed of 969 bp (combination of library clone 5 and 5′ RACE clone 3.1) and 978 bp (combination of library clone 4 and 5′ RACE clone 3.4), which translated to 323 and 326 amino acids residues, respectively (Figure 3.18). These sequences contained the methionine start codon (ATG) from the 5′ RACE product.

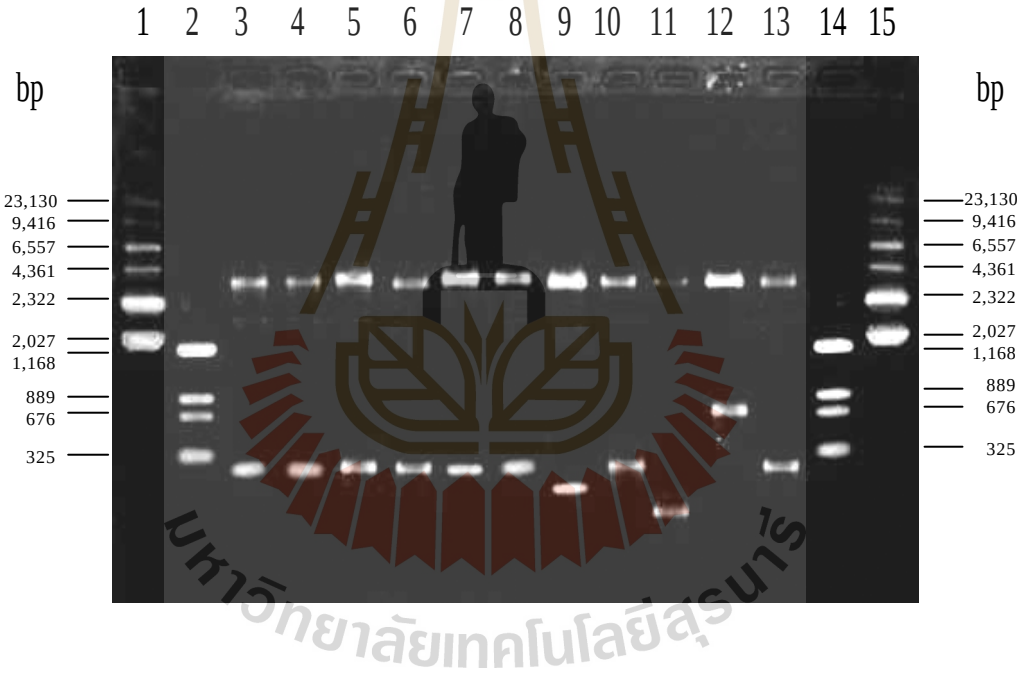

Figure 3.16 0.8% agarose gel electrophoresis of the 5′ RACE insert clone plasmids after restriction digest with *Eco*R I and *Pst* I.

Lane 1,15: λ/*Hind* III marker

Lane 2, 14 pGEM EX/*Rsa* I marker

Lane 3-13: *Eco*R I and *Pst* I digested recombinant plasmids from white colonies No.1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11 from left to right respectively.

```
S-3/1     1:ATGGATAACATGAACAAGATGCGGGTGCTCTTGTGTTTGCTGCTGAGC---CTCATGGTGGGAGGCTTAGC  68
S-3/2     1:ATGGATAACATGAACAAGATGCGGGTGCTCTTGTGTTTGCTGCTGAGC---CTCATGGTGGGAGGCTTAGC  68
S-3/6     1:ATGGATAACATGAACAAGATGCGGGTGCTCTTGTGTTTGCTGCTGAGC---CTCATGGTGGGAGGCTTAGC  68
S-3/8     1:ATGGATAACATGAACAAGATGCGGGTGCTCTTGTGTTTGCTGCTGAGC---CTCATGGTGGGAGGCTTAGC  68
S-3/3     1:ATGGATAACATGAACAAGATGCGGGTGCTCTTGTGTGTGCTGCTGTACAGCCTCATGGTGGGAGGCTTAGC  71
S-3/4     1:ATGGATAACATGAACAAGATGCGGGTGCTCTTGTGTGTGCTGCTGTACAGCTTCATGGTGGGAGGCTTAGC  71
S-3/5     1:ATGGATAACAAGAACAAGATGCGGGTGCTCTTGTGTGTGCTGCTGTACAGCCTCATGGTGGGAGGCTTAGC  71
S-3/11    1:ATGGATAACATGAGCAAGATGCGGGTGCTCTTGTGTGTGCTGCTGTACAGCCTCATGGTGGGAGGCTTAGC  71

S-3/1    69:AGAGCAATGCGGAAGACAGGCAGGGGGTGCCTTGTGCCCCGGCGGCCTCTGTTGCAGCCAATTCGGTTGGT  139
S-3/2    69:AGAGCAATGCGGAAGACAGGCAGGGGGTGCCTTGTGCCCCGGCGGCCTCTGTTGCAGCCAATTCGGTTGGT  139
S-3/6    69:AGAGCAATGCGGAAGACAGGCAGGTGGTGCCTTGTGCCCTGGCGGCCTCTGTTGCAGCCAATTCGGTTGGT  139
S-3/8    69:AGAGCAATGCGGAAGACAGGCAGGGTGTGCCTTGTGCCCCGGCGGCCTCTGTTGCAGACAATTCGGTTGGT  139
S-3/3    72:GGAGCAATGCGGCAGGCAGGCAGGGGGTGCCTTGTGCCCCGGCCGCCTCTGCTGCAGCCAATTCGGCTGGT  142
S-3/4    72:GGAGCAATGCGGCAGACAGGCAGGGGGTGCCTTGTGCCCCGGCCGCCTCTGCTGCAGCCAATTCGGCTGGT  142
S-3/5    72:GGAGCAATGCGGCAGGCAGTCAGGGGGTGCCTTGTGCCCCGGCCGCCTCTGCTGCAGCCAATTCGGCTGGT  142
S-3/11   72:GGAACAATGCGGCAGGCAGTCAGGGGGTGCCTTGTGCCCCGGCCGCCTCTGCTGCAGCCAATTCGGCTGGT  142

S-3/1   140:GTGGTTCAAATAATGATTACTGCGGCCCCGGTTGCCAGAGCCAGTGTAGCGGCAGC------GGCCCAGCC  204
S-3/2   140:GTGGTTCAAATAATGATTACTGCGGTCCCGGTTGCCAGAGCCAGTGTAGCGGCAGC------GGCCCAGCC  204
S-3/6   140:GTGGTTCAAATAATGATTACTGCGGTCCCGGTTGCCAGAGCCAGTGTAGCGGCAGC------GGTCCAGCC  204
S-3/8   140:GTGGTTCAAATAATGATTACTGCGGACCCGGTTGCCAGAGCCAGTGCAGCGGTAGC------GGTCCAGCC  204
S-3/3   143:GTGGTTCAACTAACGATTACTGCGGCCCCGGTTGCCAGAGTCAGTGTGGCGGAAGTGGCCCAGGCCCAGCC  213
S-3/4   143:GTGGTTCAACTAACGATTACTGCGGCCCCGGTTGCCAGAGCCAGTGTGGCGGAAGTGGCCCAGGCCCAGCC  213
S-3/5   143:GTGGTACCACTAACGATTACTGCGGCCCCGGTTGCCAGAGCCAGTGTGGCGGCAGTGGCCCAGGCCCAGCC  213
S-3/11  143:GTGGTACAACTAACGATTACTGCGGCCCCGGTTGCCAGAGCCAGTGTGGCGGAAGTGGCCCAGGCCCAGCC  213

S-3/1  205:CCTCCCTCCGGTGGCCTCGCCAGCATCATCTCCAGGGACACCTTCAATCAGATGCTCAAGCACCGCAACGAC  276
S-3/2  205:CCTCCCTCCGGTGGCCTCGCCAGCATCATCTCCAGGGACACCTTCAATCAGATGCTCAAGCACCGCAACGAC  276
S-3/6  205:CCTCCCTCCGGTGGCCTCGCCAGCATCATCTCAAGGGACACCTTCAATCAGATGCTTAAGCACCGCAACGAC  276
S-3/8  205:CCTCCCTCCGGTGGCCTGGCCAGCATCATCTCCAGGGACACCTTCAATCAGATGCTCAAGCACCGCAACGAC  276
S-3/3  214:CCTCCCTCCGGTGGCCTCACCGGCATCATCTCCAGGGACACCTTCAACCAGATGCTCAAGCACCGCAACGAC  285
S-3/4  214:CCTCCCTCCGGTGGCCTCACCGGCATCATCTCCAGGGACACCTTCAACCAGATGCTCAAGCACCGCAACGAC  285
S-3/5  214:CCTCCCTCCGGTGGCCTCACCGGCATCATCTCCAGGGACACCTTCAACCAGATGCTCAAGCACCGCAACGAC  285
S-3/11214:CCTCCCTCCGGTGGCCTCACCGGCATCATCTCCAGGGACACCTTCAACCAGATGCTCAAGCACCGCAACGAC  285
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A

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AA-3/1    1:MDNMNKMRVLLCLLL-SLMVGGLAEQCGRQAGGALCPGGLCCSQFGWCGSNNDYCGPGCQ  59
AA-3/2    1:MDNMNKMRVLLCLLL-SLMVGGLAEQCGRQAGGALCPGGLCCSQFGWCGSNNDYCGPGCQ  59
AA-3/6    1:MDNMNKMRVLLCLLL-SLMVGGLAEQCGRQAGGALCPGGLCCSQFGWCGSNNDYCGPGCQ  59
AA-3/8    1:MDNMNKMRVLLCLLL-SLMVGGLAEQCGRQAGGALCPGGLCCRQFGWCGSNNDYCGPGCQ  59
AA-3/3    1:MDNMNKMRVLLCLLLYSLMVGGLAEQCGRQAGGALCPGRLCCSQFGWCGSTNDYCGPGCQ  60
AA-3/4    1:MDNMNKMRVLLCLLLYSFMVGGLAEQCGRQAGGALCPGRLCCSQFGWCGSTNDYCGPGCQ  60
AA-3/5    1:MDNMNKMRVLLCLLLYSLMVGGLAEQCGRQSGGALCPGRLCCSQFGWCGTTNDYCGPGCQ  60
AA-3/11   1:MDNMSKMRVLLCLLLYSLMVGGLAEQCGRQSGGALCPGRLCCSQFGWCGTTNDYCGPGCQ  60

AA-3/1   60:SQC--SGSGPAPPSGGLASIISRDTFNQMLKHRND                            92
AA-3/2   60:SQC--SGSGPAPPSGGLASIISRDTFNQMLKHRND                            92
AA-3/6   60:SQC--SGSGPAPPSGGLASIISRDTFNQMLKHRND                            92
AA-3/8   60:SQC--SGSGPAPPSGGLASIISRDTFNQMLKHRND                            92
AA-3/3   61:SQCGGSGPGPAPPSGGLTGIISRDTFNQMLKHRND                            95
AA-3/4   61:SQCGGSGPGPAPPSGGLTGIISRDTFNQMLKHRND                            95
AA-3/5   61:SQCGGSGPGPAPPSGGLTGIISRDTFNQMLKHRND                            95
AA-3/11  61:SQCGGSGPGPAPPSGGLTGIISRDTFNQMLKHRND                            95
```

B

Figure 3.17 Multiple sequence alignment of cDNA and deduced amino acid sequences from 5′ RACE product. A, multiple sequence alignment of cDNA. B, multiple alignment of deduced amino acid sequences. They were separated into 2 groups; group 1, clones 3/1, 3/2, 3/6, and 3/8; group 2, clones 3/3, 3/4, 3/5, and 3/11.

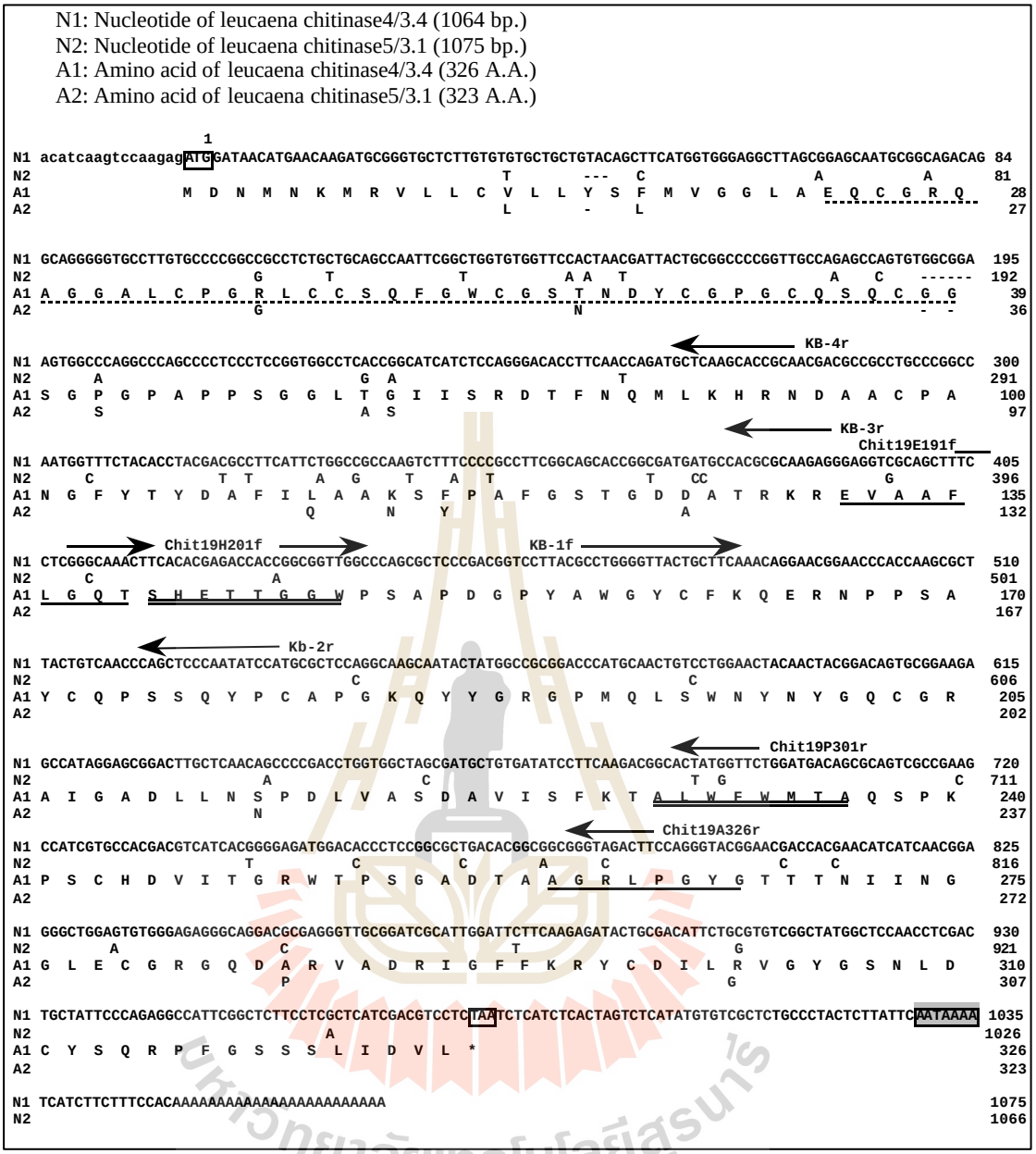


Figure 3.18 Full-length nucleotide and predicted amino acid sequences. The dotted line shows a cysteine-rich domain. The putative polyadenylation signal of leucaena chitinase 4/3.4 and leucaena chitinase 5/3.1 at 1029-1035 and 1020-1026 is in the gray box, the start codon at nucleotide 1-3 and stop codon at 979-981, 970-972 of leucaena chitinase 4/3.4 and leucaena chitinase 5/3.1, respectively, are marked with open boxes. The underline and double underline portions denote the conserved amino acid sequence regions, which were used for designing the outer and inner sets, respectively, of Nested PCR primers for initial PCR fragment amplification. The bold letters indicate the nucleotide sequences used for designing the 3′ RACE and 5′ RACE primers.

The derived protein sequences of the two clones exhibit >95% sequence identity (Figure 3.19) and code for 323 and 326 amino acid chitinase precursors. The two full-length amino acid sequences of *L. leucocephala* chitinases were analyzed using the GENETYX programs and the Expasy proteomics site (www.expasy.org). The mature protein was composed of 300 amino acid residues for clone 5/3.1, with an expected molecular weight of 31.87 kDa and predicted pI is 7.15. Clone 4/3.4 is composed of 302 amino acid residues, with an expected molecular weight of 32.21 kDa and pI of 7.53 (Table 3.1). The protein contained an N-terminal prepeptide signal for transport into the ER composed of 23 amino acid residues (M D N M N K M R V L L C V L L S F M V G G L A) for clone 5/3.1 and 24 residues (M D N M N K M R V L L C V L L Y S F M V G G L A) for clone 4/3.4.

```
   upper: leucaena chitinases 5/3.1 (323 aa)
   lower: leucaena chitinases 4/3.4 (326 aa)


  1' MDNMNKMRVL LCLLL-SLMV GGLAEQCGRQ AGGALCPGGL CCSQFGWCGS NNDYCGPGCQ
     ********** ***** *.** ********** ******** * ********** .*********
  1" MDNMNKMRVL LCLLLYSFMV GGLAEQCGRQ AGGALCPGRL CCSQFGWCGS TNDYCGPGCQ

 60' SQC--SGSGP APPSGGLASI ISRDTFNQML KHRNDAACPA NGFYTYDAFI QAANSYPAFG
     ***  **.** ********.* ********** ********** **********  **.*.****
 61" SQCGGSGPGP APPSGGLTGI ISRDTFNQML KHRNDAACPA NGFYTYDAFI LAAKSFPAFG

118' STGDAATRKR EVAAFLGQTS HETTGGWPSA PDGPYAWGYC FKQERNPPSA YCQPSSQYPC
     ****.***** ********** ********** ********** ********** **********
121" STGDDATRKR EVAAFLGQTS HETTGGWPSA PDGPYAWGYC FKQERNPPSA YCQPSSQYPC

178' APGKQYYGRG PMQLSWNYNY GQCGRAIGAD LLNNPDLVAS DAVISFKTAL WFWMTAQSPK
     ********** ********** ********** ***.****** ********** **********
181" APGKQYYGRG PMQLSWNYNY GQCGRAIGAD LLNSPDLVAS DAVISFKTAL WFWMTAQSPK

238' PSCHDVITGR WTPSGADTAA GRLPGYGTTT NIINGGLECG RGQDPRVADR IGFFKRYCDI
     ********** ********** ********** ********** ****.***** **********
241" PSCHDVITGR WTPSGADTAA GRLPGYGTTT NIINGGLECG RGQDARVADR IGFFKRYCDI

298' LGVGYGSNLD CYSQRPFGSS SLIDVL
     * ******** ********** ******
301" LRVGYGSNLD CYSQRPFGSS SLIDVL
```

Figure 3.19 Comparison of the protein sequences of leucaena chitinases 5/3.1 and leucaena chitinases 4/3.4. They have 95.5% identity of amino acid level. Identical amino acids are marked by asterisks.

Table 3.1 The prediction of protein properties of chitinases from *L. leucocephala*

Leucaena chitinase.	Precursor length (A.A.)	Precursor MW (dalton)	Precursor pI	Mature length (A.A.)	Mature MW. (dalton)	Mature pI
Clone 5/3.1	323	34,388	7.57	300	31,870	7.15
Clone 4/3.4	326	34,908	7.87	302	32,207	7.53

The deduced amino acid sequence was aligned with closely related class I chitinase protein sequences from the GENBANK database. This alignment indicated that it was homologous to a number of plant chitinase with the closest match being kidney bean, *Phaseolus vulgaris* (AC: S43926) with 74% identity; 73% for cow pea, *Vigna unguiculata* (AC: S57482); 72% for *Medicago sativa* (AC: AAB41324); 72% for *Medicago truncatula* (AC: CAA71402); and 73% for pea, *Pisum sativum* (AC: L37876) (Figure 3.20). By homology they should have a prepeptide, cysteine-rich (chitin-binding) domain, a glycine/proline-rich hinge linker, and a catalytic domain (Figure 3.21).

The deduced amino acid composition of leucaena chitinase (4/3.4) was calculated and compared with class I chitinase from tobacco (*Nicotiana tabacum*) and potato (*Solanum tuberosum*) as shown in Table 3.2. The amino acid composition of chitinase from *L. leucocephala* high content of Asx, Gly, Cys, and Pro with 33, 42, 17, and 23 residues, respectively which is like the amino acid composition of chitinase from tobacco with 36, 47, 18, 23 of these residues and potato with 40, 44, 17, and 22 of these residues respectively. These chitinases had 17-18 cysteine residues/mol and which was the typical content of class I chitinase (Yamagami *et al*., 1998).

Table 3.2 The comparison of amino acid composition between the predicted mature leucaena chitinase and plant class I endochitinases

AA	Asx	Thr	Ser	Glx	Pro	Gly	Ala	Cys	Val	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg	Trp	Tot
LL	33	17	25	16	23	42	28	17	7	3	11	17	15	13	3	8	17	7	302
PO	40	12	23	18	22	44	21	17	9	3	15	14	11	15	4	8	18	8	302
TO	34	17	24	19	26	46	20	17	8	4	15	14	11	14	4	7	18	8	306

LL: *L. leucocephala*, PO: potato, TO: Tobacco

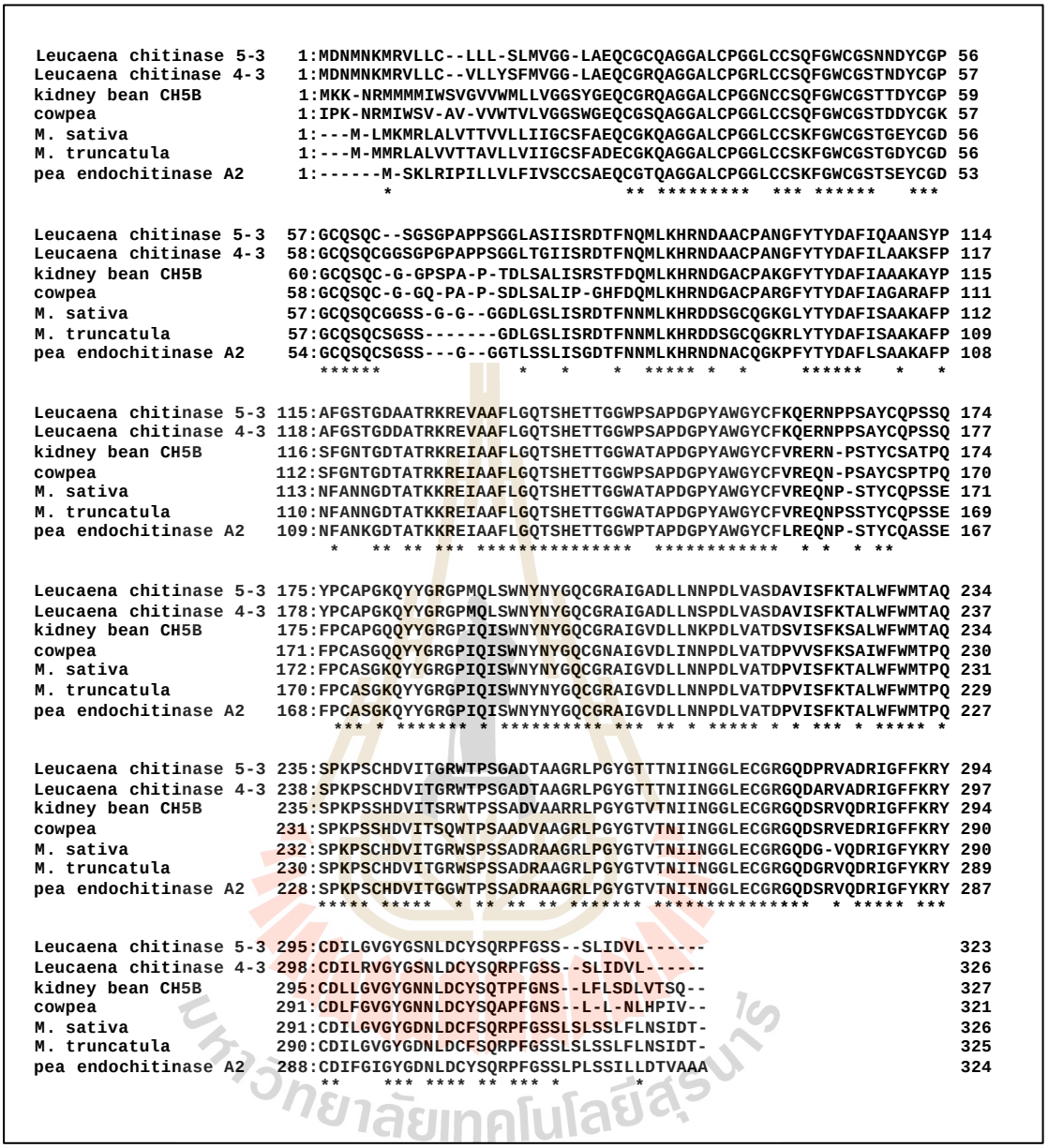


Figure 3.20 Multiple sequence alignment of deduced amino acid sequences from full-length leucaena chitinases 5/3.1 and 4/3.4 sequences. The sequences aligned include the class I chitinases from: kidney bean, *Phaseolus vulgaris* (AC: S43926); cow pea, *Vigna unguiculata* (AC: S57482); M.sativa, *Medicago sativa* (AC: AAB41324); M.trucatula, *Medicago truncatula* (AC: CAA71402); and pea, *Pisum sativum* (AC: L37876). The asterisks are conserved amino acid residues.

3.6 Expression on BL21 (DE3) and Origami (DE3) ***E.coli***

Oligonucleotide primers for DNA expression were designed from the sequence of the *L. leucocephala* class I chitinase without prepeptide (Figure 3.21). The cloning strategy was designed such that the predicted mature protein with an additional N-terminal methionine residue and a C-terminal His_6•tag would be produced in pET23d, and a chitinase with an addition N-terminal thioredoxin (Trx)•Tag, His_6•Tag, Thrombin cleavage site, S•Tag, Enterokinase cleavage site would be produced for pET32a. To construct the expression plasmid, total RNA from *L. leucocephala* seedling was reverse transcribed from a $polyT_{17}$ primer and then amplified by PCR using *Pfu* DNA polymerase using appropriate primers for cloning into the expression plasmids (Figure 3.21). The gel purified PCR product, a specific band of approximately 900 bp (lane 2 from Chit-Ex1f primer and Chit-Ex1r primer, lane 3, 4 from KBEX-2f primer and KBEX-2r primer, Figure 3.22), was cloned into the *Hinc* II site of a pUC19 vector. Recombinant plasmids were confirmed by restriction digest with *Nco* I and *Xho* I and agarose gel electrophoresis.

The expected sequences of the plasmids were confirmed by sequencing and analysis. The correct insert plasmids were digested with *Nco* I and *Xho* I, and the insert eluted. The leucaena chitinase gene was subcloned into the prokaryotic expression vectors pET23d and pET32a at the *Nco* I and *Xho* I sites and the recombinant plasmids confirmed by restriction digest with *Nco* I and *Xho* I and agarose gel electrophoresis.

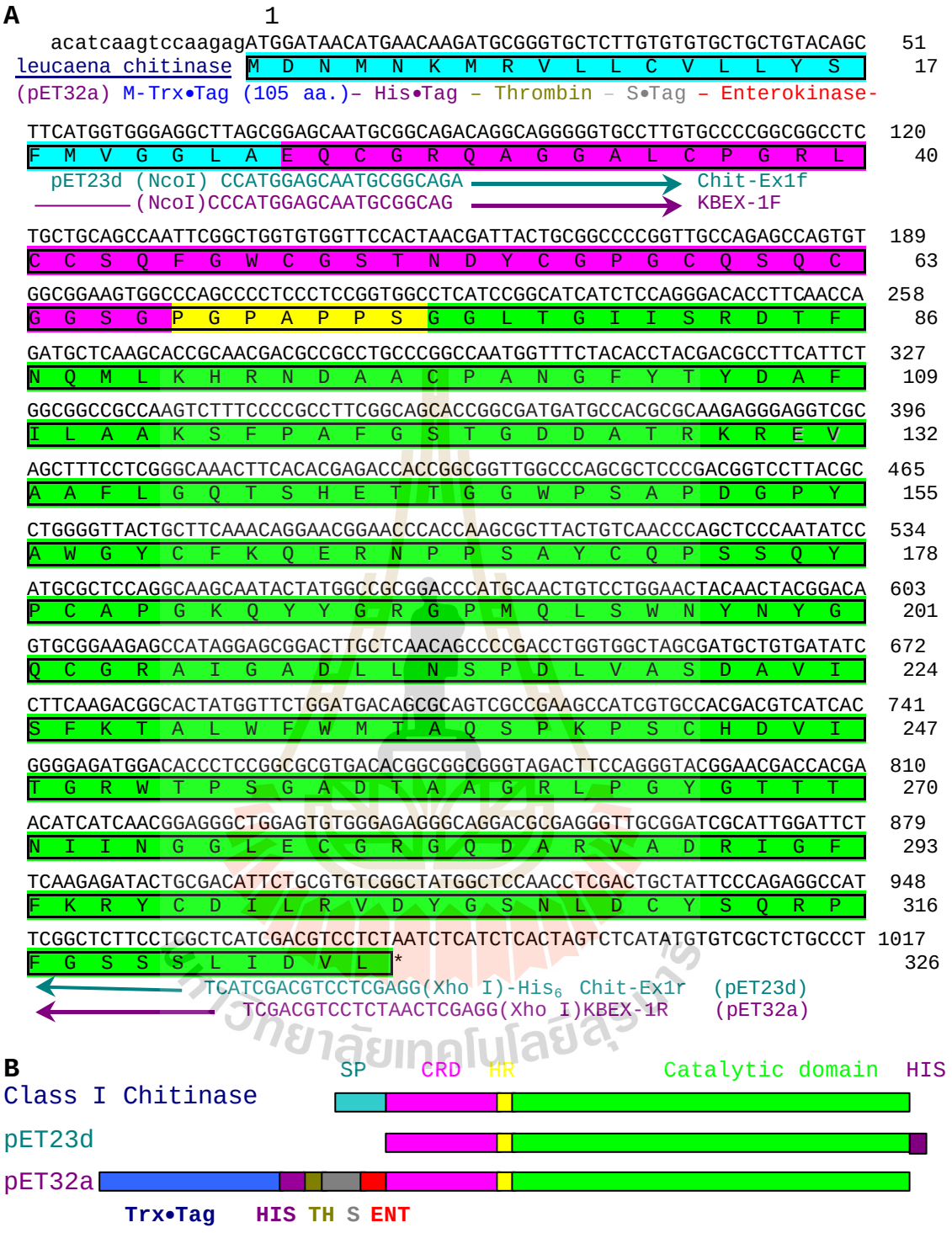


Figure 3.21 Oligonucleotide primers for DNA expression were designed from leucaena class I chitinase without prepeptide (A). The primers used were to subclone the chitinase-coding sequence into pET23d and pET32a expression plasmids to produce the proteins diagrammed in B.

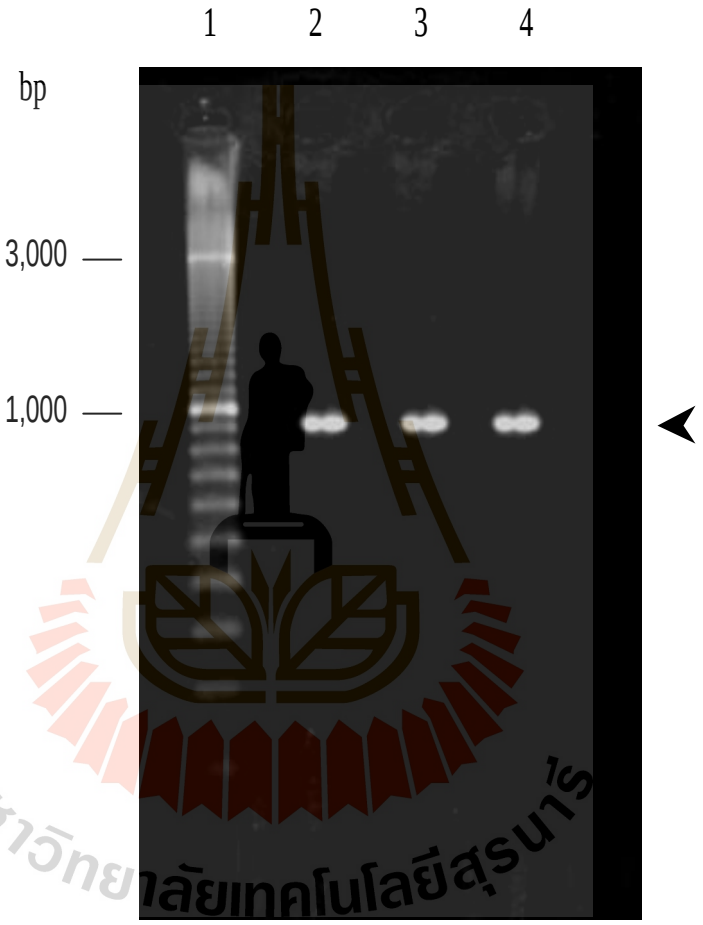


Figure 3.22 0.8% agarose gel electrophoresis of PCR product for recombinant expression.

Lane 1: 100 bp DNA marker

Lane 2: PCR product from Chit-Ex1f primer and Chit-Ex1r primer.

Lane 3-4: PCR product from KBEX-2f primer and KBEX-2r primer.

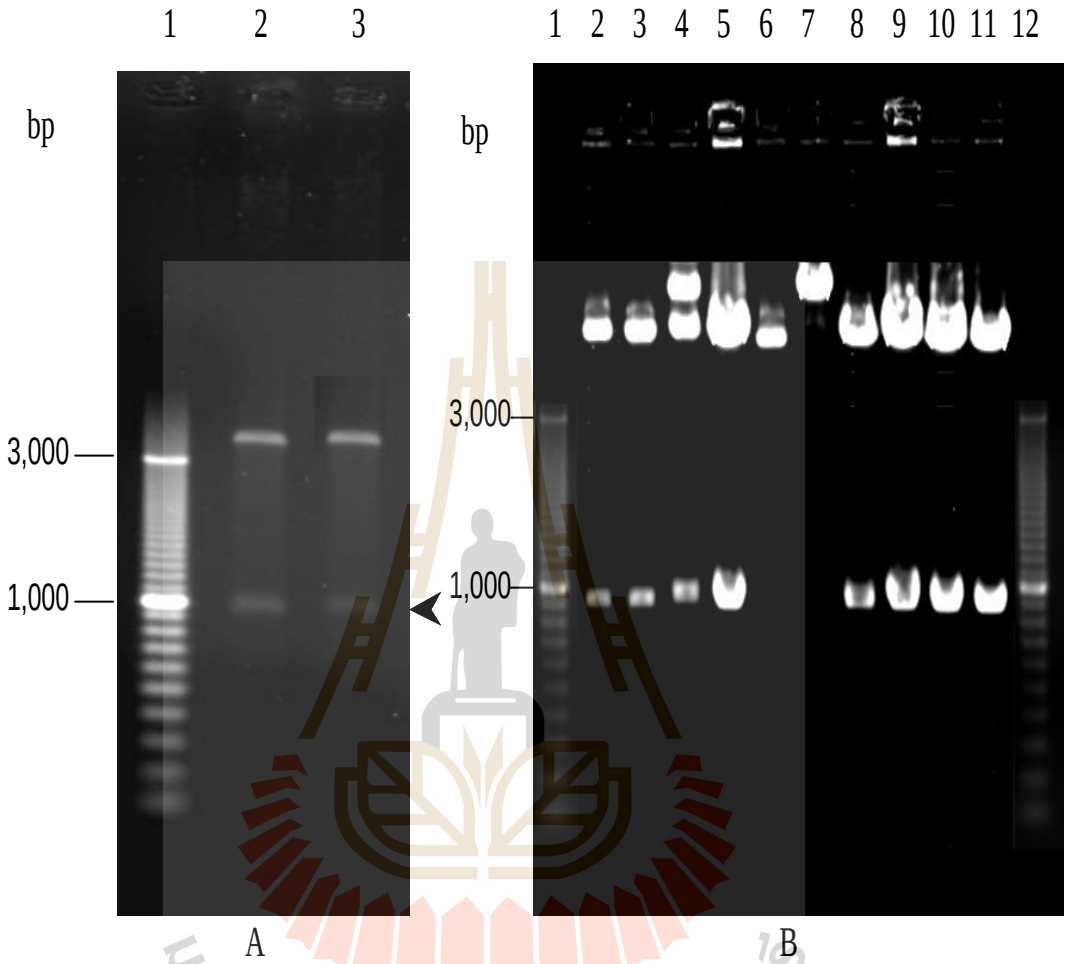


Figure 3.23 0.8% agarose gel electrophoresis of restriction enzyme analysis of pET23d (Figure 3.23 A) and pET32a (Figure 3.23 B) recombinant plasmids containing cDNA fragment of chitinase with *Nco* I and *Xho* I.

Figure 3.23 A: Lane1: 100 bp DNA marker

Lane 2, 3: *Nco* I and *Xho* I digested pET23d chitinase recombinant plasmids from white colonies No. 1, 2 from left to right, respectively.

Figure 3.23 B: Lane 1, 12: 100 bp DNA marker

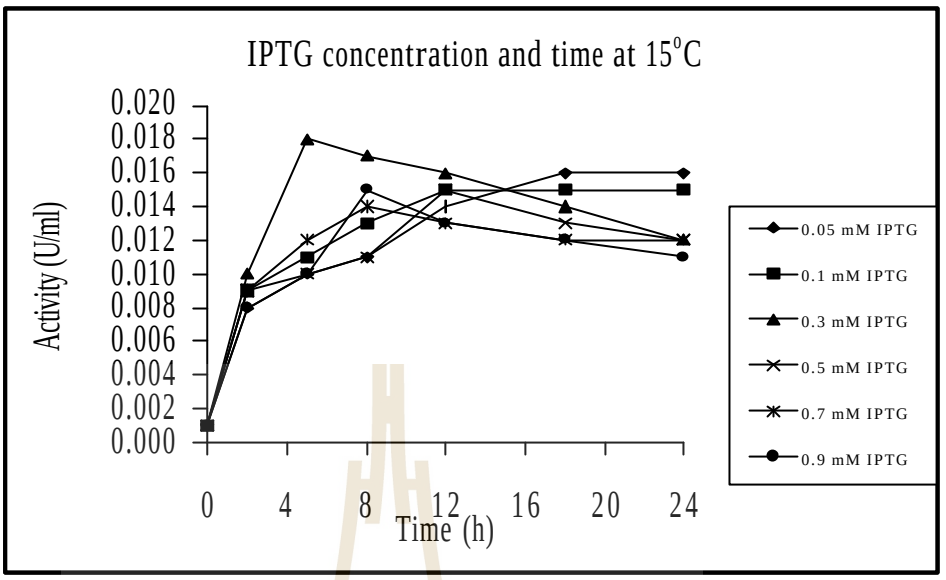
Lane 2.-11: *Nco* I and *Xho* I digested pET32a recombinant plasmids from clones No. 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 from left to right.

The size of plasmid and insert bands from recombinant plasmids No. 1 and 2 from pET23d were about 3,700 bp band and 900 bp, respectively, (lane 2 and 3) (Figure 3.23 A), and the recombinant plasmids No. 1, 2, 3, 4, 7, 8, and 9 from pET32a gave a plasmid size of about 5,900 bp and insert size of about 900 bp (lanes 2, 3, 4, 5, 8, 9, and 10, Figure 3.23 B).

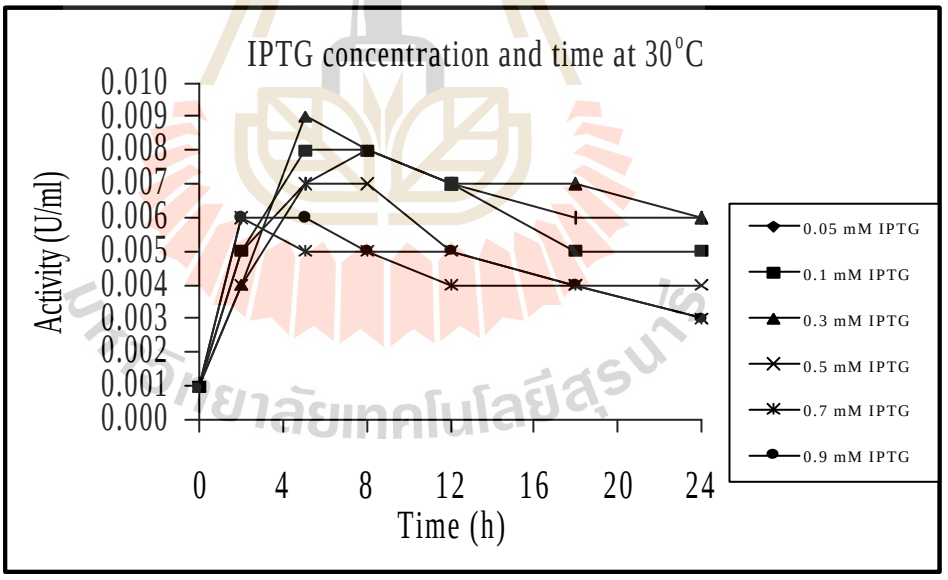
The correct insert sequences of pET23d and pET32a recombinant plasmids were confirmed by sequencing with the T7 promoter and T7 terminator primers and retransformed into competent BL21 (DE3) and Origami (DE3) cells, respectively. The optimum condition of IPTG induction and time for expression of active chitinase were determined with 0.05, 0.1, 0.3, 0.5, 0.7, and 0.9 mM IPTG for 0, 2, 5, 8, 12, 18, and 24 h at 15° and 30°C for pET23d and 0.05, 0.1, 0.3, 0.5, and 0.9 mM IPTG for 0, 4, 8, 12, 16, 20, and 24 h at 15, 30°C for pET32a. The optimum conditions are 0.3 mM IPTG and 5 h at 15°C for pET23d (Table 3.3 and Figure 3.24) which had the highest activity of 0.018 U/ml culture and 0.1 mM IPTG and 12 h for pET32a at 15°C (Table 3.4 and Figure 3.25) which had the highest activity of 0.063 U/ml.

Table 3.3 The activity (U/ml) of pET23d leucaena chitinase expressed in BL21 (DE3) *E.coli* at 15°C and 30°C for 0, 2, 5, 8, 12, 18, and 24 h at IPTG concentration of 0.05, 0.1, 0.3, 0.5, 0.7, and 0.9 mM.

Temp.	Activity at 15°C (U/ml)						Activity at 30°C (U/ml)					
IPTG (mM) Time (h)	0.05	0.1	0.3	0.5	0.7	0.9	0.05	0.1	0.3	0.5	0.7	0.9
0	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
2	0.008	0.009	0.010	0.009	0.009	0.008	0.005	0.005	0.004	0.004	0.006	0.006
5	0.010	0.011	0.018	0.010	0.012	0.010	0.007	0.008	0.009	0.007	0.005	0.006
8	0.011	0.013	0.017	0.011	0.014	0.015	0.008	0.008	0.008	0.007	0.005	0.005
12	0.014	0.015	0.016	0.015	0.013	0.013	0.007	0.007	0.007	0.005	0.004	0.005
18	0.016	0.015	0.014	0.013	0.012	0.012	0.006	0.005	0.007	0.004	0.004	0.004
24	0.016	0.015	0.012	0.012	0.012	0.011	0.006	0.005	0.006	0.004	0.003	0.003

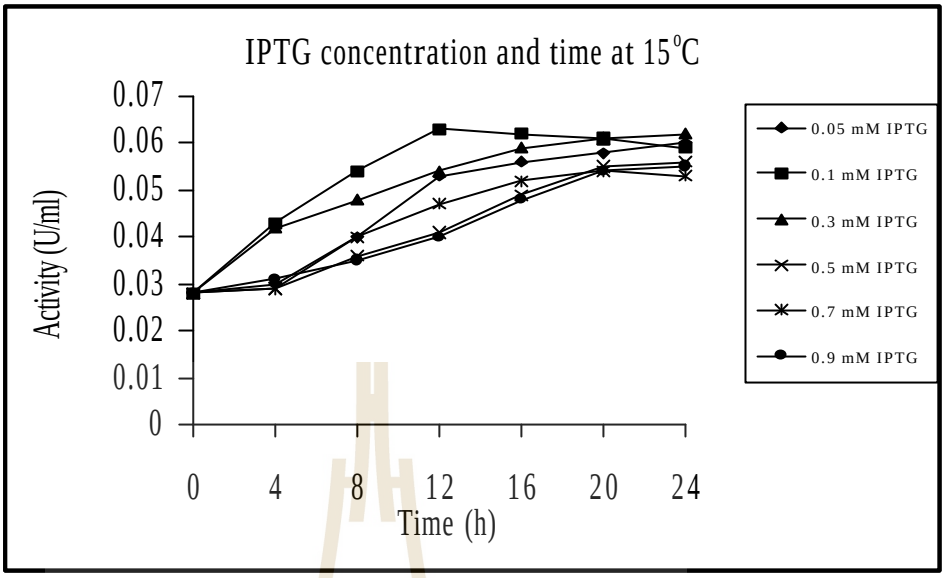


A

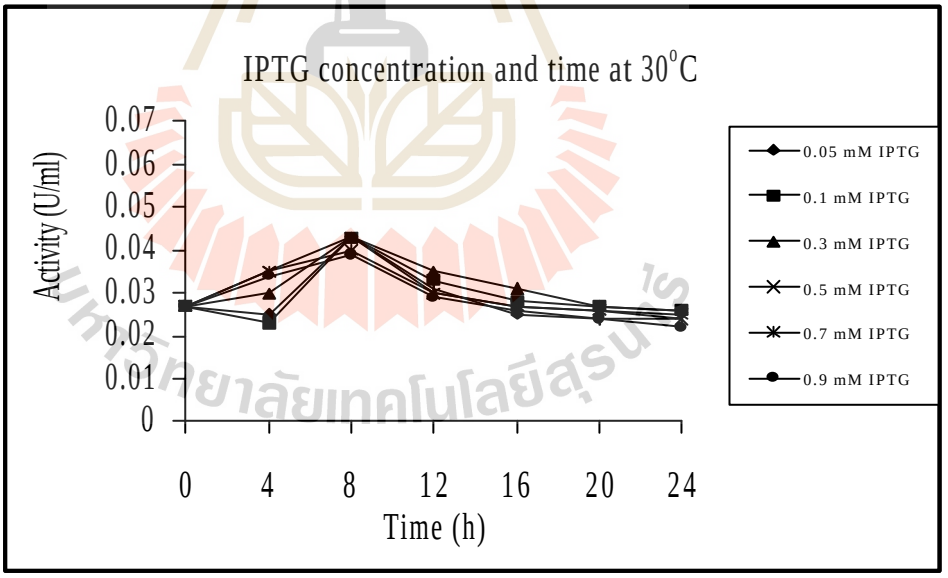


B

Figure 3.24 The comparison of chitinase activity from IPTG concentrations and incubation times from pET23d leucaena chitinase expressed in BL21 (DE3) *E.coli*. The induction temperature of A was 15°C and B was 30°C.



A



B

Figure 3.25 The comparison of chitinase activity at different inducing IPTG concentrations and induction times from pET32a leucaena chitinase expressed in Origami (DE3) *E.coli*. The *E.coli* were grown at 37°C and induced at 15°C in A and 30°C in B.

Induction of expression from pET23d leucaena chitinase at 15°C for 5 h at an IPTG concentration of 0.3 mM resulted in an approximately 32 kDa protein band on SDS-PAGE (Figure 3.26). The expressed protein was present in both insoluble inclusion bodies and soluble form. For expression using pET32a, a protein was produced with contained an additional N-terminal methionine residue, a Trx•Tag, an S•Tag, a His_6•Tag and cleavage sites for thrombin and enterokinase. Induction of expression at 15°C resulted in an approximately 46 kDa recombinant protein band on SDS-PAGE of cell extracts. The strongest band was seen with 0.1 mM IPTG induction at 15°C for 12 h (lane 7 in Figure 3.27), which had the highest activity of 0.063 units/ml culture in Table 3.4. A 14 kDa band of thioredoxin protein (Figure 3.27 lane 3) was produced from pET32a, which was induced with 0.1 M IPTG for 24 h. The recombinant protein had chitinase activity and was induced even without IPTG but accumulated less than one half the activity under these conditions compared to the optimum condition.

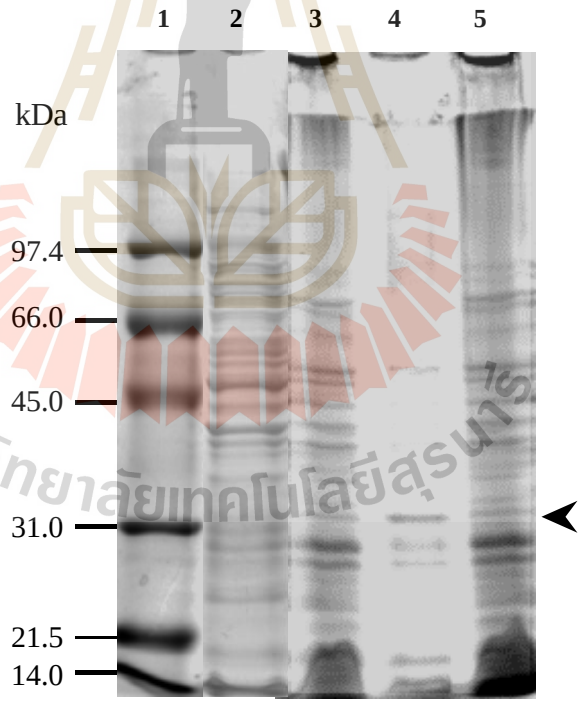


Figure 3.26 12% SDS PAGE of pET23d leucaena chitinase expression in BL21 (DE3) *E.coli* at 15°C for 5 h of induction with 0.3 mM IPTG.

Lane 1: Protein markers (kDa), Lane 2: pET23d control,

Lane 3: Crude *E.coli* extract, Lane 4: Insoluble material from *E.coli* extract,

Lane 5: soluble material from *E.coli* extract.

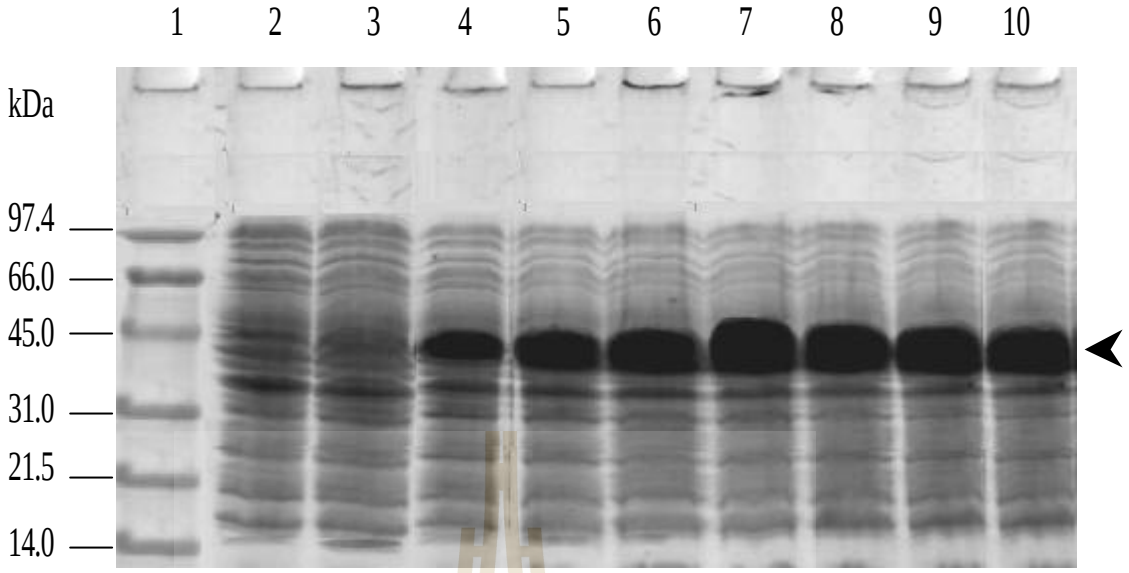


Figure 3.27 12% SDS PAGE of leucaena chitinase expression from pET32a in Origami (DE3) *E. coli* at 15°C of 0.1 mM IPTG.

Lane 1: Protein MW markers (kDa)
Lane 2: Uninduced pET32a (control) 0 h
Lane 3: 0.1 mM IPTG induced of pET32a for 24 h
Lane 4: Uninduced of pET32a leucaena chitinase
Lane 5: 0.1 mM IPTG induced for 4 h
Lane 6: 0.1 mM IPTG induced for 8 h
Lane 7: 0.1 mM IPTG induced for 12 h
Lane 8: 0.1 mM IPTG induced for 16 h
Lane 9: 0.1 mM IPTG induced for 20 h
Lane 10: 0.1 mM IPTG induced for 24 h

Table 3.4 The activity (U/ml) of leucaena chitinase expressed from pET32a in Origami (DE3) *E. coli* at 15°C and 30°C for 0, 4, 8, 12, 16, 20, and 24 h at IPTG concentrations of 0.05, 0.1, 0.3, 0.5, 0.7, and 0.9 mM.

Temp.	Activity at 15°C (U/ml)						Activity at 30°C (U/ml)					
IPTG (mM)	0.05	0.1	0.3	0.5	0.7	0.9	0.05	0.1	0.3	0.5	0.7	0.9
Time (h)												
0	0.028	0.028	0.028	0.028	0.028	0.028	0.027	0.027	0.027	0.027	0.027	0.027
4	0.030	0.043	0.042	0.029	0.029	0.031	0.025	0.023	0.030	0.035	0.035	0.034
8	0.040	0.054	0.048	0.036	0.040	0.035	0.043	0.043	0.043	0.043	0.040	0.039
12	0.053	0.063	0.054	0.041	0.047	0.040	0.031	0.033	0.035	0.030	0.030	0.029
16	0.054	0.062	0.059	0.049	0.052	0.048	0.025	0.028	0.031	0.027	0.027	0.026
20	0.058	0.061	0.061	0.055	0.054	0.054	0.024	0.027	0.027	0.026	0.026	0.024
24	0.060	0.059	0.062	0.056	0.053	0.055	0.024	0.026	0.026	0.024	0.025	0.022

3.7 Purification of recombinant protein using Ni-NTA superflow and Sephadex G-100

A *L. leucocephala* chitinase gene DNA was expressed in Origami (DE3) *E.coli* using the pET32a T7 expression vector including thioredoxin (Trx), His_6 and S•Tags, and cleavage sites for thrombin and enterokinase (Figure 3.21 B). The crude extract from this expression showed high activity, comparable to extracts of young *L. leucocephala* de Wit plants. The amount of soluble recombinant protein was dependent on the IPTG concentration, time, and temperature of induction, with optimal expression achieved on induction with 0.1 mM IPTG at 15°C for 12 h (Figure 3.27). The recombinant fusion protein has about 46 kDa, which contained approximately 14 kDa of thioredoxin fusion tag on the N-terminus and 32 kDa of chitinase. After purification using a Ni-NTA column to bind the His_6•Tag and Sephadex G-100, a single band of recombinant protein was seen on SDS-PAGE at 46 kDa (Figure 3.28).

The purified fusion protein was cleaved with enterokinase to produce the 32 kDa chitinase and 14 kDa thioredoxin fusion tag, including a thioredoxine (Trx), His_6 and S•Tags. The thioredoxin fusion tag was removed over the Ni-NTA column that bound to His_6•Tag in the thioredoxin fusion tag and released the free chitinase. Purification of the recombinant fusion protein with the Ni-NTA and Sephadex G-100 column gave a 4.6 fold purification, and cleavage of recombinant fusion protein with enterokinase and removal of the fusion tag from the chitinase increased the purification to 5.6 fold (Table 3.5). The chitinase activity recovered was 88%, 73%, and 57% from the Ni-NTA column, Sephadex G-100 column, and fusion tag removal, respectively.

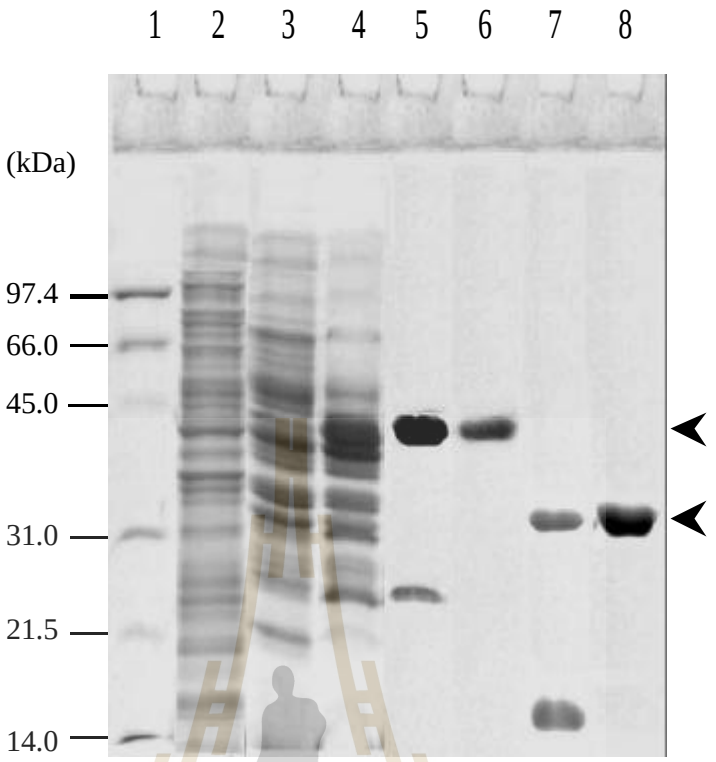


Figure 3.28 12% SDS PAGE of the pET32a *L. leucocephala* chitinase expression from *L. leucocephala* chitinase in Origami (DE3) *E.coli* containing. Either control pET32a or pET32a containing the *L. leucocephala* chitinase cDNA (pET32a/*L. leucocephala* chitinase) were induced with 0.1 mM IPTG at 15°C for 12 h. The cells were collected, extracted and purified over Ni-NTA and G-100 columns. The purified fusion protein was cleaved with enterokinase and purified over the Ni-NTA column to remove the fusion tag. Samples from the procedure were loaded in the following lanes on SDS-PAGE: lane 1: protein markers (kDa); lane 2: control pET32a; lane 3: soluble crude extract of *E.coli* with pET32a/chitinase plasmid uninduced; lane 4: soluble crude *E.coli* extract induced with 0.1 mM IPTG; lane 5: purified recombinant fusion protein from Ni-NTA column; lane 6: purified recombinant fusion protein after Sephadex G-100; lane 7: cleavage of recombinant fusion protein; lane 8: purified cleaved chitinase.

Table 3.5 Purification of leucaena chitinase after expression from pET32a was expressed in Origami (DE3) *E.coli*.

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Recovery (%)	Purification (Fold)
- Control (pET32a)	0.0045	8.27	0.0005	-	-
- Crude recombinant soluble fraction	0.4410	20.80	0.0212	100	1.0
- Ni-NTA-column	0.3882	4.11	0.0944	88	4.5
- Sephadex G-100	0.3225	3.28	0.0983	73	4.6
- Purified cleavage recombinant protein	0.2490	2.09	0.1189	57	5.6

3.8 Crude enzyme extraction and purification from ***L. leucocephala*** seedlings

The crude 0.1 M acetate buffer pH 5.0 extract from *L. leucocephala* seedlings had many bands (lane 2 in figure 3.29). Chitinase activity was detected in the extract by colorimetric assay (Table 3.6) and subjected to purification. The crude extract was precipitated with 70% ammonium sulfate and after dialysis showed two major dark bands at 18 and 40 kDa, which did not appear to correspond to chitinases (lane 3, figure 3.29). The final step was purified by affinity absorption on chitin in a beaker. The chitinase activity was eluted with 0.02 M acetic acid (pH 3.0) and dialyzed with 0.1 M acetate buffer pH 5.0. The chitinase activity was found to contain an essentially homogenous protein of 32 kDa by SDS-PAGE (lane 4, figure 3.29)

Table 3.6 shows the yield and fold purification of the chitinase from *L. leucocephala*. After 70% ammonium sulfate precipitate, the chitinase activity recovered was 65.16% with 4.3 fold purification. Further purification with chitin affinity chromatography gave higher purity up to 26.7 fold with a recovery of 15.92%. The SDS-PAGE showed a high purification of leucaena chitinase on the chitin to give a homogenous chitinase but the yield of 15.92% of chitinase activity was low. Some chitinase may be left bound on the chitin.

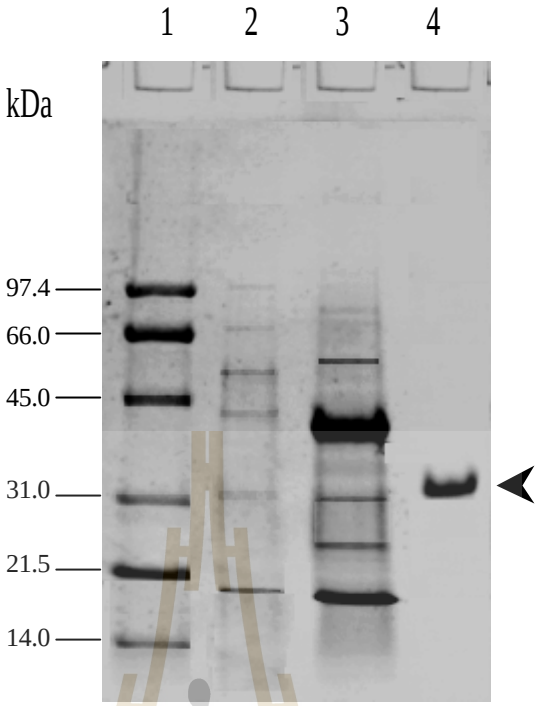


Figure 3.29 12% SDS PAGE of chitinase purification from *L. leucocephala* seedlings.
Lane 1: Protein mass makers (kDa), lane 2: Crude enzyme, lane 3: 70% Ammonium sulfate precipitate lane 4: Chitin affinity chromatography, eluted with acetic acid (pH 3.0)

Table 3.6 Purification of chitinase from *Leucaena leucocephala* de Wit

Step	Total activity (Units)	Total protein (mg)	Specific activity (Units/mg)	Recovery (%)	Purification (fold)
1. Crude extract	0.5640	202	0.0028	100	1
2. 70% $(NH_4)_2SO_4$	0.3675	30	0.0123	65	4.3
3. Chitin affinity chromatography	0.0898	1.2	0.0748	15.9	26.7

3.9 Determination of isoelectric point

To investigate isoelectric focusing point of leucaena chitinase from seedlings and purified cleaved recombinant protein, isoelectric focusing in polyacrylamide gel was performed in the pH range 3.0-9.0. The electrophoretic patterns obtained in Figure 3.30 showed a single band of chitinase from *L. leucocephala* seedlings (lane 2, Figure 3.30 A) and purified cleavage

recombinant protein (lane 2, Figure 3.30 B). The leucaena chitinases purified from seedlings and recombinant expression had pIs of approximately 7.6 and 7.5 respectively.

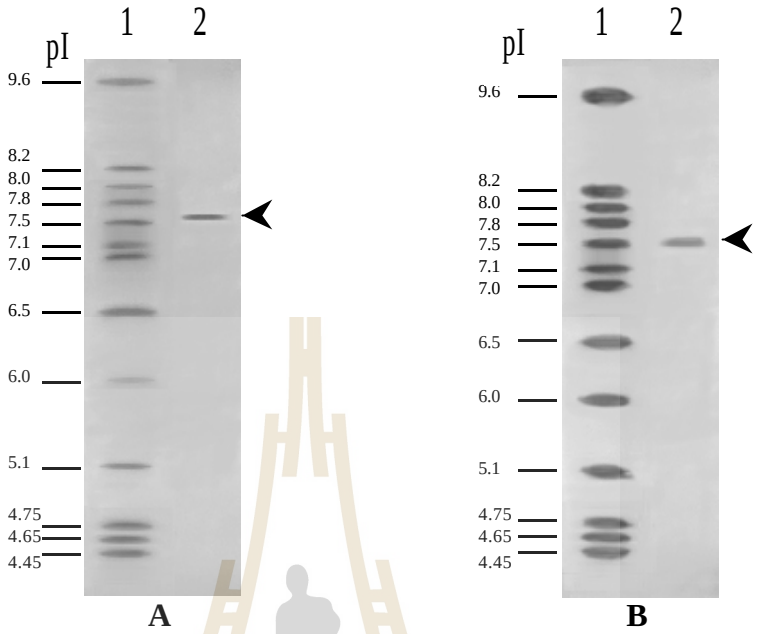


Figure 3.30 A, Isoelectric focusing of purified chitinase from *L. leucocephala* seedlings. B, Isoelectric focusing of purified chitinase from cleaved recombinant protein. They were separated by Phastgel IEF pI 3-9, and compared to pI standard markers. Lane 1: Protein pI makers (kDa), lane 2: A, purified chitinase from *L. leucocephala* seedlings. B, purified cleaved recombinant chitinase.

The cloned chitinase sequence had a calculated molecular weight of 32.21 kDa and pI of 7.53, which is close to the molecular weight and pI observed for chitinase purified from *L. leucocephala* seedlings of 32 kDa and pI of 7.6 and for purified cleaved recombinant chitinase of 32 kDa and pI of 7.5, respectively. The specific activity of the purified, cleaved recombinant chitinase from the pET32a expression vector was highest at 0.1189 U/mg (Table 3.7). The specific activity of purified recombinant chitinase without or with fusion tag removal was higher than that purified *L. leucocephala* seedlings by 1.6 fold and 1.3 fold respectively. The chitinase protein from pET23d expression vector was mostly found in the inclusion body. Then the pET32a expression vector was suitable for produce chitinase because it had a thioredoxin-fusion in redox-deficient *E.coli* to allow formation of disulfide bonds, which in class I chitinases have. The thireodoxin-fusion may catalyze the disulfide bond formation and assist in folding of the class I chitinase, resulting in better activity.

Table 3.7 Comparison of activity of leucaena chitinase purified from seedling and expressed in *E. coli*.

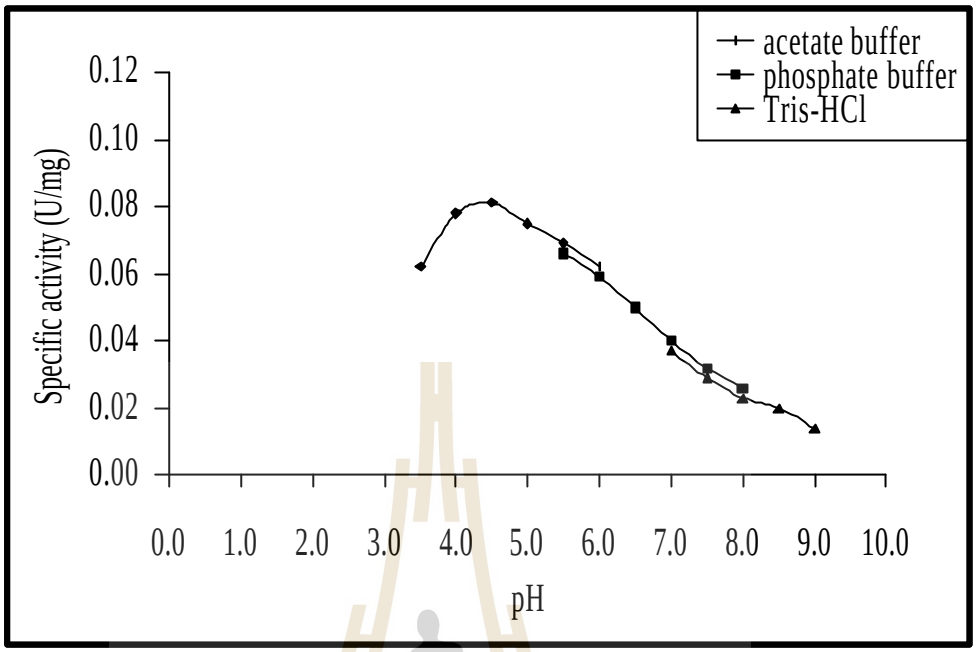
Sample	Total activity (Unit)	Total protein (mg)	Specific activity (U/mg protein)	Comparison Specific activity (Fold)
- Crude from seedlings	0.5640	202	0.0028	1.0
- Purification of seedlings	0.0898	1.20	0.0748	26.7
- Crude soluble protein pET23d	0.0146	2.10	0.0180	3.4
- Crude soluble recombinant protein pET32a	0.4410	20.80	0.0212	7.6
- Purified recombinant fusion protein pET32a	0.3225	3.28	0.0983	35.1
- Purified, cleaved recombinant chitinase pET32a	0.2490	2.09	0.1189	42.5

3.10 Effect of pH on chitinase activity

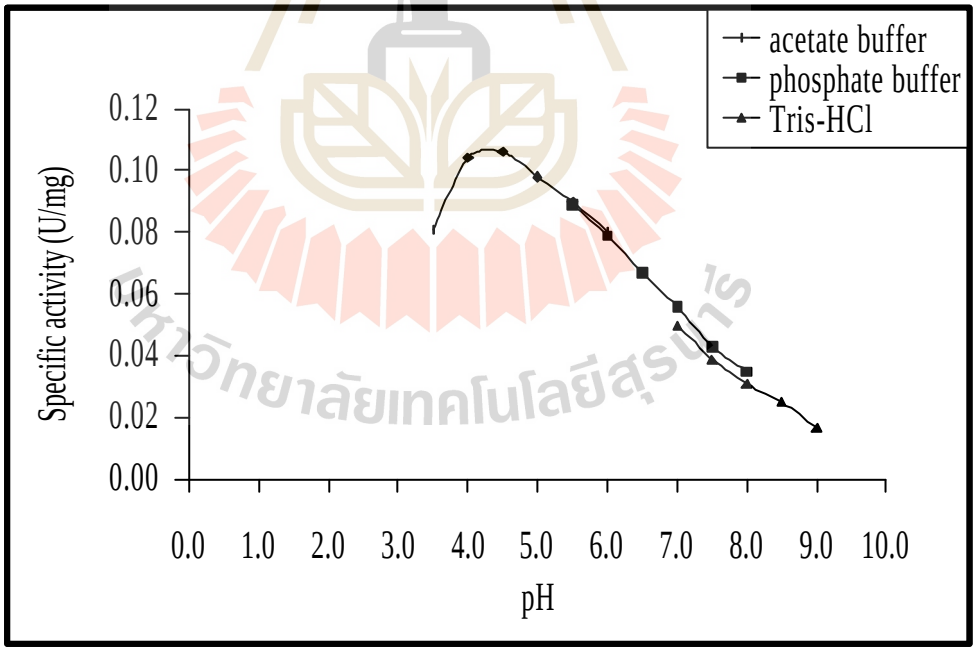
The optimum pH for the activity of the chitinase from seedlings and recombinant fusion protein was determined. The enzyme was incubated at 40°C for 10 min at various pH of 0.1 M acetate buffer pH 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0; 0.1 M phosphate buffer pH 5.5, 6.0, 6.5, 7.0, and 7.5; and 0.1 M Tris-HCl pH 7.0, 7.5, 8.0, 8.5, and 9.0. The chitinase isolated from seedlings and recombinant fusion protein both exhibited maximal activity at pH 4.5 as shown in Figure 3.31.

3.11 Effect of temperature on chitinase activity

The optimum temperature for the activity of chitinase from seedlings and recombinant fusion protein was determined. The enzyme was incubated in 0.1 M acetate buffer pH 4.5 for 10 min at various temperatures: 25, 30, 35, 40, 45, 50, 55, 60, 65, and 70°C. The enzyme from seedlings and recombinant fusion protein both exhibited maximal activity at 55°C (Figure 3.32). Therefore, the suitable condition for a 10 min chitinase assay should be at pH 4.5 and 55°C. The pH and temperature optimums indicated the some properties of chitinases from seedlings and the recombinant fusion protein were the same.

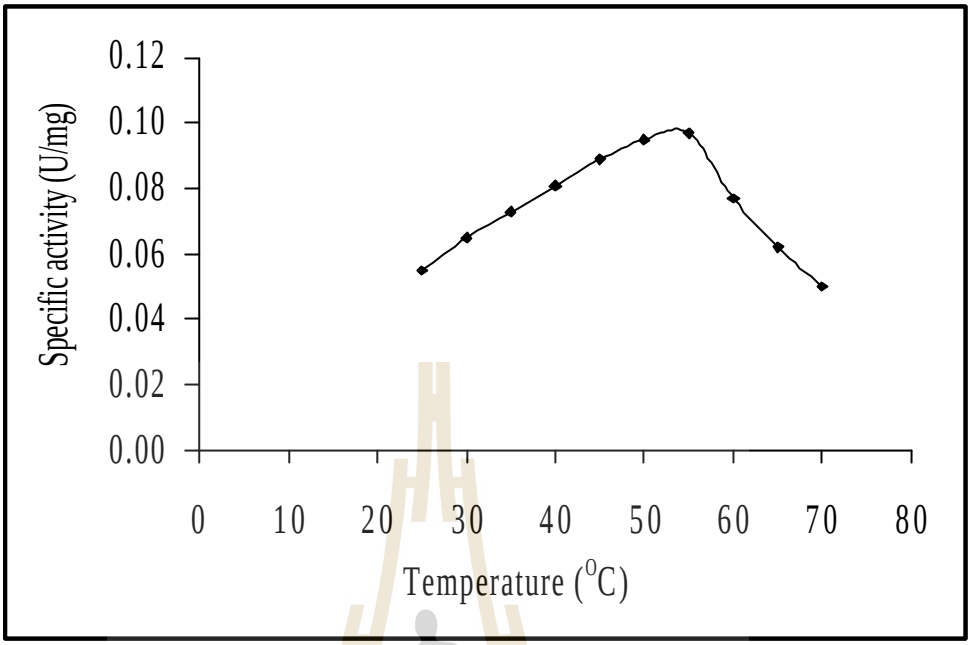


A

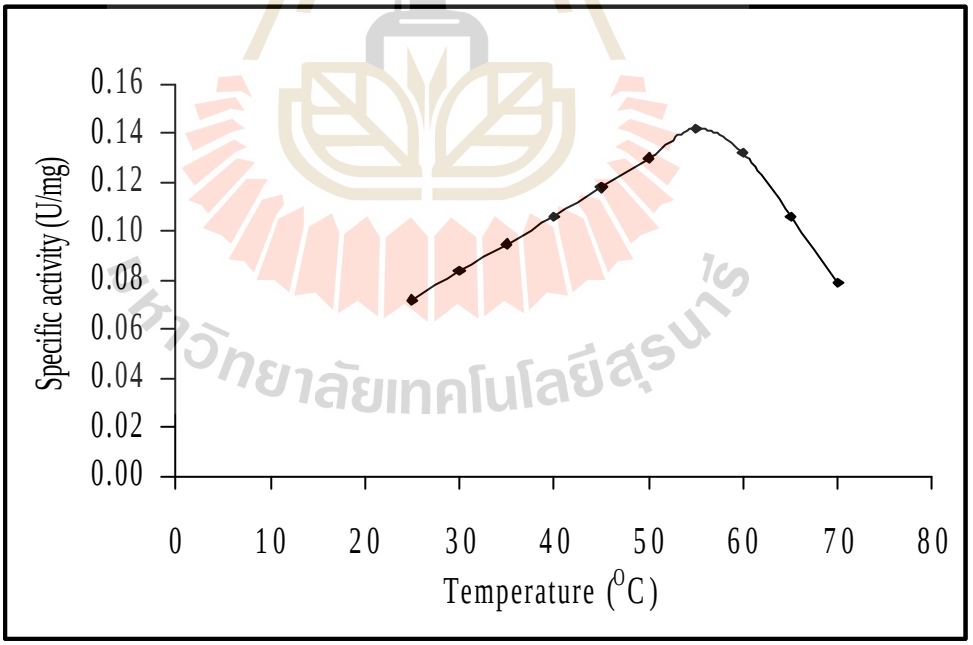


B

Figure 3.31 Effect of pH on purified leucaena chitinase activity. A shows the effect of pH on chitinase purified from *L. leucocephala* seedlings and B shows the effect of pH on chitinase activity of recombinant fusion protein.



A



B

Figure 3.32 Effect of temperature on purified leucaena chitinase activity. A shows the effect of temperature on chitinase purified from *L. leucocephala* seedlings and B shows the effect of temperature on the chitinase activity of recombinant fusion protein.

3.12 Substrate specificity test

The substrate specificity of purified chitinase was tested with 1% of each substrate, colloidal chitin, swollen chitin, purified chitin, glycol chitin, and chitosan, in 0.1 M acetate buffer, pH 4.5 for 10 min at 40◦C the same temperature for determined chitinase activity. The results show that using colloidal chitin as substrate gave the highest activity for both chitinase purified from *L. leucocephala* seedlings and recombinant protein of 0.075 and 0.098 units/mg, respectively (Table 3.8). Purified chitinases hydrolyzed chitosan slowly because chitinases hydrolyzed chitin and its derivatives, at *N*-acetylglucosamine residues to give chitooligosaccharides and chitosan residues are largely unacetylated. Chitinase is the best to hydrolyzed colloidal chitin because colloidal chitin is a somewhat different structure than purified chitin and swollen chitin, but for glycol chitin (6-O-hydroxyethyl-chitin) has hydroxyethyl group at O of C6 so which has effect to bind and hydrolyze at chitinase.

Table 3.8 Substrate specificity of chitinase activity from *L. leucocephala*

	Enzyme activity (U/mg)		Relative activity (%)	
Substrate	*L. leucocephala* seedlings	Recombinant Protein	*L. leucocephala* seedlings	Recombinant Protein
- Colloidal chitin	0.075	0.098	100	100
- Purified chitin	0.038	0.049	50.7	50.0
- Swollen chitin	0.025	0.030	33.3	30.6
- Glycol chitin	0.017	0.022	22.7	22.4
- Chitosan	0.014	0.018	18.7	18.4

3.13 Enzymatic assay

The purified chitinase was tested to see whether it was an endochitinase or exochitinase with *p*-nitrophenyl-*N*-acetyl-D-glucosaminide (pNP(GlcNAc)$_1$), *p*-nitrophenyl-*N*,*N*′-diacetyl-chitobiose (pNP(GlcNAc)$_2$), and *p*-nitrophenyl-*N*,*N*′,*N*″-triacetylchitotriose (pNP(GlcNAc)$_3$). The results showed highest activity on *p*-nitrophenyl-*N*,*N*′,*N*″-triacetylchitotriose as substrate of 0.023 units/ml and low activity on *p*-nitrophenyl-*N*-acetyl-D-glucosaminide, *p*-nitrophenyl-

N,*N*′-diacetylchitobiose (Table 3.9). From these results, chitinase from *L. leucocephala* is likely an endochitinase because it hydrolyze *p*-nitrophenyl-*N*,*N*′,*N*″-triacetylchitotriose to produce *N*,*N*′,*N*″-triacetylchitotriose that like hydrolyze chitin to produce *N*,*N*′,*N*″-triacetylchitotriose, while exochitinase (chitobiosidase) can hydrolyze *p*-nitrophenyl-*N*,*N*′-diacetylchitobiose to produce *N*,*N*′-diacetylchitobiose and *N*-acetyl-D-glucosaminidase hydrolyze *p*-nitrophenyl-*N*-acetyl-D-glucosaminide to produce *N*-acetyl-D-glucosamine (Roberts and Selitrennikoff 1988).

Table 3.9 Enzymatic assay of chitinase activity from *L. leucocephala*

Substrate	Enzyme activity (units/ml)	
	seedlings	Recombinant protein
- pNP$(GlcNAc)_1$	0.002	0.002
- pNP$(GlcNAc)_2$	0.002	0.003
- pNP$(GlcNAc)_3$	0.018	0.023

3.14 Kinetic constants

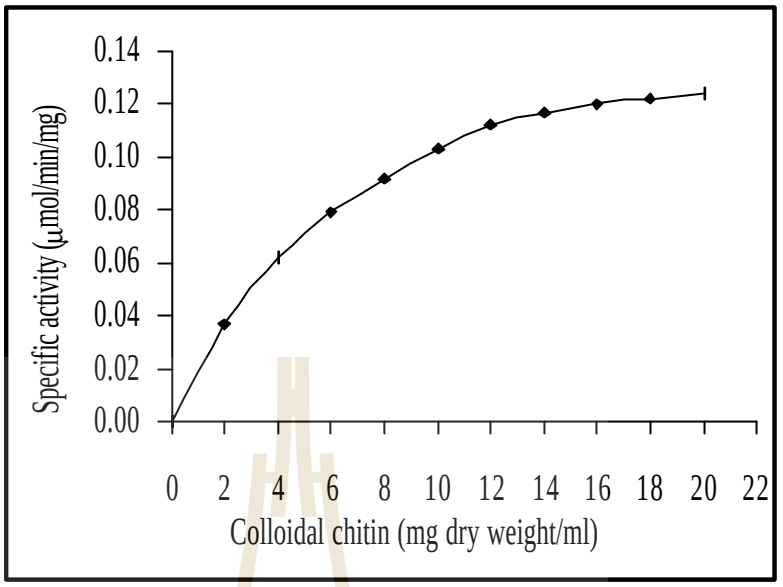
To study the kinetic properties, chitinase purified from recombinant fusion protein was assayed in 0.1 M sodium acetate buffer pH 4.5 at 55°C for 10 min with colloidal chitin, *p*-nitrophenyl-*N*,*N*′,*N*″-triacetylchitotriose, and *N*,*N*′,*N*″,*N*‴-triacetylchitotriose as substrate (Table 3.10). The results were plotted on Lineweaver-Burk plot to determine K_m and k_{cat} values of 7.60 mg dry weight chitin/ml and 8.28 min^{-1}, respectively for colloidal chitin. The k_{cat}/K_m was calculated to be 1.09 (mg dry weight chitin/ml)$^{-1}$ min^{-1} as was shown in Figure 3.33 and Table 3.13. For *p*-nitrophenyl-*N*,*N*′,*N*″-triacetylchitotriose as substrate (Table 3.11), K_m and k_{cat} were 48.78 μM and 35.42 min^{-1}, respectively. The kcat/Km was calculated to be 0.73 μM^{-1} min^{-1} as shown in Figure 3.34 and Table 3.13. For *N*,*N*′,*N*″,*N*‴-tetraacetylchitotetraose as substrate (Table 3.12), K_m and k_{cat} were 2.05 μM, 95.22 min^{-1}, respectively. The kcat/Km was calculated to be 46.44 μM^{-1} min^{-1} as shown in Figure 3.35 and Table 3.13.

Table 3.10 Kinetic properties of recombinant chitinase fusion protein was tested activity with colloidal chitin as substrate

[Colloidal chitin] (mg dry weight/ml)	Specific activity (μmol/min/mg)	1/[colloidal chitin] (mg dry weight/ml) $^{-1}$	1/ specific activity (μmol/min/mg)$^{-1}$
0	0	-	-
2	0.037	0.500	27.027
4	0.062	0.250	16.129
6	0.079	0.167	12.658
8	0.092	0.125	10.870
10	0.103	0.100	9.709
12	0.112	0.083	8.929
14	0.117	0.071	8.547
16	0.120	0.063	8.333
18	0.122	0.056	8.197
20	0.124	0.050	8.065

Table 3.11 Kinetic properties of recombinant chitinase fusion protein was tested activity with *p*-nitrophenyl *N,N′,N″*-triacetylchitotriose as substrate

[pNP$(GlcNAc)_3$] (μM)	Specific activity (μmol/min/mg)	1/ [pNP$(GlcNAc)_3$] (μM) $^{-1}$	1/specific activity (μmol/min/mg)$^{-1}$
0	0	-	-
20	0.225	0.0500	4.441
60	0.409	0.0167	2.443
100	0.512	0.0100	1.954
150	0.596	0.0067	1.679
200	0.637	0.0050	1.571
250	0.655	0.0040	1.527
300	0.665	0.0033	1.503
350	0.675	0.0029	1.480
400	0.686	0.0025	1.458
450	0.696	0.0022	1.437



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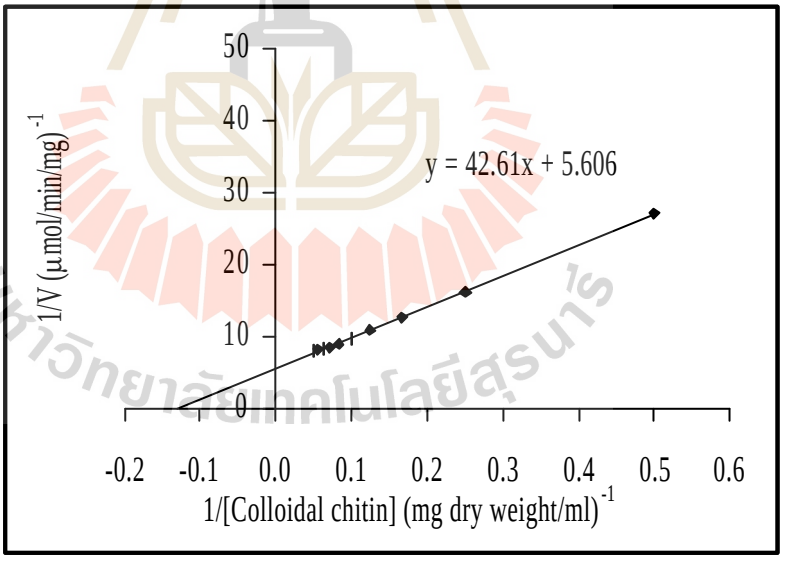
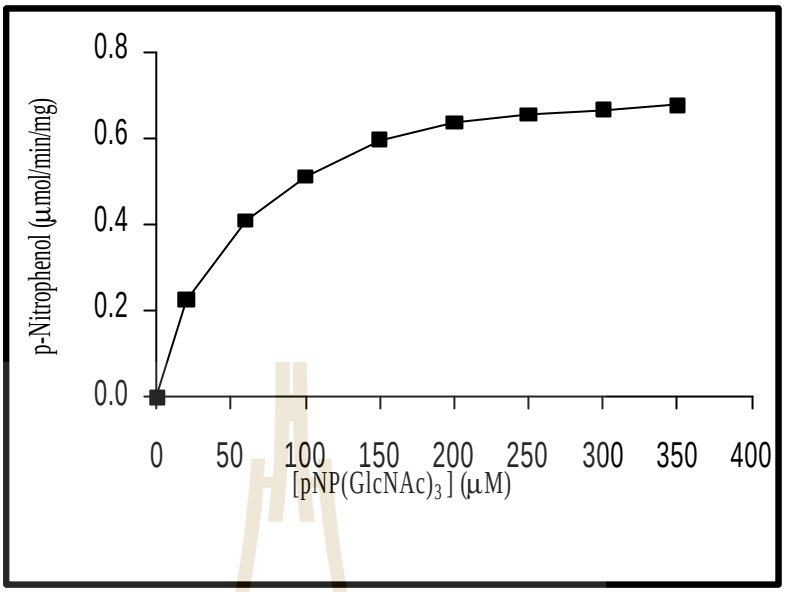


B

Figure 3.33 Kinetic analysis of purified chitinase from recombinant protein activity with colloidal chitin as substrate. A is a Michaelis-Menten plot of chitinase hydrolysis of colloidal chitin. B is a Lineweaver-Burk plot of chitinase hydrolysis of colloidal chitin.



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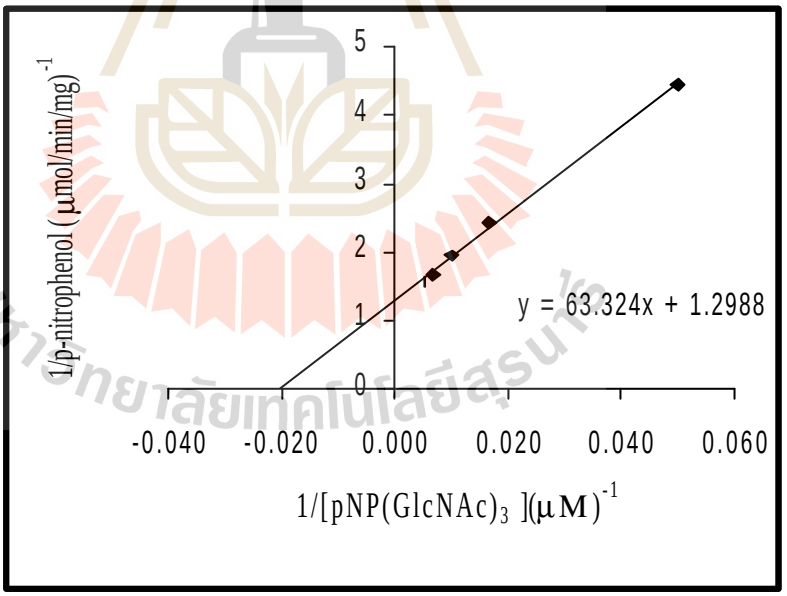
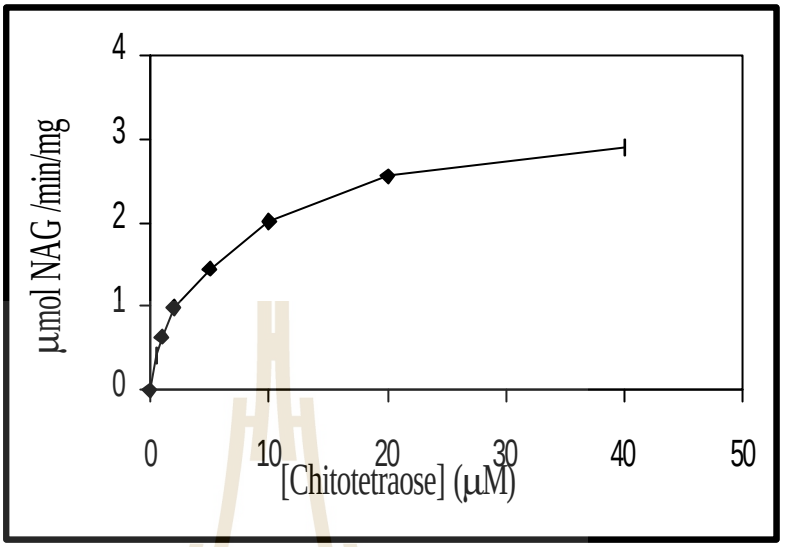
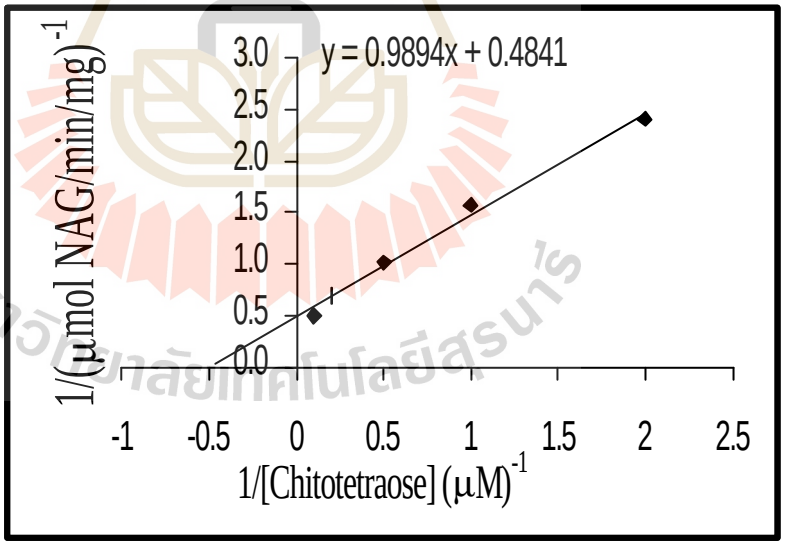


B

Figure 3.34 Kinetic analysis of purified chitinase from recombinant protein activity with pNP $(GlcNAc)_3$ as substrate. A is a Michaelis-Menten plot of chitinase hydrolysis of pNP$(GlcNAc)_3$. B is a Lineweaver-Burk plot of chitinase release of pNP from pNP-$(GlcNAc)_3$.



A



B

Figure 3.35 Kinetic analysis of purified chitinase from recombinant protein activity with chitotetraose as substrate. A is a Michaelis-Menten plot of chitinase hydrolysis of chitotetraose. B is a Lineweaver-Burk plot of chitinase hydrolysis of chitotetraose.

Table 3.12 Kinetic properties of recombinant chitinase fusion protein was tested activity *N,N′,N″,N‴*-tetraacetylchitotetraose as substrate

[Chitotetraose] (μM)	Specific activity (μmol/min/mg)	1/ [Chitotetraose] $(\mu M)^{-1}$	1/activity $(\mu mols/min/mg)^{-1}$
0	0	-	-
0.5	0.415	2.000	2.410
1.0	0.637	1.000	1.570
2.0	0.989	0.500	1.011
5.0	1.446	0.200	0.691
10.0	2.009	0.100	0.498
20.0	2.549	0.050	0.392
40.0	2.888	0.025	0.346

Table 3.13 Kinetic properties of recombinant chitinase fusion protein

Substrate	K_m	V_{max} (μmol/min/mg)	k_{cat} (min^{-1})	k_{cat}/K_m
- Colloidal chitin	7.60 mg dry weight chitin/ml	0.18	8.28	1.09 (mg dry weight chitin/ml)$^{-1}$ min^{-1}
- pNP$(GlcNAc)_3$	48.78 μM	0.77	35.42	7.26 x 10^5 M^{-1} min^{-1}
-$(GlcNAc)_4$	2.05 μM	2.07	95.22	46.44 x 10^6 M^{-1} min^{-1}

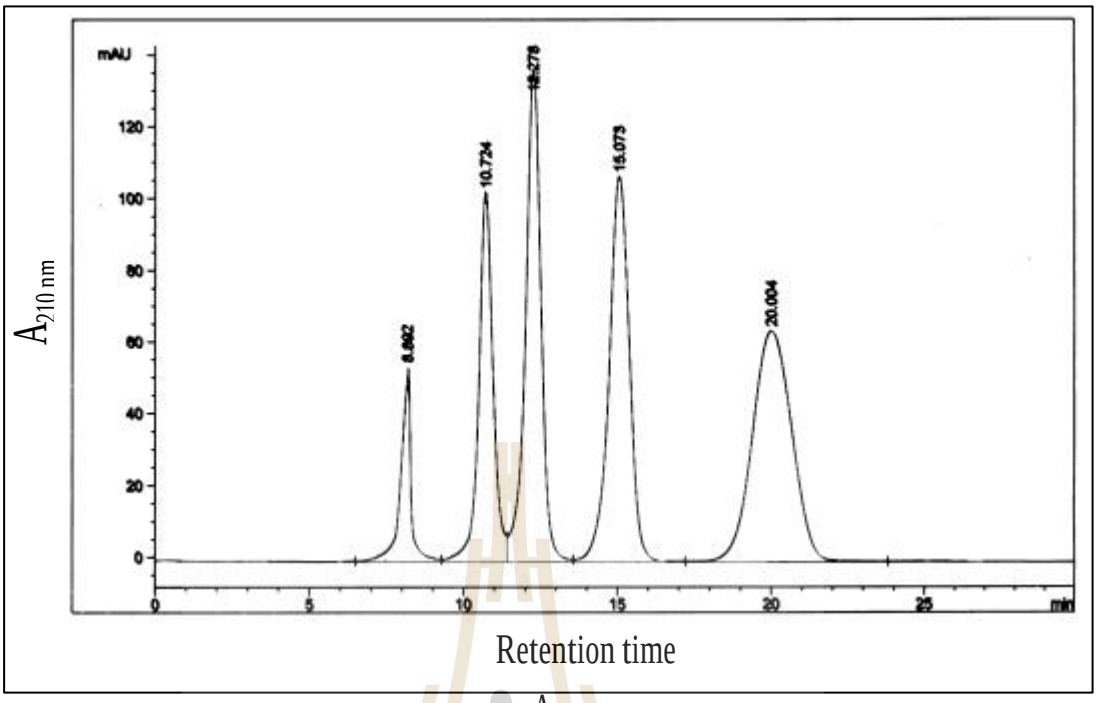
The K_m value of recombinant chitinase fusion protein with *N,N′,N″,N‴*-tetraacetyl-chitotetraose was lower than *p*-nitrophenyl-*N,N′,N″*-triacetylchitotriose. This results indicated purified leucaena chitinase binds to *N,N′,N″,N‴*-tetraacetylchitotetraose better than *p*-nitrophenyl-*N,N′,N″*-triacetylchitotriose. The V_{max} value for *N,N′,N″,N‴*-tetraacetyl-chitotetraose was higher than p-nitrophenyl-*N,N′,N″*-triacetylchitotriose and colloidal chitin, which indicated the chitinase hydrolyzed chitotetraose fastest. This may be because the chitinase can hydrolyze chitotetraose to produce chitotriose and *N*-acetyl-D-glucosamine, and hydrolyze chitotriose to produce chitobiose and *N*-acetyl-D-glucosamine but *p*-nitrophenyl-*N,N′,N″*-triacetylchitotriose can detect only *p*-nitrophenol which was produced. Colloidal chitin had

polyoligosaccharide and mixed form of insoluble and soluble substrate so chitinase hydrolyzed colloidal chitin to produce soluble *N*-acetyl-D-glucosamine slower than chitotetraose and p-nitrophenyl-*N*,*N*′,*N*″-triacetylchitotriose.

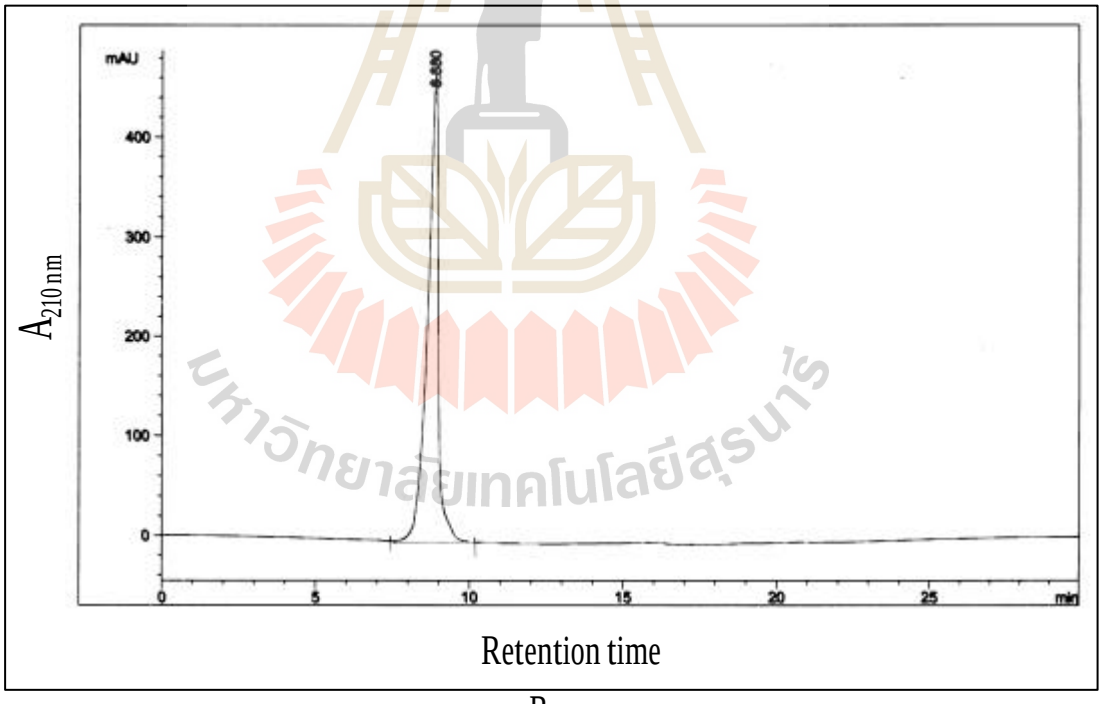
K_m value of recombinant chitinase fusion protein with *N*,*N*′,*N*″,*N*‴-tetraacetyl-chitotetraose was slightly lower than that published for the purified chitinase from barley (*Hordeum vulgare* L.) of 3 μM and k_{cat} value was higher than that of barley, 35 min^{-1}. K_m value of purified leucaena chitinase with colloidal chitin was slightly lower than that purified chitinase from tomato (*Lycopersicon esculentum*) of 10.46 mg dry weight chitin/ml. Overall, this shows the values obtained were similar to those from other plant chitinases.

3.15 Analysis of product reaction using HPLC

To analyze the cleavage specificities of the recombinant chitinase, colloidal chitin in 0.1M acetate buffer pH 4.5 was used as substrate at 40°C for 0 min, 30 min, 1 h, 2 h, 4 h, 8h, 16h, and 24 h. The hydrolysis products obtained from various times of incubation were separated by HPLC on a CARBOSep CHO-411 oligosaccharide column. The primary mode of separation of higher oligomers on this column is gel filtration, which separated according to size differences so the large molecules leave the column first followed by the smaller molecules. Chitin oligomers were quantified by UV absorbance at 210 nm. The oligosaccharide products obtained at 30 min that could be identified on this column were 12.81% *N*-acetyl-D-glucosamine, 53.28% *N*,*N*′-diacetyl-chitobiose, 27.41% *N*,*N*′,*N*″-triacetylchitotriose, and 6.50% *N*,*N*′,*N*″,*N*‴-tetraacetylchitotetraose (Figure 3.36). From the results, *N*,*N*′-diacetylchitobiose and *N*,*N*′,*N*″-triacetylchitotriose were predominant after short incubation times. After 24 h, the products obtained were 25.77% *N*-acetyl-D-glucosamine, 62.84% *N*,*N*′-diacetylchitobiose, 11.24% *N*,*N*′,*N*″-triacetylchitotriose, 0.15% *N*,*N*′,*N*″,*N*‴-tetraacetylchitotetraose, *N*,*N*′-diacetylchitobiose increased in quantity and represented 62.84% of the hydrolysis products after incubation for 24 h. From these results, *N*-acetyl-D-glucosamine and *N*,*N*′-diacetylchitobiose were predominant after long incubation times may be because chitinase hydrolyzed chitotetraose to chitobiose and hydrolyzed chitotriose to chitobiose and *N*-acetyl-D-glucosamine. These results are like those obtained with the class I chitinase from tobacco, *Nicotiana tabacum* cv. Samsun (NN) as shown in Figure 3.37 (Brunner *et al*., 1998) because class I chitinases were most active on chitin and chitin oligomers as substrate and they are both these are class I chitinases from plants.

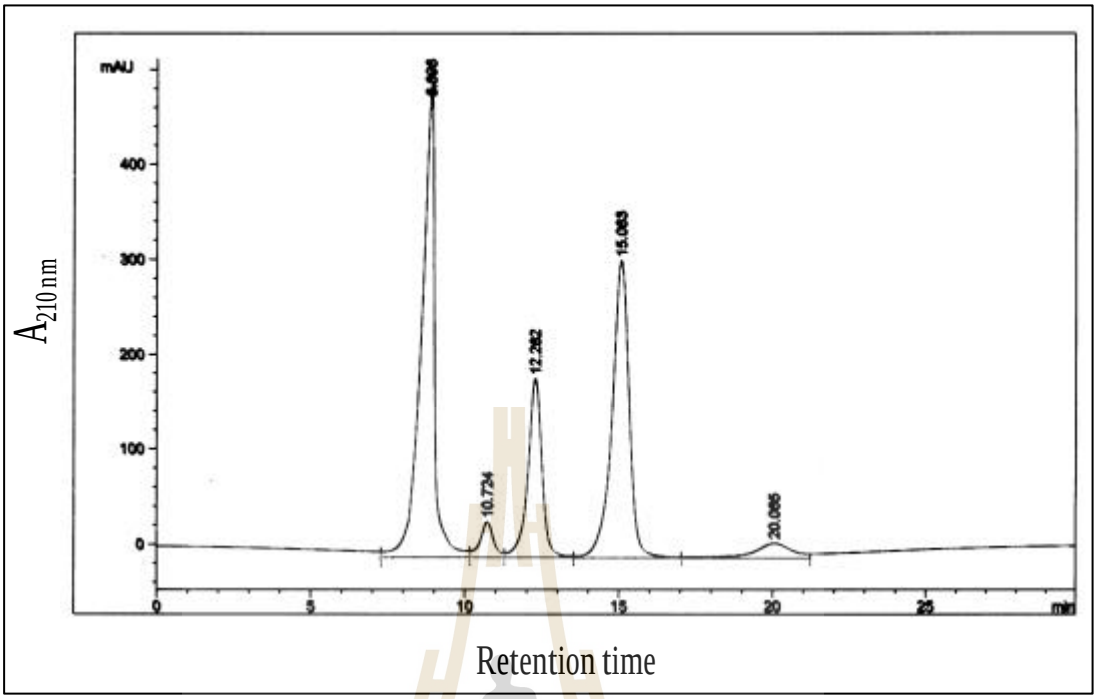


A

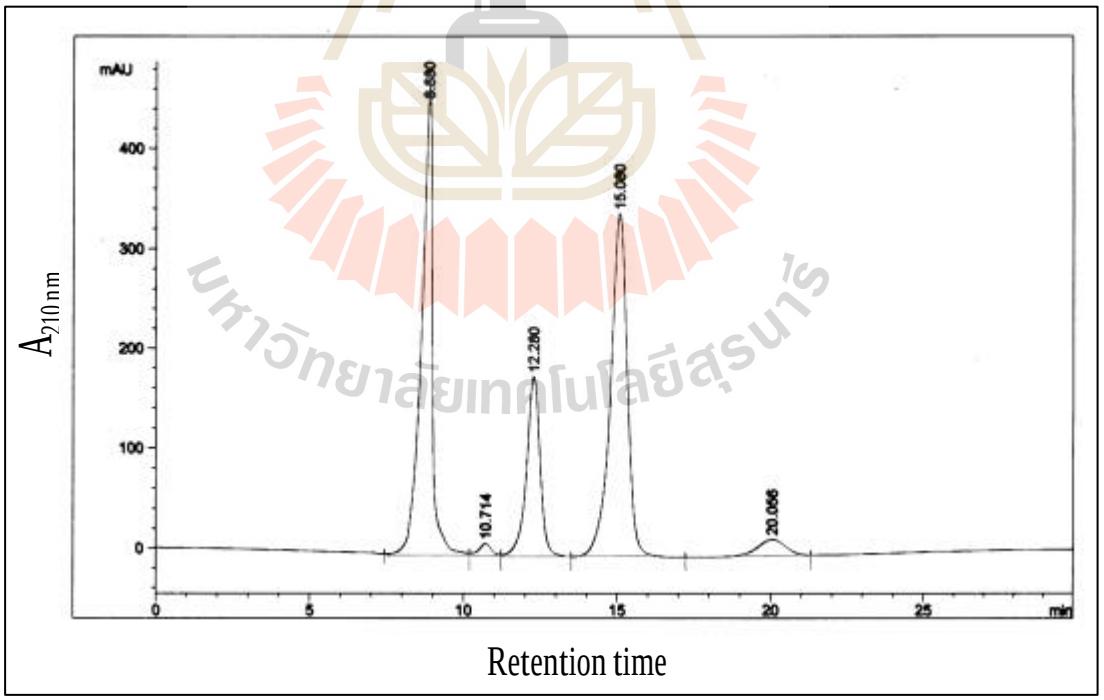


B

Figure 3.36 HPLC analysis of hydrolysis products released from colloidal chitin by leucaena chitinase. A is the Chromatograph of a standard of 20 μl (0.1 mg/ml) chitotetraose, chitotriose, chitobiose, and *N*-acetyl-D-glucosamine at retention times of 10.7, 12.3, 15.1, and 20.0 min, respectively. B, C, D, E, F and G are products released at incubation time 0 min, 30 min, 2 h, 5 h, 12 h, and 24 h respectively.

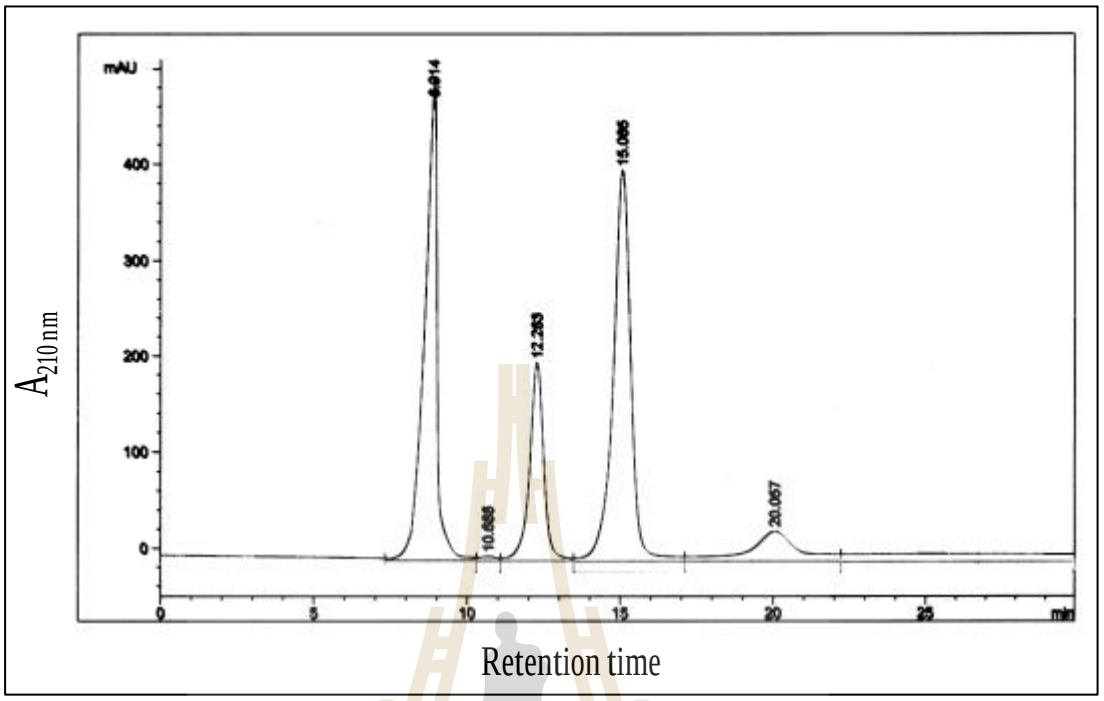


C



D

Figure 3.36 (continued)



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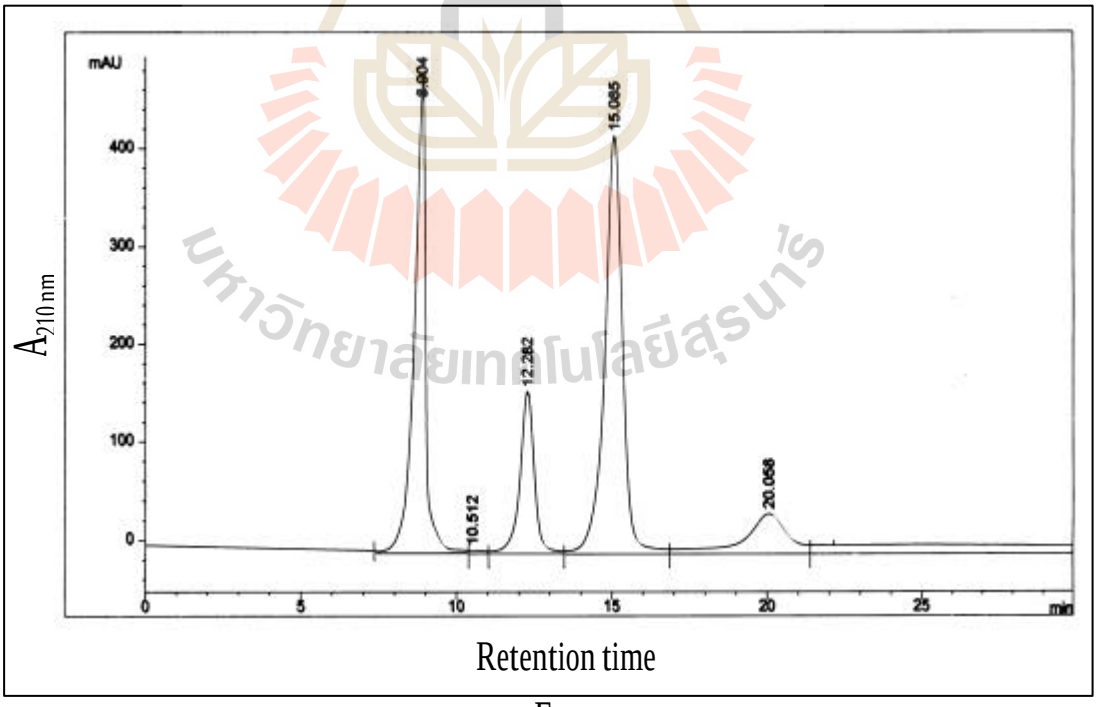
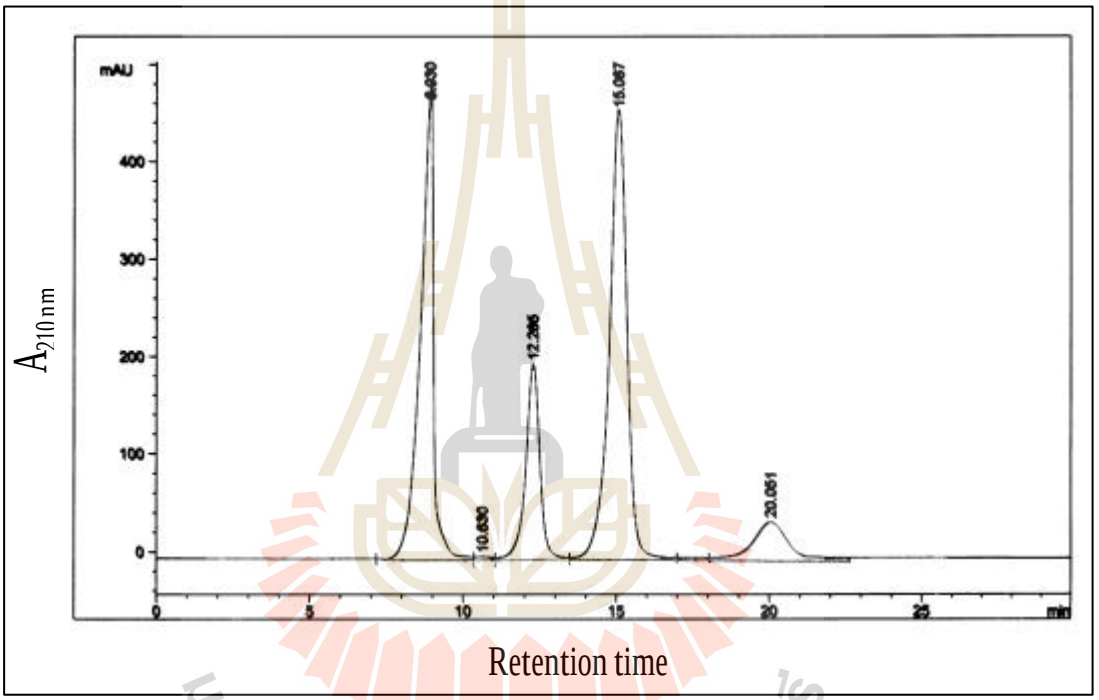


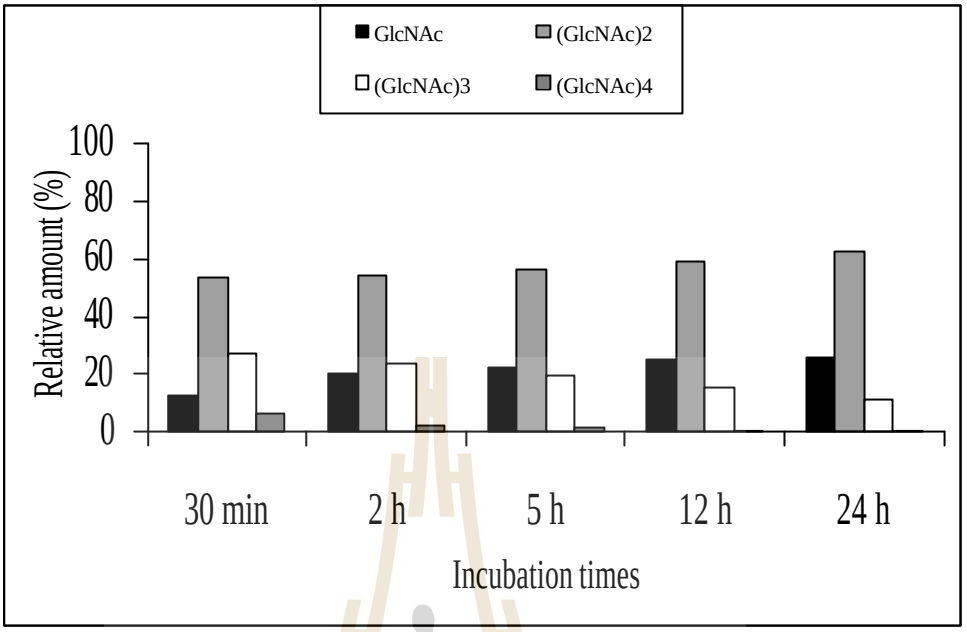
F

Figure 3.36 (continued)

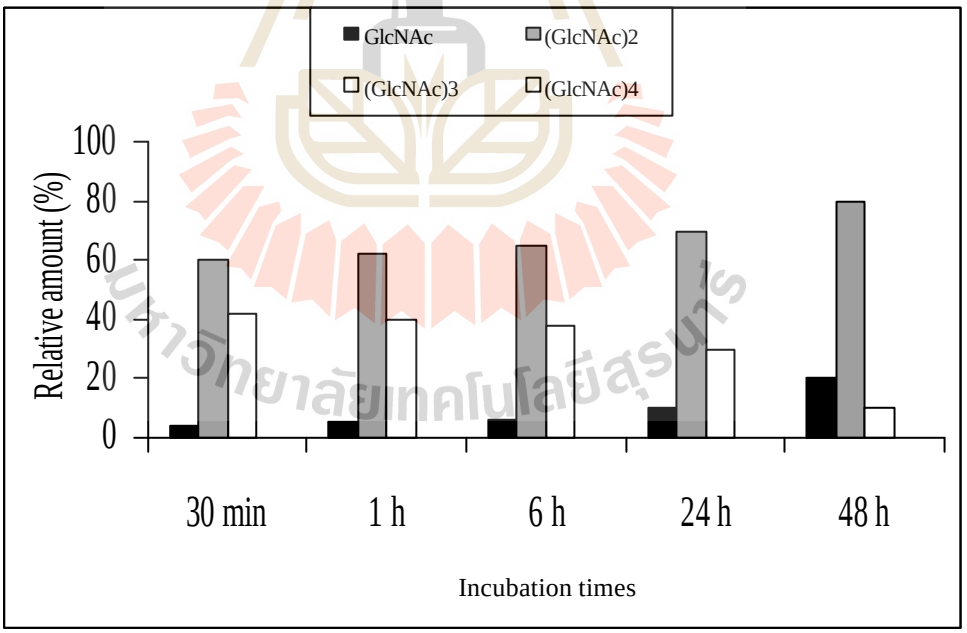


G

Figure 3.36 (continued)



A



B

Figure 3.37 The comparison of hydrolysis products released from colloidal chitin between leucaena chitinase and tobacco class I chitinase. A shows the products from leucaena chitinase and B shows the products from tobacco class I chitinase (Brunner *et al*., 1998).

3.16 Antifungal activity

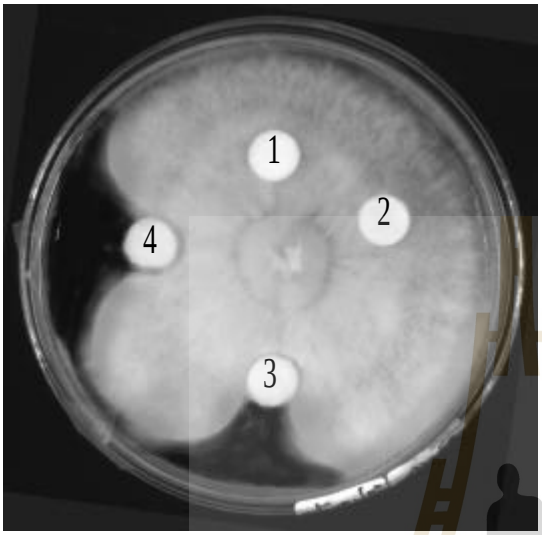
To study antifungal activity, purified recombinant chitinase at concentrations of 0.5, 1, and 2 μg/disc, was tested in a disc inhibition assay with 14 fungal strains. Most of fungi were inhibited by recombinant protein as seen in Figure 3.38. Purified recombinant chitinase inhibit *Collectotrichum* sp. 1 and 2, and *Pestalestiopsis* sp. 1 at a concentration of 0.5 μg per disc (Figure 3.38 D, E, and J); *Anthanose collectotrichum*, *Fusarium* sp. 1, *Fusarium monoliforme*, *Fusarium* oxysporum, *Pestalestiopsis* sp. 2, 3, and 4 (Figure 3.38 A, G, H, I, K, L, and M) at a concentraion of 1 μg per disc; *Cercospora* sp., *Drechslera* sp., and *Sclerotium* sp. (Figure 3.38 B, F, and N) at a concentraion of 2 μg per well, whereas *Cladosporium* sp. seem could not inhibited at these levels (Figure 3.38 C). *Cladosporium* sp. may need a higher concentration of purified recombinant chitinase than the 2 μg level, or may not be inhibited by chitinase due to a lack of appropriate chitin substrate in its cell wall (Roberts and Selitrennikoff 1988). These results indicate the recombinant chitinase protein caused growth inhibition of several types of fungi, likely those which have chitin in the cell wall.

To comparison of inhibition with other plant chitinase, purified chitinase from *L. leucocephala* inhibited *Fusarium moni*liforme, *Fusarium oxysporum* at a concentration of 1.0 μg/disc which is better than chitinases from *Arabidopsis thaliana*, which inhibited at a concentration of 8 μg/disc and sorghum seeds at 5 μg/disc level, respectively (Huynh 1991; Krishnaveni *et al*., 1999). Purified chitinase from *L. leucocephala* inhibited *Sclerotium* sp. at concentration 2.0 μg which is better than, that reported for *Arabidopsis thaliana* which inhibited at concentration of 8 μg/disc (Huynh 1991).

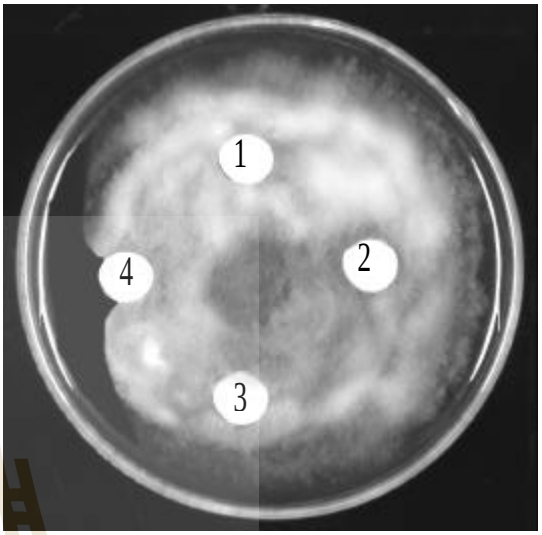
Some plant class I chitinases have also been reported to inhibit fungal growth, however, the concentrations required for inhibitor were in the range of 1 to 10 μg, and more than 50 μg for bacterial chitinases, and the range of 10-30 μg for fungal chitinases. The chitinase from *L. leucocephala* has been shown to exhibit antifungal activity by causing in vitro growth inhibition of 13 strains fungal, it would be interesting to prove the overexpression of this gene in transgenic plants such as rice and grape. It may substantiate its role in plant defense.

Chitinases against fungi containing chitin in cell wall which newly synthesized chitin fibres in the hyphal apex because apical growth in filamentous fungi, chitin and β-1,3-glucan fibres are synthesized simultaneously in the apex of the growing hypha. But in the mature cell wall distant from the apex, the polysaccharides are cross-linked to from mixed chitin-glucan fibres and may

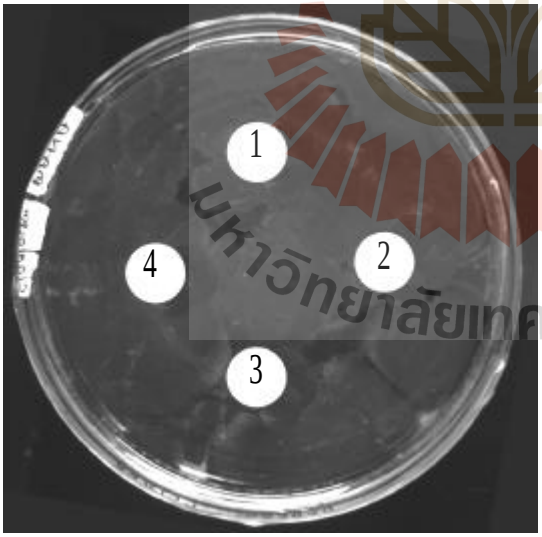
be overlaid by other polysaccharides and protein layers (Mikkelsen *et al*., 1992; Roberts and Selitrennikoff 1988).



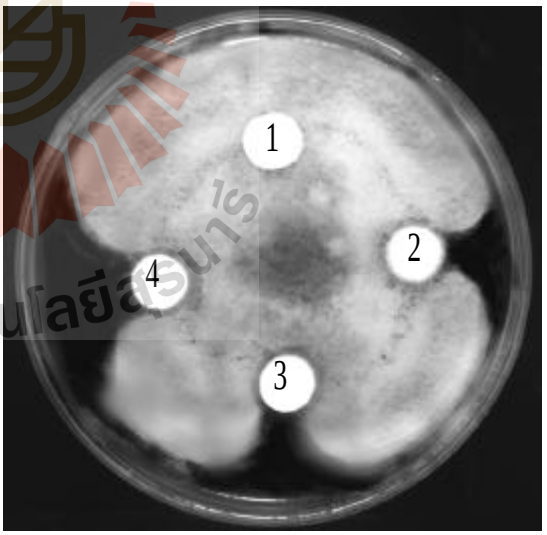
A: *Anthanose collectotrichum*



B: *Cerospora* sp.

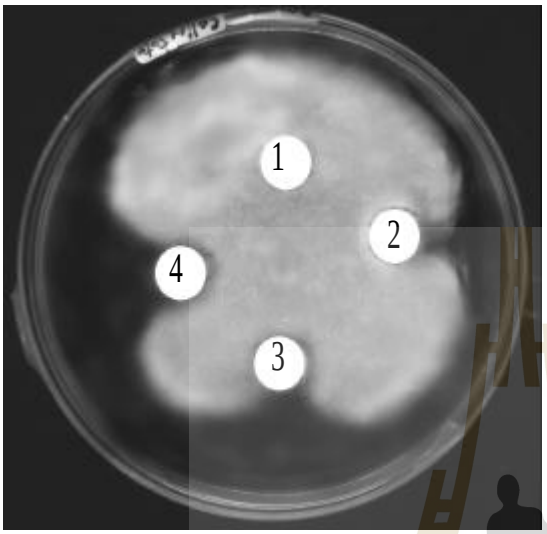


C: *Cladosporium* sp. 1

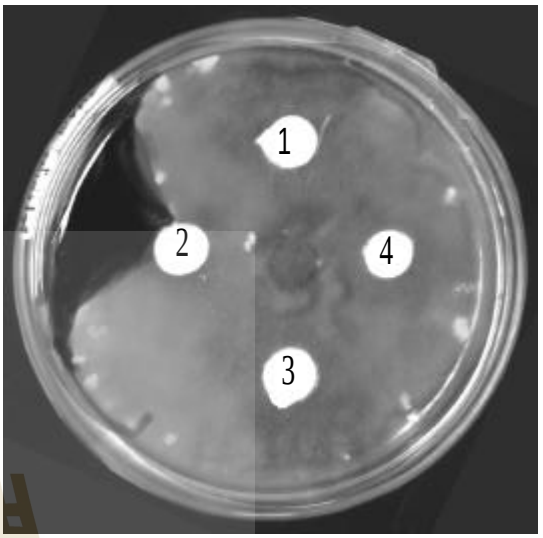


D: *Colletotrichum* sp. 1

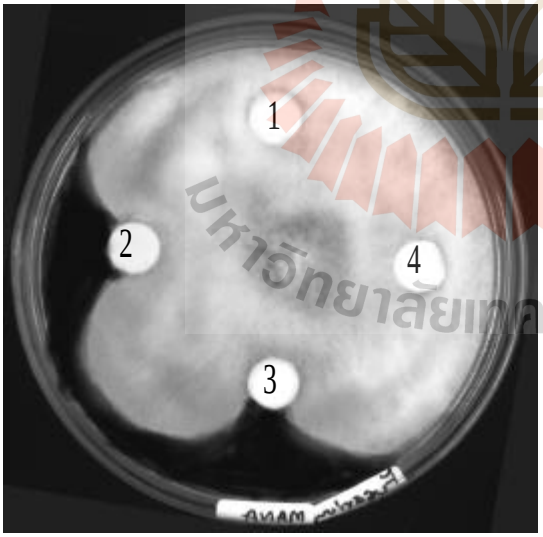
Figure 3.38 Antifungal activity of *L.leucocephala* chtitinase from recombinant protein against 14 strains of fungi. Number 1, 2, 3, and 4 are control (pET32a), 0.5, 1, 2 μg per well of purified recombinant protein, respectively.



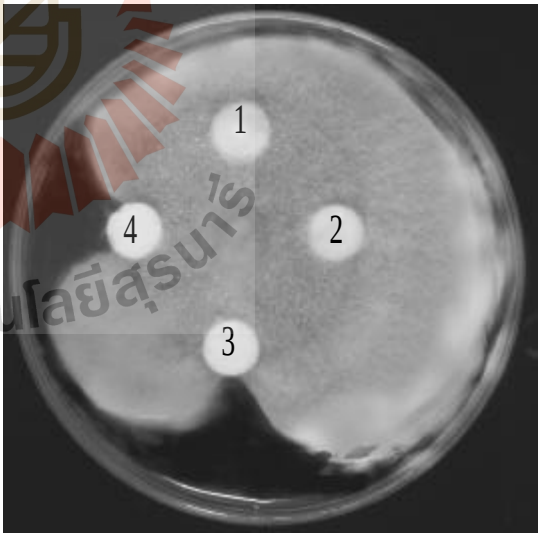
E: *Colletotrichum* sp. 2



F: *Drechslera* sp.

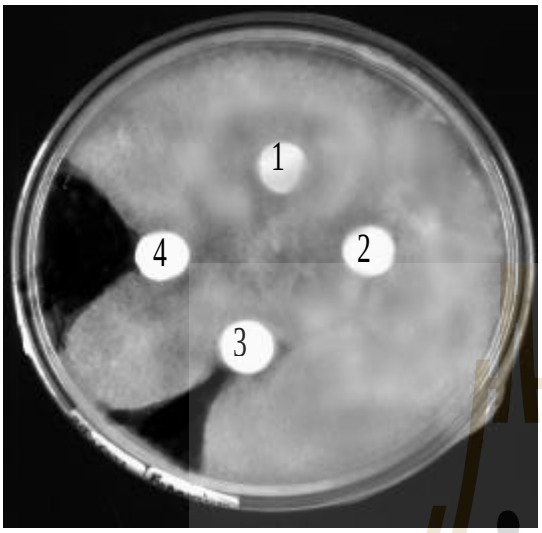


G: *Fusarium* sp. 1

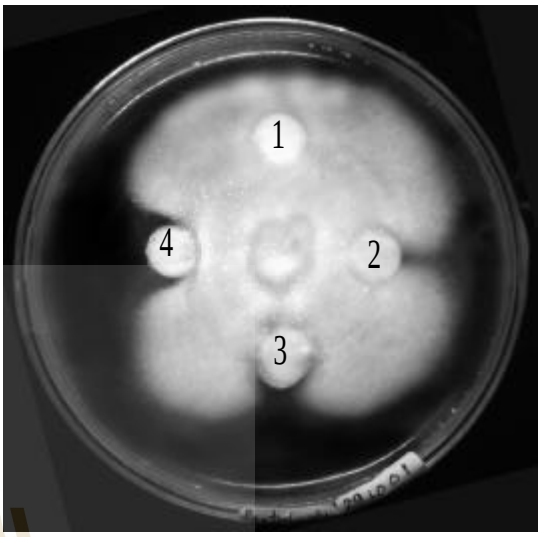


H: *Fusarium monoliforme*

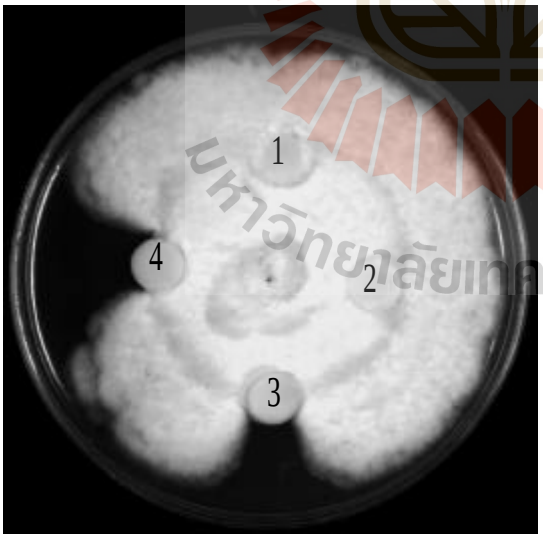
Figure 3.38 (continued)



I: *Fusarium oxysporum*



J: *Pestalestiopsis* sp. 1



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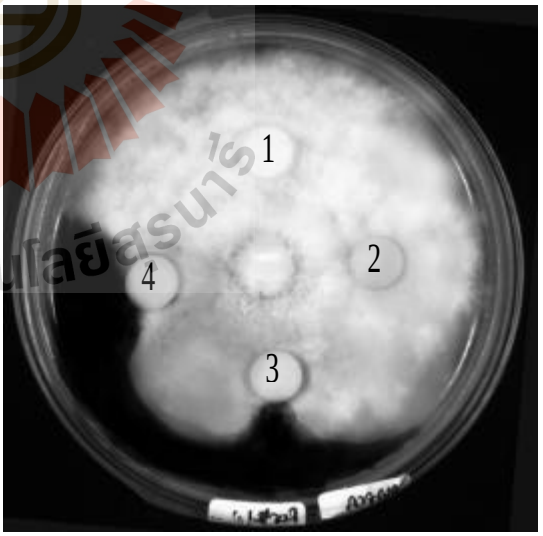
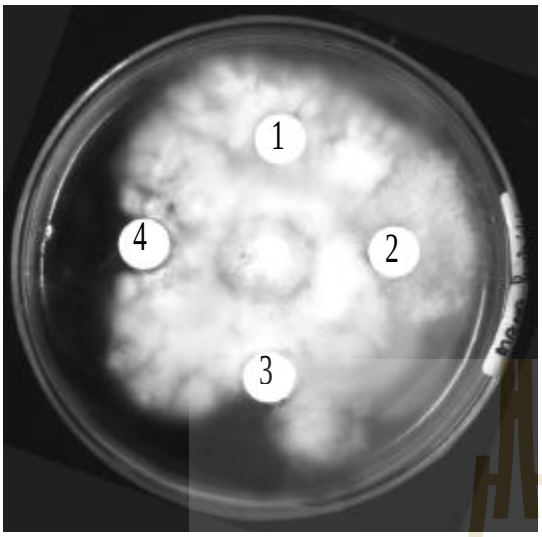


L: *Pestalestiopsis* sp. 3

Figure 3.38 (continued)



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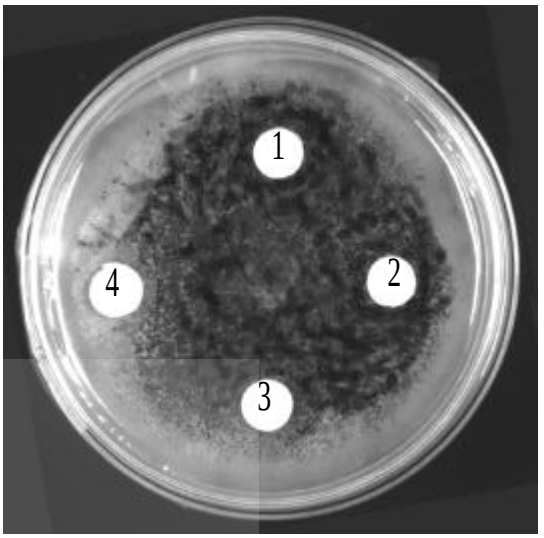

N: *Sclerotium* sp.

Figure 3.38 (continued)

3.17 General discussion

3.17.1 Chitin-binding domain

The chitin-binding activity of many plant proteins residues is found in so-called hevein domains, structural units that closely resemble hevein, the small chitin-binding latex protein from the rubber tree (*Hevea brasiliensis*), with respect to their amino acid sequence and three-dimensional structure (Van Damme *et al*., 1999). Hevein itself is a 43-amino acid polypeptide containing eight Cys residues that are all involved in disulfide bridges that stabilize the protein (*Waljuno et al.*, 1975). Hevein is considered a lectin because it has carbohydrate-binding activity. Evidence has accumulated that chitin-binding domains similar to hevein occur in various types of plant proteins (Van Parijs *et al*., 1991). Class I chtinases consist of an N-terminal hevein domain linked through a short, variable Gly/Pro-rich hinge domain to a catalytically active chitinase domain (Collinge *et al*., 1993; Beintema, 1994; Ponstein *et al*., 1994). Molecular cloning further revealed that several chitin-binding merolectins and hololectins are derived from chimeric precursors. For example, hevein is the final processing product of a large precursor consisting of a signal peptide, a 43-residue sequence corresponding to mature hevein, and a large (144-residue) C-terminal peptide (Broekaert *et al*., 1990). Leucaena chitinase is composed of a signal peptide,

a 43-residue sequence homologous to mature hevein, a Gly/Pro-rich linker, and a large (252-residue) C-terminal catalytic domain. So the CRD region in the leucaena chitinase may act to bind chitin like hevein.

Most of the amino acid residues of hevein and wheat germ agglutinin (WGA) that are involved in the binding of either *N*-acetylneuraminic acid or GlcNAc are conserved in *Sambucus nigra* hevein-like fruit protein (SN-HLPf) and correspond to 4 amino acid residues which occur in the small chitin-binding antimicrobial polypeptides from *Amarathus caudatus* and in one of the two hevein-like domains of UDA (Peumans *et al*., 1984; Beintema and Peumans, 1992; Martins *et al*., 1996). Chitin-binding proteins, conservation of these amino acid residues in WGA and hevein, strongly suggests that they play a similar role in the binding of GlcNAc and GlcNac-containing glycoproteins. SN-HLPf exhibits chitin-binding activity but poor antifungal activity, especially compared with the hevein-like protein from *P. nil* (Koo *et al*., 1998). These residues conserved in leucaena chitinase which exhibited 73% identity with hevein. So they would play role in the binding of the GlcNAc residues in chitin.

The results of the modeling studies strongly suggest that *Sambucus nigra* hevein-like fruit protein (SN-HLPf) exhibits the same overall fold as hevein and other chitin-binding lectins possing hevein-like domains. Moreover, the extreme conservation of the Cys residues located along the polypeptide chains, the conformational role of the resulting disulfide bonds in the stabilization of the three-dimensional structure of the chains. This is especially true for β-stretch consisting of two stands of antiparallel β-sheet interconnected by a short turn, in which most of the residues responsible for the specific binding of GlcNAc are located.

The function of chitin-binding domain in the chitinases is presumably to anchor the enzyme tightly onto the substrate, thereby facilitating the hydrolytic process (Blaxter, 1996). The role of the N-terminal Cys-rich domain of class I chitinases have been a matter of interest. The similarity of this domain to hevein, a polypeptide from the latex of the rubber tree, and to each of the four domains of the chitin-binding lectin WGA, first described by Lucas *et al*. (1985) for bean chitinase, has suggested a role in the affinity of chitinase for its substrate. It was found that hevein as well as UDA, a chitin-binding lectin built of two domains homologous to hevein, inhibit fungal growth (Broekaert *et al*., 1989; Van Parijs *et al*., 1991). This, and the lack of antifungal activity of tobacco class II chitinases, which lack of N-terminal Cys-rich domain might be responsible for antifungal activity of chitinase. However, the mechanism of inhibition was different for the

chitinases, which caused lysis of the hyphal tip, and hevein and UDA, which caused swelling of the hyphae (Broekaert *et al*., 1989). The spectrum of affected fungi was also different for two types of proteins. Iseli *et al*. (1993) investigated the role of the N-terminal Cys-rich, hevein domain, in catalytic activity, chitin-binding, and antifungal activity by comparing a class I chitinase and class II chitinase. They summarized as followed: class II chitinase failed to bind to the affinity matrix of regenerated chitin, demonstrating directly that the N-terminal domain acts as the CBD. The specific hydrolytic activity of the two enzymes toward colloidal chitin was very similar under normal assay conditions, showing that the basal catalytic activity is independent of the chitin-binding properties. So these results showed that the CBD in class I chitinase has a considerably higher affinity for chitin as a substrate than the catalytic domain of chitinases that lack the CBD as in class II chitinases. Both class I and class II chitinases had antifungal activity but class II chitinase was about three times lower than that of the class I chitinase. These results suggested that the catalytic domain of class I chitinase was sufficient to inhibit fungal growth, but that the CBD improved its antifungal activity, perhaps by attaching the enzyme to its substrate in the fungal cell wall. The binding of leucaena chitinase to chitin during purification and its high antifungal activity would thus suggest that the N-terminal CRD acts as a functional chitin-binding domain.

3.17.2 Catalytic properties and amino acids that act as catalytic acid–base

The major function of endochitinases is depolymerization of the insoluble polymeric substrate. Brameld and Goddard (1998) have examined the binding of a hexaGlcNAc substrate and to a family 19 barley chitinase. They found the hexaGlcNAc substrate binds with all sugars in a chair confromation, unlike the family 18 chitinase which causes substrate distortion. Tight binding of hexaGlcNAc substrate is achieved through well defined hydrogen bonds which are constant during the simulations. Figure 1.9 shows a schematic of these interactions and highlights residues that are conserved strongly in family 19 chitinases. The N-acetyl amide of sugar A donates a hydrogen bond to the side chain of Gln 162, and O3′ accepts a hydrogen bond from Trp 103. HO3′ and HO6′ of sugar B donate hydrogen bonds to the backbone carbonyls of Tyr 123 and Asn 124, respectively. In addition, the *N*-acetyl carbonyl forms a hydrogen bond with the charged side chain of Lys 165. The *N*-acetyl amide of sugar C donates a hydrogen bond to the backbone carbonyl of Ile 198, and the carbonyl of the *N*-acetyl group accepts a hydrogen bond

from the backbone amide of Ser 120. Asn 124 also forms a hydrogen bond to O3′ of sugar C. Two critical hydrogen-bonding interactions are observed for sugar D. The first is between Asn 199 and the *N*-acetyl carbonyl, which serves to constrain the *N*-acetyl geometry. The second hydrogen bond is transiently formed between the protonated Glu 67 and O5′, O6′ or O1′. Sugar residues E and F make the fewest specific contacts. HO6′ of sugar E donates a hydrogen bond to amide of Arg 215. Sugar F forms only one hydrogen bond between O6′ and the side chain of Thr 69. Hart *et al*. (1995) suggested tetraGlcNAc substrate binding causes the molecule to undergo a conformational change incompatible with maintenance of crystal contacts since crystals dissolved when tetraGlcNAc was added. The crystals could reform in the native conformation upon cleavage of tetraGlcNAc to disaccharide, which remains unbound. Since leucaena chitinase has identical amino acids at all of these interacting positions, except Trp 103 which is substituted with the similar polar aromatic amino acid tyrosine, it would be expected to bind chitin substrate in a similar manner.

Leucaena chitinase was compared amino acid sequence with mature hevein (Chrispeel and Raukhel, 1991), barley class II chitinase (Andersen *et al*., 1997), and chestnut class I chitinase (Gareia-Casado *et al*., 1998), revealed the similarity between the catalytic domains (Figure 3.39). The barley enzyme is the member of family 19 glycosyl hydrolases for which cystallographic data are available. On the basis of its x-ray structure and alignment of figure 3.39, a model was constructed for the catalytic domain of leucaena chitinase (residues 51-302, Figure 3.39). Barley class II chitinase has its catalytic acid at Glu 67 and its catalytic base at Glu 89, chestnut class I chitinase has catalytic acid at Glu 124 and catalytic base at Glu 146 which are the same positions in the multiple sequence alignment (bold alphabet). Based on these conserved positions in the sequence alignment then catalytic acid-base of leucaena chitinase are likely to be at Glu 118 and Glu 140, as shown in the figure 3.39.

Gareia-Casado *et al.* (1998) studied the structure-activity analysis using a class I enzyme from chestnut (*Castanea sativa* Mill.) seeds as their model (Collada *et al*., 1992; Allona *et al*., 1996). Site-directed mutagenesis was performed to identify residues involved in chitin hydrolysis. Glu 124 and Glu 146, then only carboxylic residues properly located into the active site cleft to participate in catalysis, were both mutated to Gln and Asp. These results suggest that Glu 124 function as the general acid catalyst whereas Glu 146 is likely to act as a general base. Other mutations involving three highly conserved active site residues, Gln 173, Thr 175, and Asn 254

(shown by asterisks in Figure 3.39), also impaired the chitinolytic activity of chestnut class I chitinase. These three mutagenesis positions for chitin-binding chitinase are the same amino acids at 2 of 3 residues as those that Brameld and Doddard (1998) said were important for chitin binding in barley which included H 66, T 69, W 103, S120, Y 123, N124, Q162, K165, N199, and R215 as shown in Figure 3.39. Mutations of three other residues, Gln173, Thr175, and Asn 254, impaired the chitinolytic activity of chestnut class I chitinase. The effects of these variants on the fungus *Trichoderma viride* revealed that catalysis is not necessary for antifungal activity. Similar to its homologous nonenzymatic polypeptides hevein and stinging nettle lectin, the *N*-terminal chitin-binding domain of chestnut appears to interfere itself with hyphal growth.

Figure 3.39 Comparison of the amino acid sequences of chitinase from mature hevein (43 aa), barley class II chitinase (243 aa), chestnut class I chitinase (297 aa), and leucaena chitinase (302 aa). Bolds are amino acids catalytic residues, asterisks are mutagenesis residues, and arrows show chitin-binding residues.

3.17.3 Assay of chitin hydrolysis

Brunner *et al*. (1998) showed that chitinases activities decreased with shorter oligomers as substrates but the rates of hydrolysis of chito-oligomers of given chain length were found to be strikingly variable. The half-life of an oligomer would vary considerably depending both on the type of chitinase it faced and on the length of the GlcNAc chain.

Chitinase recognizes only GlcNAc residues in chitin and chitosan. The hydrolysis rate by chitinase increases with increased degree of acetylation (Aiba 1994). GlcNAc can be determined by a color reaction with the *p*-dimetylaminobenzaldehyde, DMAB (Boller *et al*., 1983). If chitooligosaccharides are released rather than *N*-acetylglucosamine, they can be digested by *N*-acetyl-***b***-glucosaminidase to give *N*-acetyl-D-glucosamine. By comparing the amount of *N*-acetyl-D-glucosamine released in the presence or absence of addition *N*-acetyl-***b***-glucosaminidase, some idea of the relative activity of *N*-acetyl-***b***-glucosaminidase versus chitobiosidase or endochitinase can be obtained. However, Domard and Vasseur (1991) showed that the colorimetric method was not specific for quantification of *N*-acetyl-*D*-glucosamine. The assay is more sensitive to monomers, but it recognizes all soluble *N*-acetyl-*D*-glucosamine oligomers; therefore caution should be used when interpreting results of assays using this method.

3.17.4 The usage of leucaena chitinase

Leucaena chitinase was shown to have high antifungal activity and to rapidly hydrolyze chitin forms compared with other plant endochitinases. It may be a good candidate for the biological control of plant pathogenic fungi, and may also be useful in degradation of chitin-containing waste, such as shrimp and crab shells to produce shorter chitin and chitosan chains, oligosaccharides and GlcNAc. Many papers and reviews have discussed the advantages of using chitinases for plant protection because these enzymes are fungicidal, part of plant defense system, and nontoxic to plants, animals, and higher vertebrates. Transgenic expression in plant of chitinase genes has been shown to improve disease resistance in various crops, and patents have been issued on related methods (Lorito *et al*., 1998). A few reports concerning transgenic plants with elevated expression levels of chitinase have been published recently. Transgenic tobacco and rape plants containing a bean chitinase gene with a constitutive promoter have been shown to exhibit higher basal levels of chitinase and concomitant increased resistance to *Rhizoctonia solani*

when compared with control plants (Broglie *et al*., 1991). Oilseed rape (*Brassica napus* var. *oleifera*) containing a tomato chitinase gene with a constitutive promoter exhibited an increased tolerance to disease as compared with the nontransgenic parental plants (Grison *et al*., 1996). Japonica rice cultivars, Nipponbare and Koshihikari transformed with chitinase from rice (*Oryza sativa* L.) with a constitution promoter exhibited an 12-fold increased tolerance to rice blast pathogen (*Magnaporth grisea* race 007.0) over control (Nishizawa *et al*., 1999). Since leucaena chitinase displayed relatively high activity against several strains of phytopathogenic fungi, future studies may include expression of this chitinase in transgenic rice and other crops.

Chapter IV
Conclusions

Chitinase cDNA from *Leucaena leucocephala* were cloned, and the mRNA and derived protein sequences were determined. The full coding sequence was completed by Nested PCR, 3′ RACE, cDNA library screening, and 5′ RACE. Two closely related chitinases cDNA were composed of 1064 (clone 5/3.1) and 1075 (clone 4/3.4) base pairs of cDNA sequence, which translated to 323 and 326 amino acid proteins, respectively. The derived protein sequences of the two clones exhibit >95% sequence identity and code for 326 and 323 amino acid chitinase precursors. The deduced amino acid composition of the predicted mature form of leucaena chitinases was high in aspartic acid + asparagine , glycine, cysteine, and proline with 31, 41, 18, 23 for clone 5/3.1 and 33, 42, 17, 23 4/3.4 for clone 4/3.4, respectively. This amino acid composition closely resembles amino acid compositions of potato (*Solanum tuberosum*) and tobacco (*Nicotiana tabacum*) class I chitinases. The sequences were closely related to plants class I chitinases with 74% identity to that from kidney bean, *Phaseolus vulgaris* (AC: AAB41324). By homology they have a prepeptide, cysteine-rich (chitin-binding) domain, a glycine/proline-rich hinge linker, and a catalytic domain. Precursor proteins have predicted molecular weights of 34.39 and 34.91 kDa and pI of 7.87 and 7.55 for clone 5/3.1 and clone 4/3.4 respectively. The predicted pI of the predicted mature proteins of clone 5/3.1 and clone 4/3.4 were 7.15 and 7.53 and predicted molecular weights were 31.87 and 32.21 kDa.

Active chitinase without prepeptide (clone 4/3.4) from *L. leucocephala* was expressed in T7 expression systems in BL21 (DE3) and Origami (DE3) *E.coli* from the cloned cDNA using the expression vectors pET23d and pET32a, respectively. Optimum conditions for expression in *E.coli* were 15°C, 5 h at 0.3 mM concentration of inducing IPTG for pET23d, and 15°C, 12 h at 0.1 mM IPTG for pET32a. The expressed protein from pET23d in BL21 (DE3) had an apparent MW of approximately 32 kDa on SDS-PAGE, which was present in both insoluble inclusion bodies and the soluble fractions, but most was in inclusion bodies. A thioredoxin-chitinase fusion protein was expressed in redox-deficient Origami (DE3) *E.coli* from the cloned cDNA in pET32a. It had an apparent MW of approximately 46 kDa on SDS-PAGE, was mostly soluble and had

higher activity than chitinase expressed in redox-competent bacteria. Purified recombinant protein that was cleaved with enterokinase, was active and had an apparent MW of approximately 32 kDa on SDS-PAGE. The purification of cleaved recombinant chitinase obtained 5.6 fold purification and 55.69% yield, compared to crude *E. coli* extract.

Complete purification of chitinase from *L. leucocephala* seedlings was obtained in 3 steps, including extraction of crude enzyme, 70% ammonium sulfate precipitation, and affinity chromatography on chitin. The purified chitinase was obtained with 26.7 fold purification and 15.9% yield. Purification of chitinase from expression in *E. coli* using pET32a had highest specific activity of 0.1119 U/mg, 1.6 fold higher than chitinase purified from seedlings.

Characterization of purified chitinase from germinated seeds and purified cleaved recombinant chitinase both had molecular weights of about 32 kDa and pI around 7.5-7.6 which is close to the calculated molecular weight from the deduced amino acid sequence of the predicted mature protein of 32.21 kDa and the calculated pI of 7.53. The optimum pH of 4.5 and optimum temperature of 55°C in a 10 min assay, was the same for both the chitinase purified from seedlings and purified recombinant enzyme from expression in Origami (DE3) *E. coli* using the expression vector pET32a. Chitinase from seedlings and expression in Origami *E. coli* had higher activity on colloidal chitin (0.074 and 0.098 U/mg, respectively) compared to other chitin forms. Purified chitinase from both seedlings and from expression in Origami (DE3) *E. coli* had activity on *p*-nitrophenyl-*N*,*N*′,*N*′′-triacetylchitotriose of 0.018 and 0.023 U/mg, respectively, but almost zero in *p*-nitrophenyl-*N*,*N*′-diacetylchitobiose and *p*-nitrophenyl-*N*-acetyl-D-glucosamine. These results indicated purified chitinase from *L. leucocephala* acts like an endochitinase.

Kinetic studies of purified recombinant chitinase expressed in Origamin (DE3) *E. coli* determined a K_m and k_{cat} of 7.60 mg dry weight chitin/ml and 8.28 min^{-1} with colloidal chitin, 48.78 μM and 35.42 min^{-1} with *p*-nitrophenyl-*N*,*N*′,*N*′′-triacetylchitotriose, 2.05 μM and 95.22 min^{-1} with *N*,*N*′,*N*′′,*N*′′′-tetraacetylchitotetraose, respectively. Chitinase products specifies analyzed using HPLC obtained 12.8% *N*-acetyl-D-glucosamine, 53.3% *N*,*N*′-diacetylchitobiose, 27.4% *N*,*N*′,*N*′′-triacetylchitotriose, and 6.5% *N*,*N*′,*N*′′,*N*′′′-tetraacetylchitotetraose at an incubation time of 30 min. *N*,*N*′-diacetylchitobiose and *N*,*N*′,*N*′′-triacetylchitotriose were predominant, after short incubation times. After prolonged incubation times (to 24 h), the products obtained were 25.8% *N*-acetyl-D-glucosamine, 62.8% *N*,*N*′-diacetylchitobiose, 11.2%

N,*N*′,*N*′′-triacetylchitotriose, and 0.15% *N*,*N*′,*N*′′,*N*′′′-tetraacetylchitotetraose. These results were similar to those from class I chitinase from tobacco, *Nicotiana tabacum* cv. Samsun (NN).

Purified recombinant chitinase inhibited *Collectotrichum* sp. 1, and 2, and *Pestalestiopsis* sp. 1 at a concentration of 0.5 μg/disc; *Anthanose collectotrichum*, *Fusarium* sp. 1, *Fusarium monoliforme*, *Fusarium* oxysporum, *Pestalestiopsis* sp. 2, 3, and 4 at a concentraion of 1 μg per disc; *Cercospora* sp., *Drechslera* sp., and *Sclerotium* sp. at a concentraion of 2 μg per well, whereas *Cladosporium* sp. was not inhibited at these levels.

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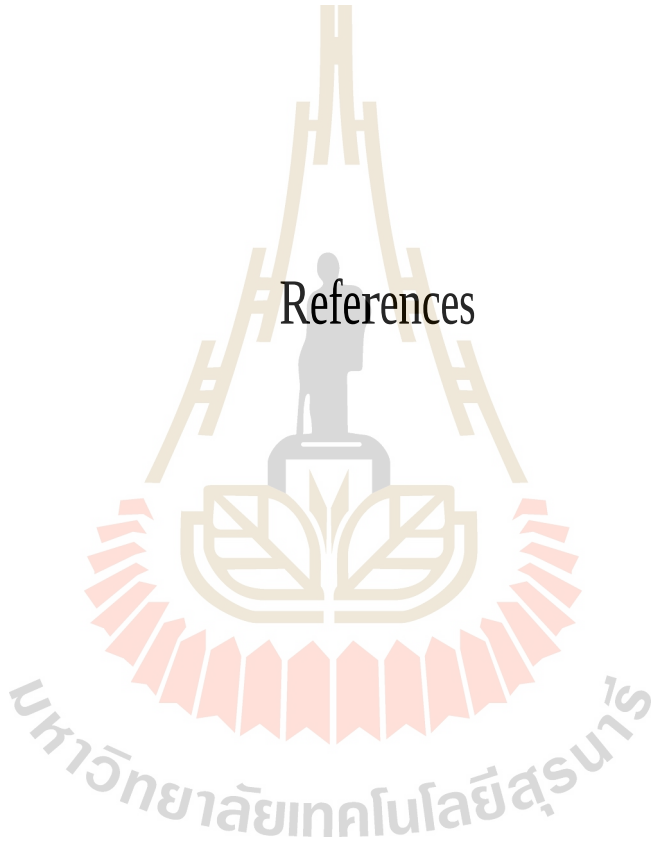

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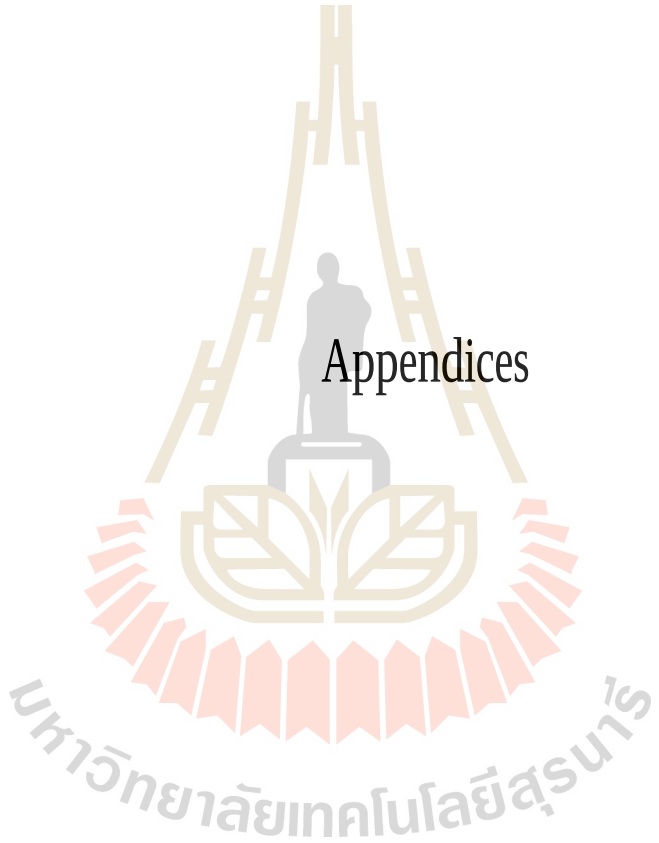
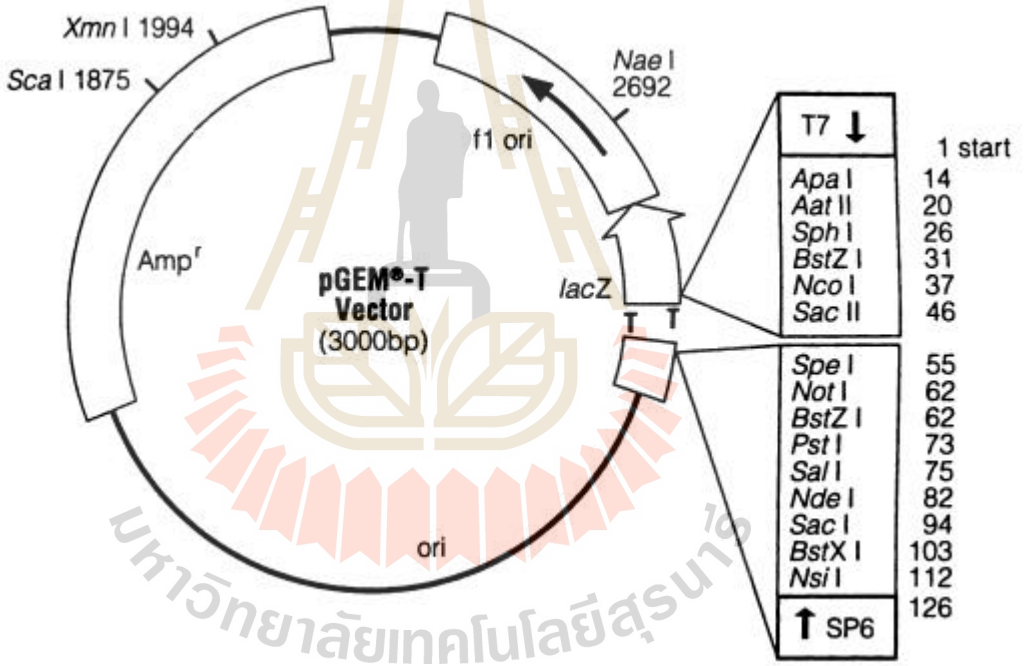
Appendices

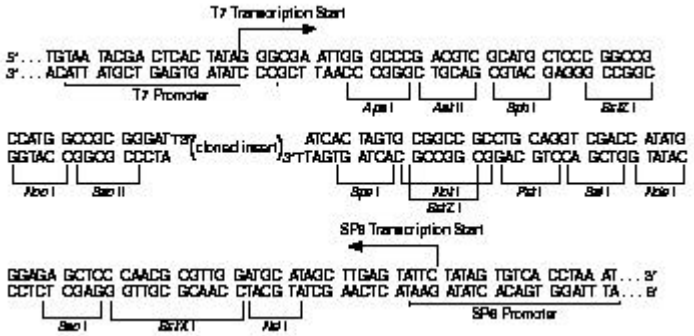


Appendix A

1. pGEM-T Vector

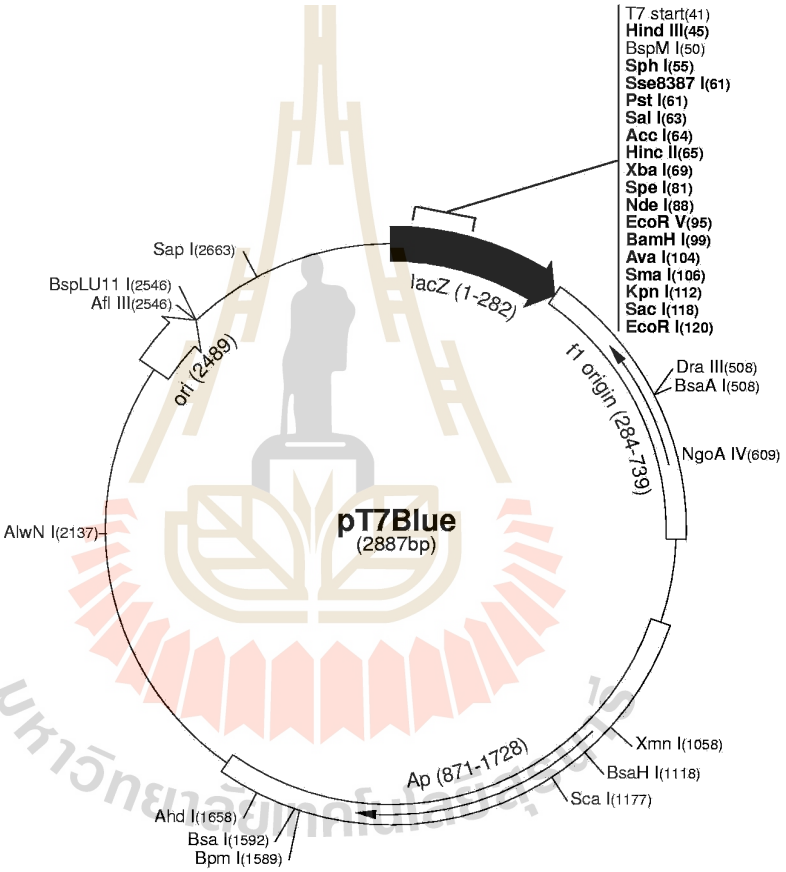
The pGEM-T vector is convenient system for the cloning of PCR products. The vector is prepared by cutting Promega's pGEM-5Zf(+) vector with *Eco*R V and adding a 3' terminal thymidine to the end.

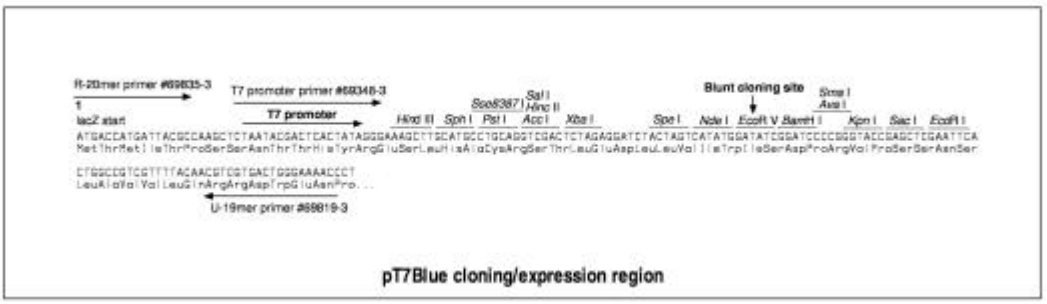




2. pT7Blue T-Vector

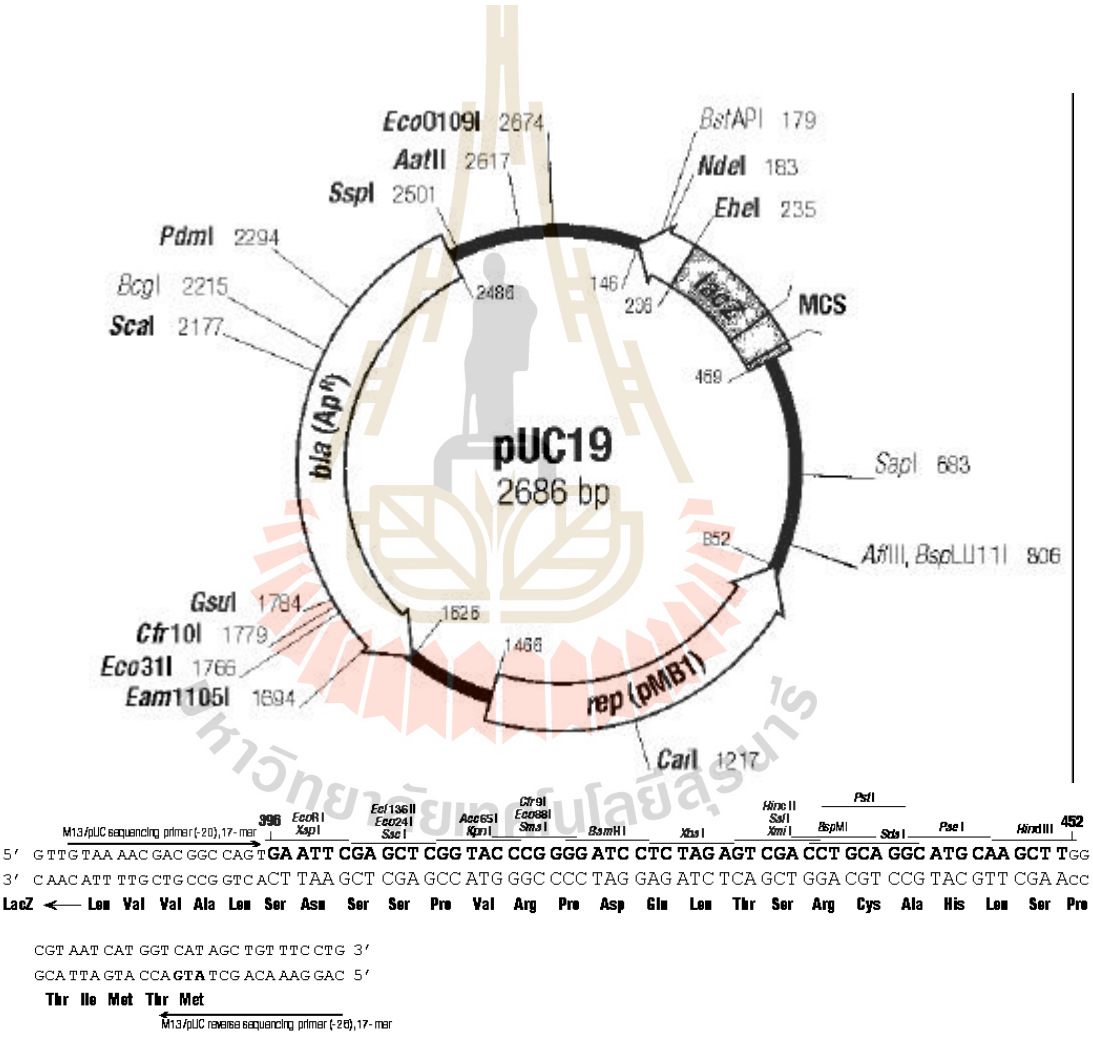
The pT7Blue T-vector contains the pUC19 backbone (including high-copy number origin of replication and *lac* sequence), a T7 promoter, f1 origin of replication, and modified multiple cloning region. The multiple cloning region contains an *Eco*R V site used for T-cloning flanked by an *Nde* I site, which allows insert to be conveniently subcloned into the *Nde* I site of many pET vectors.





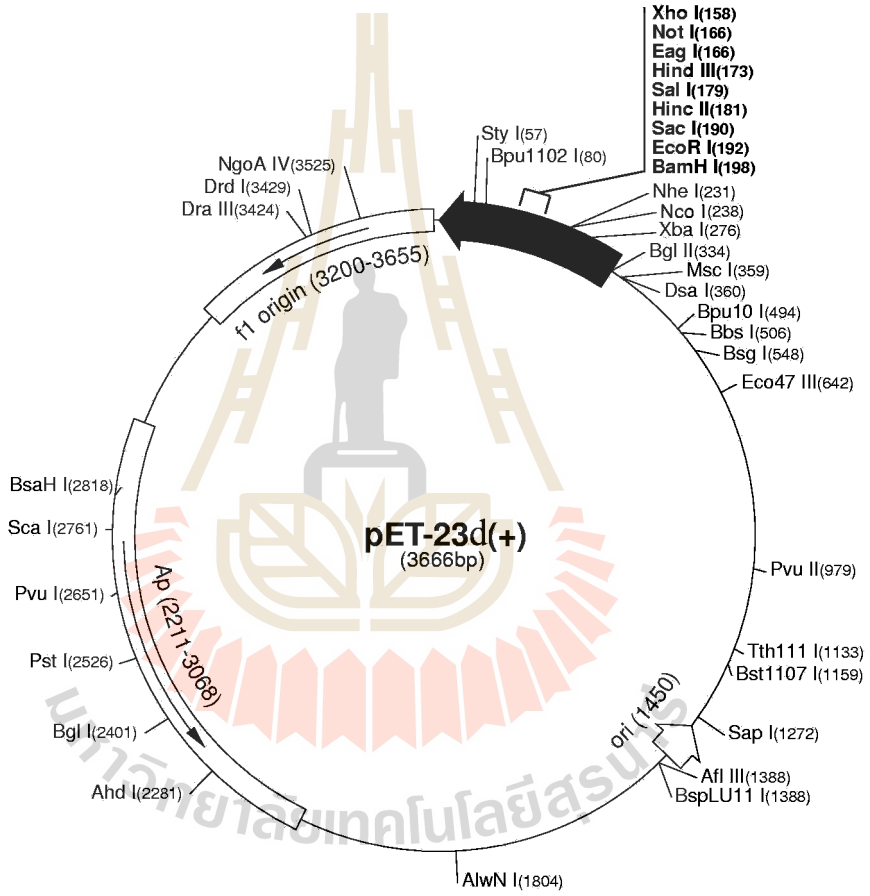
3. pUC19 Vector

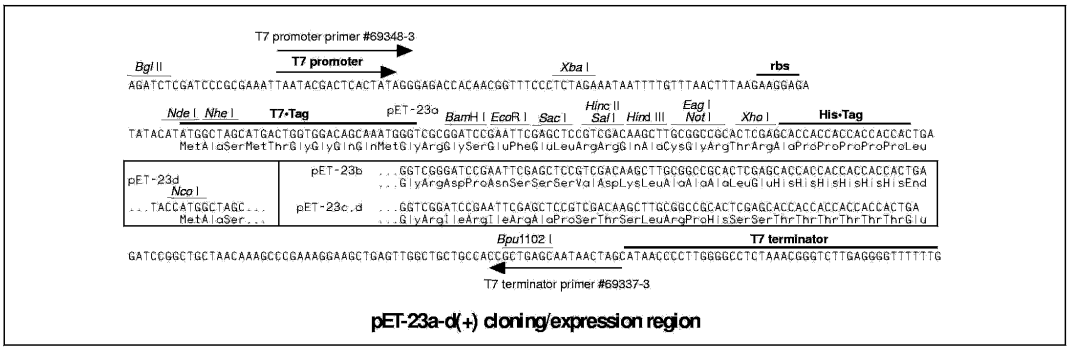
The pUC19 vector is a commonly used plasmid cloning vector in *E. coli*. The molecule is double-stranded circle, 2686 base pairs in length, and has a high copy number. pUC19 carries a 54 base-pair multiple cloning site polylinker that contains unique sites for 13 different hexanucleotide-specific restriction endonucleases. The molecular weight of pUC19 is 1.75×10^6 daltons.



4. pET-23d(+) Vector

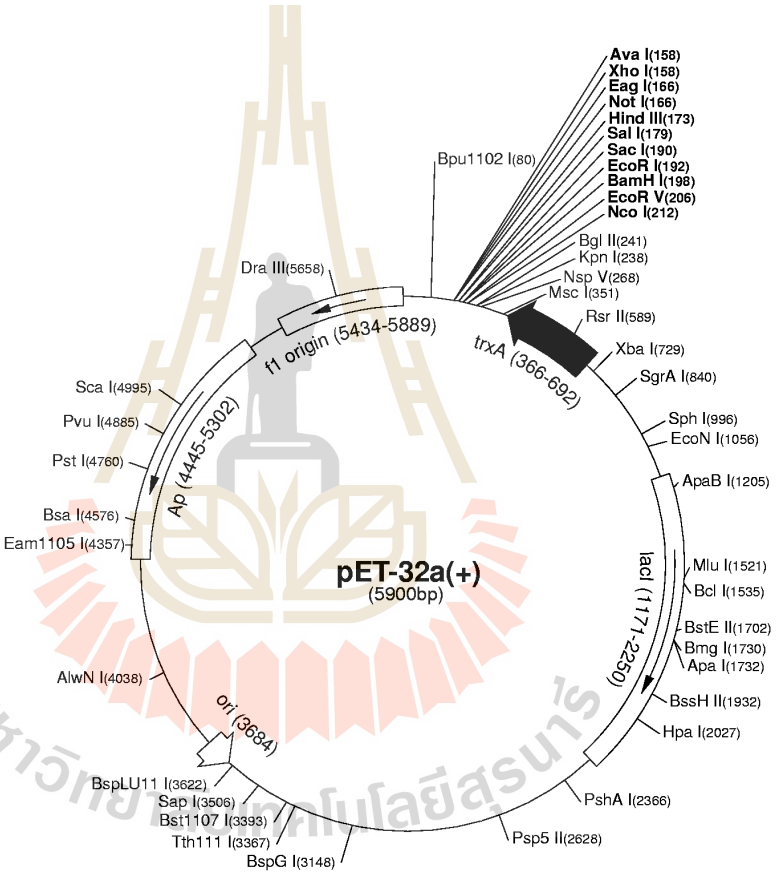
The pET-23d(+) vector carry an N-terminal T7•Tag sequence plus an optional C-terminal His•Tag sequence. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below. The f1 origin is oriented so that infection with helper phage will produce virions containing single-stranded DNA that corresponds to the coding strand. Therefore, single-stranded sequencing should be performed using the T7 terminator primer.

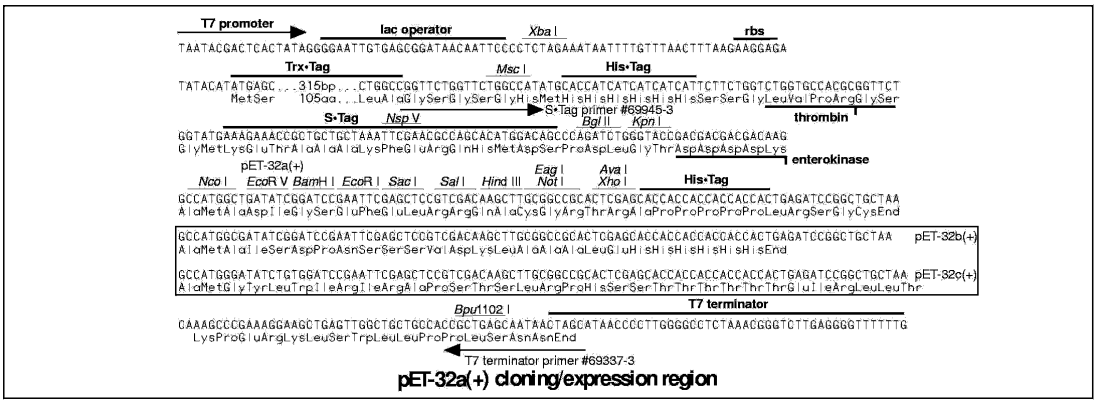




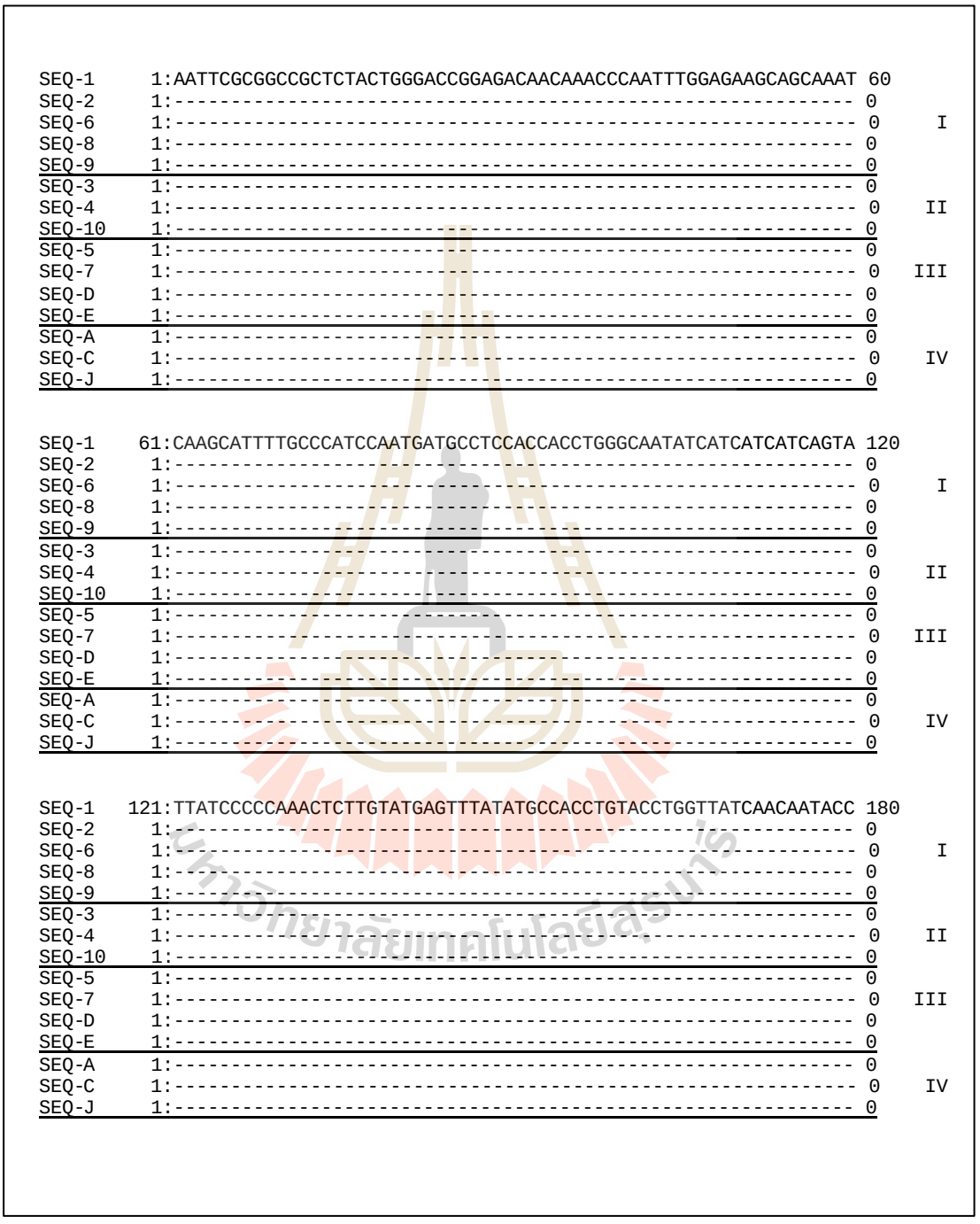
5. pET-32a(+) Vector

The pET-32a(+) vector is designed for cloning and high-level expression of peptide sequences fused with the 109 amino acids of thioredoxin protein (Trx•Tag). Cloning sites are available for producing fusion proteins also containing cleavable His_6•Tag and S•Tag sequences for detection and purification. The sequence is numbered by the pBR322 convention, so the T7 expression region is reserved on the circle map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below.

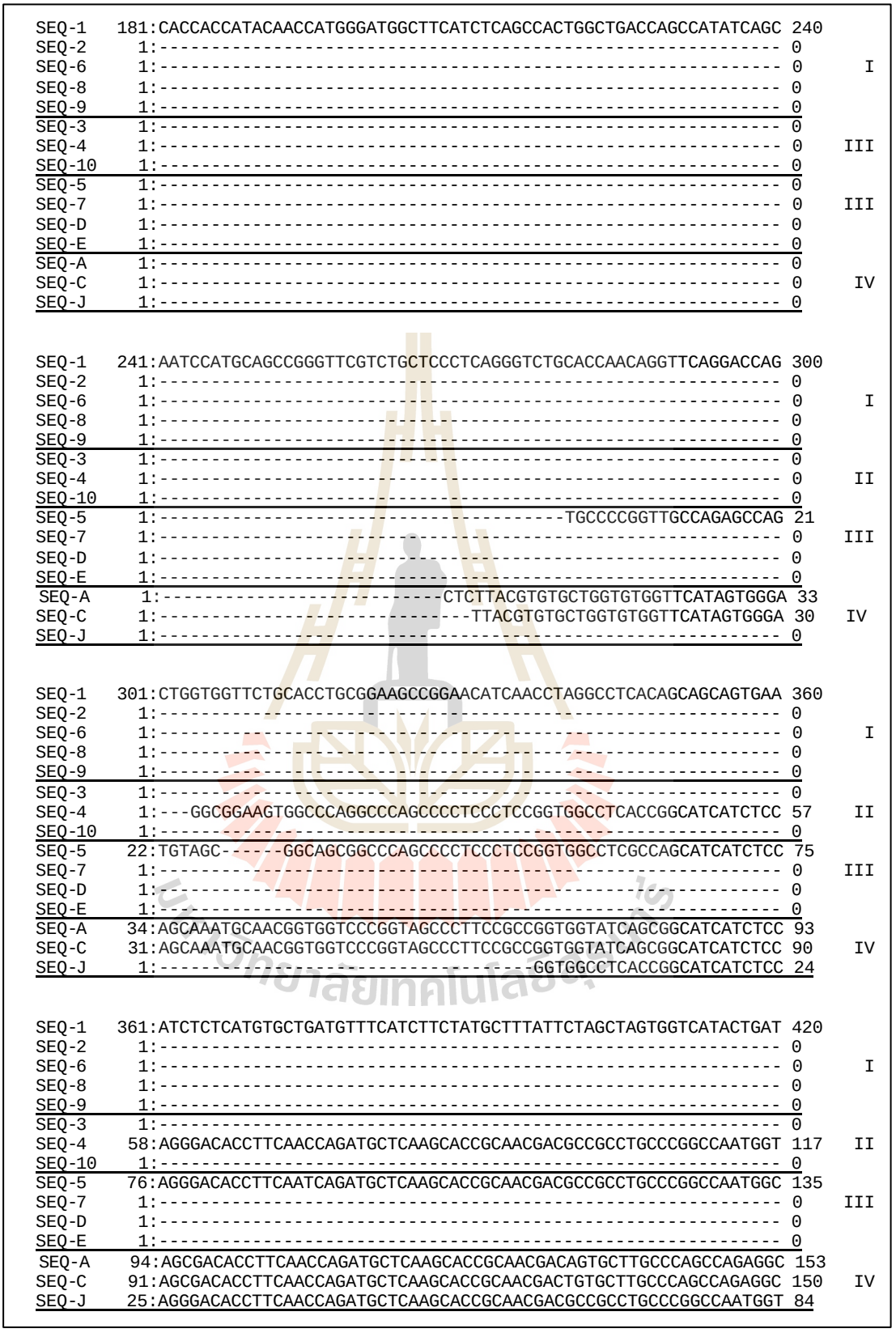




Appendix B



Multiple alignment of nucleotide sequences from screening the *L. leucocephala* shoots library. They were separated 4 groups; group 1, clones 1; group 2, clones 3, 4, and 10; group 3 ,clones 2, 5, 6, 7, 8, 9, and D; and group 4 ,clones A, C, and J.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SEQ-8 1:---------- 0
SEQ-9 1:---------- 0
SEQ-3 1:---------- 0
SEQ-4 1:---------- 0 III
SEQ-10 1:---------- 0
SEQ-5 1:---------- 0
SEQ-7 1:---------- 0 III
SEQ-D 1:---------- 0
SEQ-E 1:---------- 0
SEQ-A 1:---------- 0
SEQ-C 1:---------- 0 IV
SEQ-J 1:---------- 0
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SEQ-6 1:---------- 0 I
SEQ-8 1:---------- 0
SEQ-9 1:---------- 0
SEQ-3 1:---------- 0
SEQ-4 1:---------- 0 II
SEQ-10 1:---------- 0
SEQ-5 1:----------TGCCCCGGTTGCCAGAGCCAG 21
SEQ-7 1:---------- 0 III
SEQ-D 1:---------- 0
SEQ-E 1:---------- 0
SEQ-A 1:----------CTCTTACGTGTGCTGGTGTGGTTCATAGTGGGA 33
SEQ-C 1:----------TTACGTGTGCTGGTGTGGTTCATAGTGGGA 30 IV
SEQ-J 1:---------- 0
SEQ-1 301:CTGGTGGTTCTGCACCTGCGGAAGCCGGAACATCAACCTAGGCCTCACAGCAGCAGTGAA 360
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SEQ-6 1:---------- 0 I
SEQ-8 1:---------- 0
SEQ-9 1:---------- 0
SEQ-3 1:---------- 0
SEQ-4 1:---GGCGGAAGTGGCCCAGGCCCAGCCCCTCCCTCCGGTGGCCTCACCGGCATCATCTCC 57 II
SEQ-10 1:---------- 0
SEQ-5 22:TGTAGC------GGCAGCGGCCCAGCCCCTCCCTCCGGTGGCCTCGCCAGCATCATCTCC 75
SEQ-7 1:---------- 0 III
SEQ-D 1:---------- 0
SEQ-E 1:---------- 0
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SEQ-C 31:AGCAAATGCAACGGTGGTCCCGGTAGCCCTTCCGCCGGTGGTATCAGCGGCATCATCTCC 90 IV
SEQ-J 1:----------GGTGGCCTCACCGGCATCATCTCC 24
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SEQ-6 1:---------- 0 I
SEQ-8 1:---------- 0
SEQ-9 1:---------- 0
SEQ-3 1:---------- 0
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SEQ-10 1:---------- 0
SEQ-5 76:AGGGACACCTTCAATCAGATGCTCAAGCACCGCAACGACGCCGCCTGCCCGGCCAATGGC 135
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SEQ-D 1:---------- 0
SEQ-E 1:---------- 0
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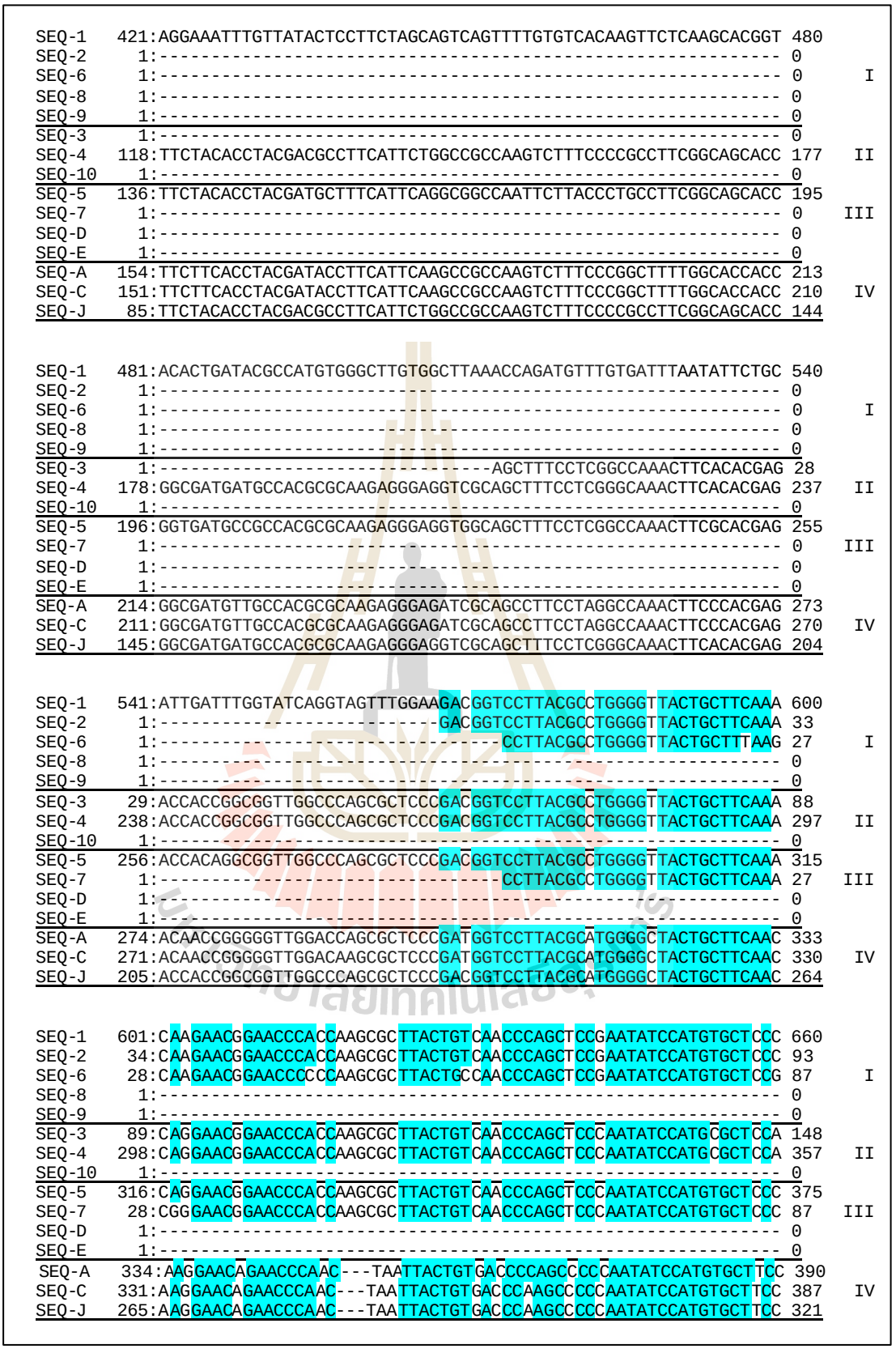
(Continued)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SEQ-9 1:---------- 0
SEQ-3 1:---------- 0
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SEQ-D 1:---------- 0
SEQ-E 1:---------- 0
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SEQ-C 151:TTCTTCACCTACGATACCTTCATTCAAGCCGCCAAGTCTTTCCCGGCTTTTGGCACCACC 210 IV
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SEQ-8 1:---------- 0
SEQ-9 1:---------- 0
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SEQ-5 196:GGTGATGCCGCCACGCGCAAGAGGGAGGTGGCAGCTTTCCTCGGCCAAACTTCGCACGAG 255
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SEQ-6 1:----------CCTTACGCCTGGGGTTACTGCTTTAAG 27 I
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SEQ-6 28:CAAGAACGGAACCCCCCAAGCGCTTACTGCCAACCCAGCTCCGAATATCCATGTGCTCCG 87 I
SEQ-8 1:---------- 0
SEQ-9 1:---------- 0
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SEQ-4 298:CAGGAACGGAACCCACCAAGCGCTTACTGTCAACCCAGCTCCCAATATCCATGCGCTCCA 357 II
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SEQ-7 28:CGGGAACGGAACCCACCAAGCGCTTACTGTCAACCCAGCTCCCAATATCCATGTGCTCCC 87 III
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SEQ-E 1:---------- 0
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SEQ-C 331:AAGGAACAGAACCCAAC---TAATTACTGTGACCCAAGCCCCCAATATCCATGTGCTTCC 387 IV
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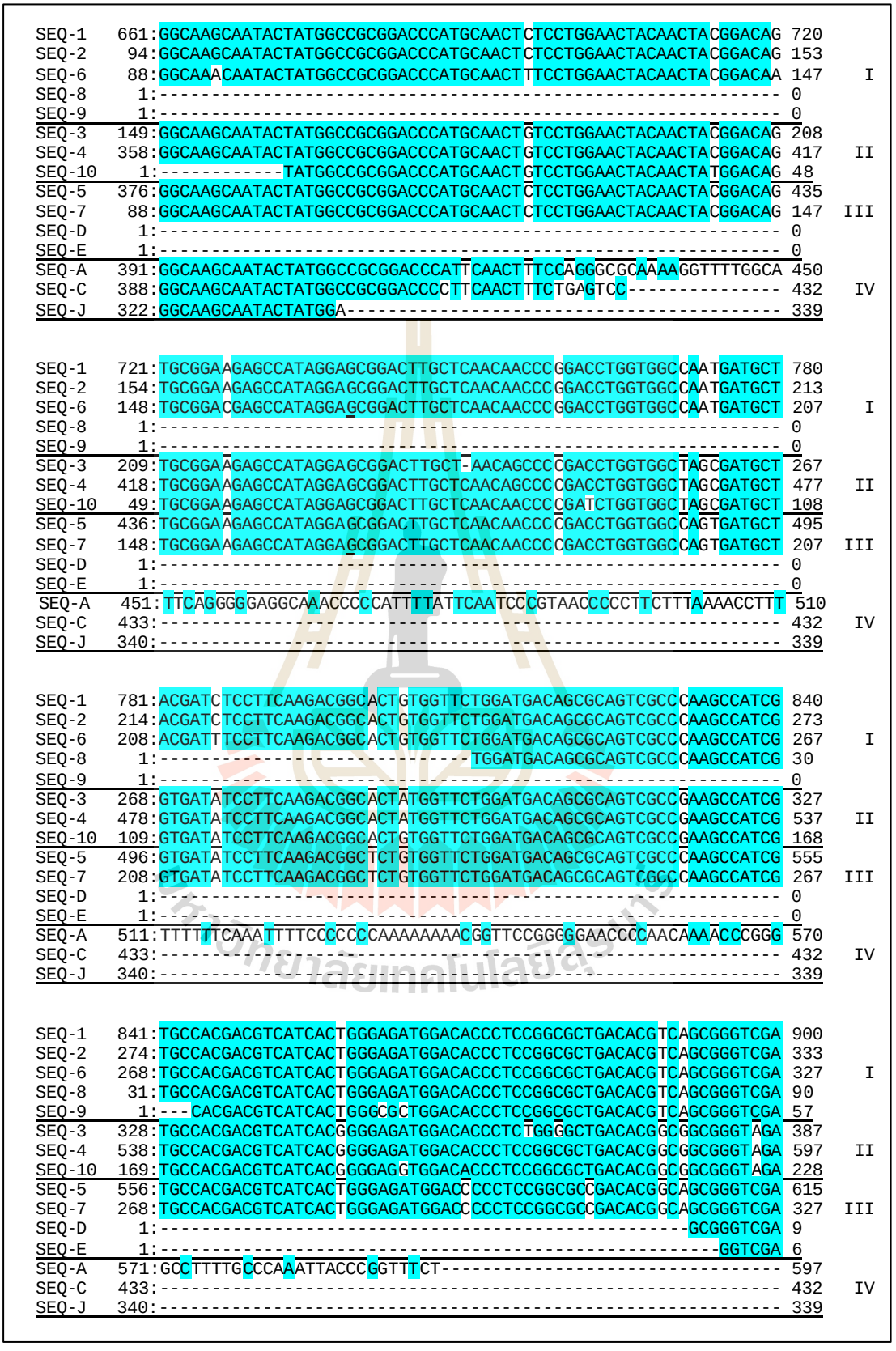
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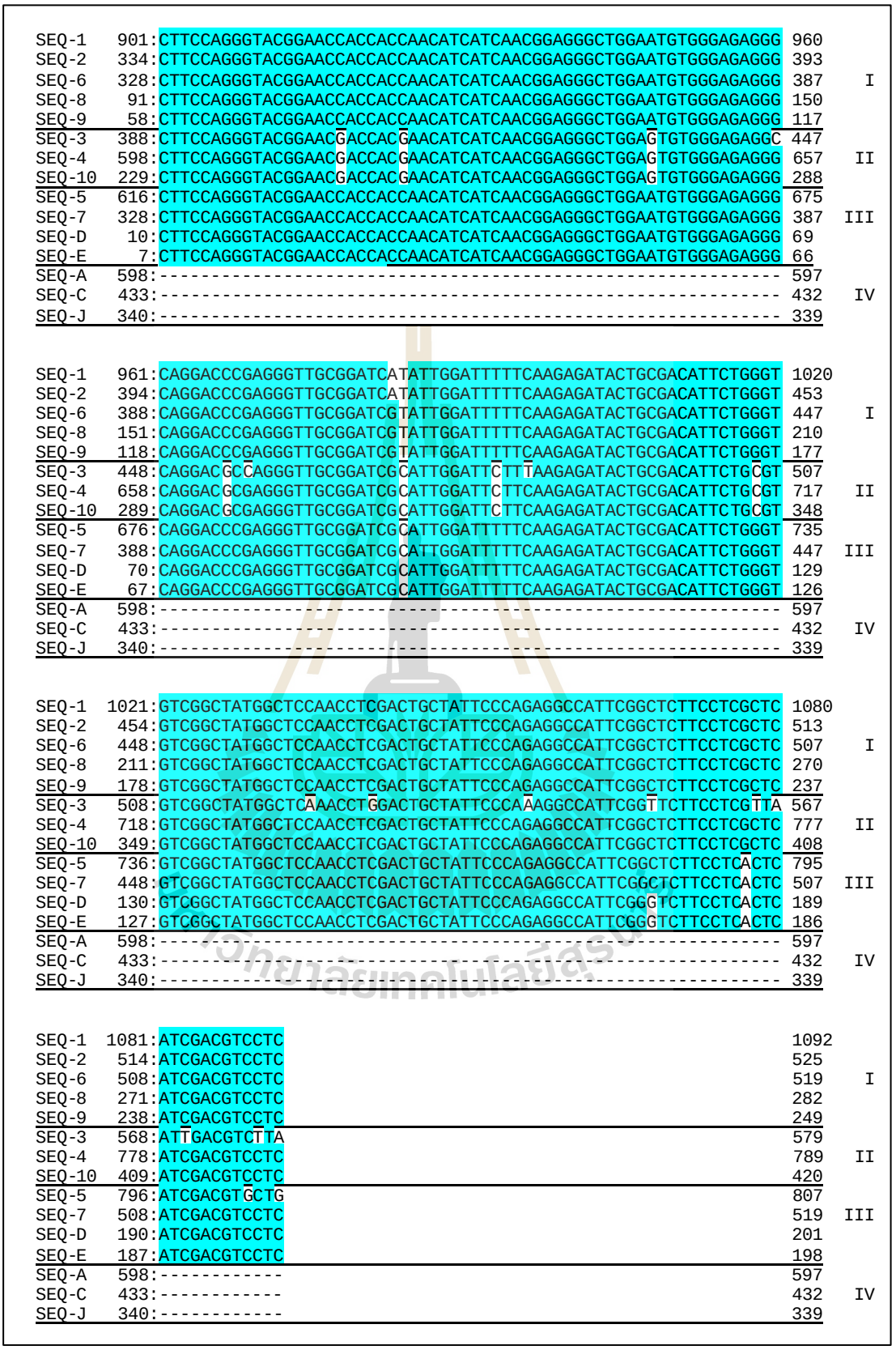
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SEQ-J 340:---------- 339

(Continued)

Appendix C

1. DEPC treated water

Add 0.1 ml of diethyl pyrocarbonate (DEPC) to 100 ml of distilled water to be treated, and shake vigorously. Allow it let sit overnight and autoclave the solution at 121◦C for 15 min to inactivate the remaining DEPC.

2. IPTG stock solution (0.1M)

Dissolve 0.12 g IPTG (Isopropyl thio-*b*-D-galactoside) to final volume of 5 ml distilled water. Sterilize by filter sterilize and store at 4◦C.

3. LB broth

Bacto Tryptone	10 g
Bacto Yeast Extract	5 g
NaCl	5 g
Agar	15 g

Dissolve in 800 ml dH_2O. Adjust pH to 7.2 with NaOH and the volume to 1liter with dH_2O. Autoclave the solution at 121◦C for 15 min.

4. LB plate with 100 mg/ml of ampicillin

Bacto Tryptone	10 g
Bacto Yeast Extract	5 g
NaCl	5 g
Agar	15 g

Dissolve in 800 ml dH_2O. Adjust pH to 7.2 with NaOH and the volume to 1l with dH_2O. Sterilize by autoclaving at 121◦C for 20 min. Allow the medium to cool to 50◦C before adding ampicillin to a final concentration 100 μg/ml. Pour medium into Petri-dishes. Allow the agar to harden.

5. LB plate with 100 mg/ml of ampicillin/IPTG/X-Gal

Make the LB plates with ampicillin as above. Then spread 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-Gal over the surface of the plates and allow to absorb for 30 min at 37°C before use.

6. Preparation of SOC media 1L

Bacto Tryptone	20 g
Bacto Yeast Extract	5 g
1 M of NaCl (5.85 g/100 ml)	10 ml
1 M of KCl ((7.44 g/100 ml)	2.5 ml

Adjust the volume to 980 ml with distilled waster. Sterilize by autoclaving at 121°C for 20 min. Allow the medium cool to room temperature before adding 5 ml 1 M of $MgCl_2 \cdot 6H_2O$ (20.33 g/100 ml), 5 ml 1 M of $MgSO_4 \cdot 7\ H_2O$ (12.30 g/100 ml), 10 ml 2 M of Glucose 10 ml (36.00 g/100 ml) which was filter sterilized by 0.2 µm filter.

7. Reagent for prepared competent cells

TB (transformation buffer)

PIPES (piperazine-1,4-bis(2-ethanesulfonic acid)	0.30 g
Calcium chloride	0.22 g
Potassium Chloride	1.86 g
Mili Q water	90 ml

Adjust pH to 6.7 with KOH and add 1.09 g maganese chloride. Adjust the volume to 100 ml with dH_2O. Sterilize by 0.2 µm filter.

8. Reagent for extraction of DNA from polyacrylamide gel

8.1 Preparation of Gilbert buffer (200 ml)

0.5 M $AcONH_4$	7.7 g
10 mM $MgCl_2$	0.41 g
1 mM EDTA	0.07 g
0.1 % SDS	0.29 g

Adjust the volume to 200 ml with dH_2O. Sterilize by autoclaving at 121°C for 20 min.

8.2 10X TBE

Tris	108.0 g
Boric acid	55.0 g
0.5 M EDTA (pH 8.0)	40 ml

Adjust the volume to 1 liter with dH_2O.

8.3 30% of acrylamide gel

Acrylamide	30 g
N,*N*′-methyllene bisacrylamide	0.8 g

Adjust the volume to 100 ml with dH_2O and filter with Whatman filter paper No 0.

8.4 Preparation of 5% of Polyacrylamide gel (15 ml)

30% of acrylamide gel (29 g acrylamide, 1 g Bris-acrylamide)	2.50 ml
10X TBE	1.50 ml
dH_2O	10.89 ml
10% Ammonium persulphate (0.1 g/ml)	0.10 ml
TEMED	0.015 ml

8.5 3 M of CH_3COONa pH4.8

$CH_3COONa.3H_2O$	408.1 g
dH_2O	800 ml

Adjust pH to 4.8 with glacial acetic acid and the volume to 1 liter with dH_2O. Sterilize by autoclaving at 121°C for 20 min.

9. Reagents for agarose gel electrophoresis

9.1 50 X TAE

Tris	242.0 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100.0 ml

Adjust the volume to 1 liter with dH_2O.

9.2 10X TAE

Tris	48.4 g
Glacial acetic acid	11.42 ml
0.5 M EDTA (pH 8.0)	20.0 ml

Adjust the volume to 1 liter with dH_2O.

10. 6X dye for northern (100 ml)

0.5% of Bromophenol blue	0.5 g
50% of Glycerol	50 ml

Adjust the volume to 100 ml with DEPC treated dH_2O.

11. Reagents for isolation plasmid DNA (alkaline lysis)

11.1 Preparation of PEG 600 /2.5 M NaCl

2.5 M NaCl	73.125 g

Adjust the volume to 500 ml with dH_2O. Sterilize by autoclaving at 121◦C for 20 min. Allow the medium to cool to room temperature before adding polyethylene glycol (MW 6,000) to 20% final concentration (100 g).

11.2 Lysozyme buffer (100 ml)

1 M of Tris/HCl (pH 8.0)	5 ml
0.5 M EDTA (pH 8.0)	2 ml

Adjust the volume to 95 ml with dH_2O. Sterilize by autoclaving at 121◦C for 20 min and add 5 ml 1 M Glucose which was sterilized by 0.2 μm ultrafilter.

11.3 Phenol / Chloroform

TE (pH 8.0)	300 ml
Melted Phenol at 50◦C	300 ml
8-Hydroxyquinoline	4.5 g

Incubate at 4◦C overnight and then, remove the supernatant and add 300 ml chloroform.

11.4 0.5 M EDTA (pH 8.0)

EDTA (disodium ethylene diamine tetraacetate •$2H_2O$)	186.1 g
Distilled water	800 ml

Adjust pH to 8.0 with NaOH (about 20 g)

Adjust the volume to 1 liter with dH_2O. Sterilize by autoclaving at 121◦C for 20 min.

11.5 10 mM TE pH 8.0

Tris (10 mM)	0.121 g
EDTA (1 mM)	0.3722 g
water	80 ml

Adjust pH to 8.0 with HCl and the volume to 100 ml. Sterilize by autoclaving at 121◦C for 20 min.

11.6 1% of SDS /0.2 M of NaOH

SDS 10 % (10 g/100 ml)	10 ml
NaOH 1 N (40 g/l)	20 ml

Adjust the volume to 100 ml with dH_2O.

11.7 3M of CH_3COOK pH4.8

5 M CH_3COOK (510 g/l)	120 ml
Glacial acetic acid	23 ml

Adjust the pH to 4.8 with acetic acid and the volume to 200 ml with dH_2O. Sterilize by autoclaving at 121◦C for 20 min.

12. Reagents for total RNA isolation by using CTAB

12.1 2X CTAB solution (l)

2% Cetyltrimethylamonium bromide (CTAB)	
0.1 M Tris/HCl (pH 9.5)	1.21 g
20 mM EDTA	7.44 g
1.4 M NaCl	8.19 g

Adjust the volume to 90 ml. Sterilize by autoclaving at 121◦C for 40 min and add 1 ml *b*-mercaptoethanol to 1% final concentration.

12.2 10 M LiCl (1 l)

LiCl	423.90 g

Adjust the volume to 1 liter with dH_2O. Sterilize by autoclaving at 121◦C for 40 min.

12.3 3 M AcONa (pH5.2)

$CH_3COONa \cdot 3H_2O$	408.1 g
Add water up to	800 ml

Adjust pH 5.2 with Glacial acetic acid and the volume to 1 liter. Sterilize by autoclaving at 121◦C for 40 min.

12.4 TE saturated phenol (pH9.0)

TE (pH 8.0)	300 ml
Melted Phenol at 50◦C	300 ml
8-Hydroxyquinoline	4.5 g

Mix and incubate at 4◦C overnight. Remove supernatant.

12.5 Chloroform solution (Chloroform : Isoamylalcohol = 24:1)

Chloroform	960 ml
Isoamyalcohol	40 ml

13. Reagents for northern blot analysis

13.1 20X SSC (1 l)

Sodium chloride	175.3 g
Sodium citrate	88.2 g
Distilled water	800 ml

Adjust pH to 7.0 with NaOH and the volume to 1 l. Sterilize by autoclaving at 121◦C for 20 min.

13.2 2X SSC + 1%SDS (200 ml)

10% SDS	20 ml
20X SSC	108 ml

13.3 TES buffer (1 l)

10 mM Tris.HCl	1.21 g
1 mM EDTA (pH 8.0)	3.72 g
150 mM NaCl	8.82 g

Adjust the volume to 1 liter with dH_2O. Sterilize by autoclaving at 121◦C for 20 min.

13.4 20X SSPE (1 l)

Sodium chloride	174.0 g
Sodium dihydrogen phosphate	27.6 g
EDTA	7.4 g
dH_2O	800 ml

Adjust pH to 7.4 with NaOH and the volume to 1 liter with dH_2O. Sterilize by autoclaving at 121◦C for 20 min.

13.5 50X Denhardt's solution (500 ml)

Ficoll	5 g
Polyvinylpyrrolidone	5 g
BSA (Pentax Fraction V)	5 g

Adjust the volume to 500 ml with dH_2O and filter through a disposable Nalgene filter. Dispense into 25 ml aliquots and stored at –20◦C.

13.6 Hybridization buffer (20 ml)

20X SSPE	5 ml
10% SDS	0.2 ml
50X Denhardt's solution	0.4 ml

Adjust the volume to 20 ml with autoclave dH_2O.

14. Reagent of Screening cDNA library

14.1 SM buffer

NaCl	5.8 g
$MgSO_4 \cdot 7H_2O$	2.0 g
1 M of Tris-HCl (pH 7.0)	50.0 ml
2 % of gelatin	5 ml

Adjust the volume to 1 liter with dH_2O. Sterilize by autoclaving at 121◦C for 20 min.

14.2 Top agarose

Bacto Tryptone	10 g
Bacto Yeast Extract	5 g
NaCl	5 g

Adjust the volume to 1liter with dH_2O. Autoclave the solution at 121◦C for 15 min. Add 10 mM $MgSO_4$ and 0.8% agarose. Before use add ampicillin to final concentration 10μl/ml.

14.3 Medium for ***E.coli*** Y1090r-

Bacto Tryptone	10 g
Bacto Yeast Extract	5 g
NaCl	5 g

Adjust the volume to 1 liter with dH_2O. Autoclave the solution at 121◦C for 15 min. Add 500 μl of 20 % maltose and 200 μl of 25 mg/ml ampicillin.

15. Buffers for expression

15.1 Extraction buffer

20 mMTris	0.242 g
2 mM EDTA	0.7444 g
dH_2O	80 ml

Adjust pH to 8.0 with HCl and the volume to 100 ml with dH_2O. Sterilize by autoclaving at 121◦C for 20 min. Add 1 mg of lysozyme (100 μl/ml), 1 ml of Triton X-100 (1%), and 0.018 g of PMSF (1 mM).

15.2 Lysis buffer

50 mM NaH_2PO_4	6.90 g
300 mM NaCl	17.54 g

Adjust pH to 8.0 with NaOH and the volume to 1 liter with dH_2O.

15.3 Wash buffer 1

50 mM NaH_2PO_4	6.90 g
300 mM NaCl	17.54 g
10 mM Imidazole	0.68 g

Adjust pH to 8.0 with NaOH and the volume to 1 liter with dH_2O.

15.4 Wash buffer 2

50 mM NaH_2PO_4	6.90 g
300 mM NaCl	17.54 g
50 mM Imidazole	3.40 g

Adjust pH to 8.0 with NaOH and the volume to 1 liter with dH_2O.

15.4 Elution buffer

50 mM NaH_2PO_4	0.69 g
300 mM NaCl	1.75 g
250 mM Imidazole	1.70 g

Adjust pH to 8.0 with NaOH and the volume to 100 ml with dH_2O.

16. 10X buffer for Enterokinase

200 mM Tris	0.2422 g
500 mM NaCl	0.2925 g
20 mM $CaCl_2$	0.0220 g

Adjust pH to 7.4 with HCl and the volume to 10 ml with dH_2O.

17. Solutions for protein

17.1 1.5 M Tris pH 8.0

1.5 M Tris	18.17 g

Adjust pH to 8.0 with HCl and the volume to 100 ml with dH_2O.

17.2 1.0 M Tris pH 6.8

1 M Tris	12.11 g

Adjust pH to 6.8 with HCl and the volume to 100 ml with dH_2O.

17.3 SDS-gel loading buffer (5X stock)

2.5 M Tris	0.30 g
10% SDS	1.0 g
0.5% Bromophenol blue	0.05 g
50% glycerol	5 ml

Adjust pH to 6.8 with HCl and the volume to 8 ml with dH_2O. Before use add 20 μl of *b*-mercapthoethanol to 80 μl of solution mixture.

17.4 Tris-Glycine electrode buffer (5X stock)

250 mM Tris	30.29 g
1.25 M glycine	144 g
0.5% SDS	5 g

Adjust pH to 8.3 with HCl and the volume to 1liter with dH_2O.

17.5 12% separation gel SDS-PAGE (10 ml)

1.5 M Tris (pH 8.0)	1.3 ml
dH_2O	3.3 ml
10% SDS	0.1 ml
30% acrylamide solution	4.0 ml
10% ammonium persulfate	0.1 ml
TEMED	0.004 ml

17.6 15% separation gel SDS-PAGE (10 ml)

1.5 M Tris (pH 8.0)	1.3 ml
dH_2O	2.3 ml
10% SDS	0.1 ml
30% acrylamide solution	5.0 ml
10% ammonium persulfate	0.1 ml
TEMED	0.004 ml

17.7 5% stacking gel SDS-PAGE (5 ml)

1.0 M Tris (pH 6.8)	0.63 ml
dH_2O	3.4 ml
10% SDS	0.05 ml
30% acrylamide solution	0.83 ml
10% ammonium persulfate	0.05 ml
TEMED	0.005 ml

17.8 Destaining solution for Coomassie Stain

Methanol	400 ml
Water	500 ml
Glacial acetic acid	100 ml

17.9 Staining solution with Coomassie brillant blue for protein

Coomassie brilliant blue R-250	1 g
Methanol	400 ml
Water	500 ml
Glacial acetic acid	100 ml

Filter the solution through a Watman No. 1 filter to remove any particulate matter.

17.10 Staining solution for isoelectric focusing polyarylamide gel.

PhasGel Blue R (0.02%)	200 mg
0.1% $CuSO_4$	1 g
Methanol	300 ml
Water	600 ml
Glacial acetic acid	100 ml

Filter the solution through a Watman No. 1 filter to remove any particulate matter.

17.11 Destraining solution for isoelectric focusing polyarylamide gel.

Methanol	300 ml
Water	600 ml
Glacial acetic acid	100 ml

18. Buffers and Reagents for enzyme assay

18.1 0.8 M Sodium tetraborate

Sodium tetraborate	1.6098 g

Adjust the volume to 10 ml with dH_2O.

18.2 ***p***-dimethylaminobenzaldehyde (DMAB, 10X stock)

Glacial acetic acid	70 ml
HCl	10 ml
DMAB	8 g

Before use, add 90 ml glacial acetic acid in 10 ml DMAB solution.

18.3 200 m M ***N***-acetyl-D-glucosamine

N-acetyl-D-glucosamine	0.2212 g

Adjust the volume to 5 ml with dH_2O.

18.4 10 mM ***p***-nitrophenol

p-nitrophenol	0.01391

Adjust the volume to 10 ml with dH_2O.

18.5 0.1 M sodium acetate buffer pH 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0

Sodium acetate	9.53 g

Dissolve in 800 ml dH_2O. Adjust pH to 3.5, 4.0, 4.5, 5.0, 5.5, or 6.0 with glacial acetic acid and the volume to 1liter with dH_2O.

18.6 0.1 M sodium phosphate buffer pH 5.5

Disodium hydrogen phosphate	0.85 g
Sodium dihydrogen phosphate	17.3 g

Adjust pH to 5.5 and the volume to 1 liter with dH_2O.

18.7 0.1 M sodium phosphate buffer pH 6.0

Disodium hydrogen phosphate	1.9 g
Sodium dihydrogen phosphate	13.6 g

Adjust pH to 6.0 and the volume to 1 liter with dH_2O.

18.8 0.1 M sodium phosphate buffer pH 6.5

Disodium hydrogen phosphate	5.2 g
Sodium dihydrogen phosphate	9.9 g

Adjust pH to 6.5 and the volume to 1 liter with dH_2O.

18.9 0.1 M sodium phosphate buffer pH 7.0

Disodium hydrogen phosphate	8.5 g
Sodium dihydrogen phosphate	6.2 g

Adjust pH to 7.0 and the volume to 1 liter with dH_2O.

18.10 0.1 M sodium phosphate buffer pH 7.5

Disodium hydrogen phosphate	11.8 g
Sodium dihydrogen phosphate	2.5 g

Adjust pH to 7.5 and the volume to 1 liter with dH_2O.

18.11 0.1 M Tris-HCl buffer pH 7.0, 7.5, 8.0, 8.5, and 9.0

Tris	12.14 g

Adjust pH to 7.0, 7.5, 8.0, 8.5, or 9.0 with HCl and the volume to 1liter with dH_2O.

18.12 10 mM ***p***-nitrophenyl-***N***-acetylglucosamine

p-nitrophenyl-*N*-acetylglucosamine 3.4231 mg

Adjust the volume to 1 ml with dH_2O.

18.13 10 mM ***p***-nitrophenyl-***N,N¢***-diacetylchitobiose

p-nitrophenyl-*N,N¢*-diacetylchitobiose 5.455 mg

Adjust the volume to 1 ml with dH_2O.

18.14 10 mM ***p***-nitrophenyl-***N,N¢,N¢¢***-triacetylchitotriose

p-nitrophenyl-***N***,*N¢,N¢¢*-triacetylchitotriose 7.4869 mg

Adjust the volume to 1 ml with dH_2O.

18.15 40 mM ***N,N¢,N¢¢***-tetraacetylchitotetraose

N,N¢,N¢¢-tetraacetylchitotetraose 8.308 mg

Adjust the volume to 250 µl with dH_2O.

19. Preparation of Glycol chitin

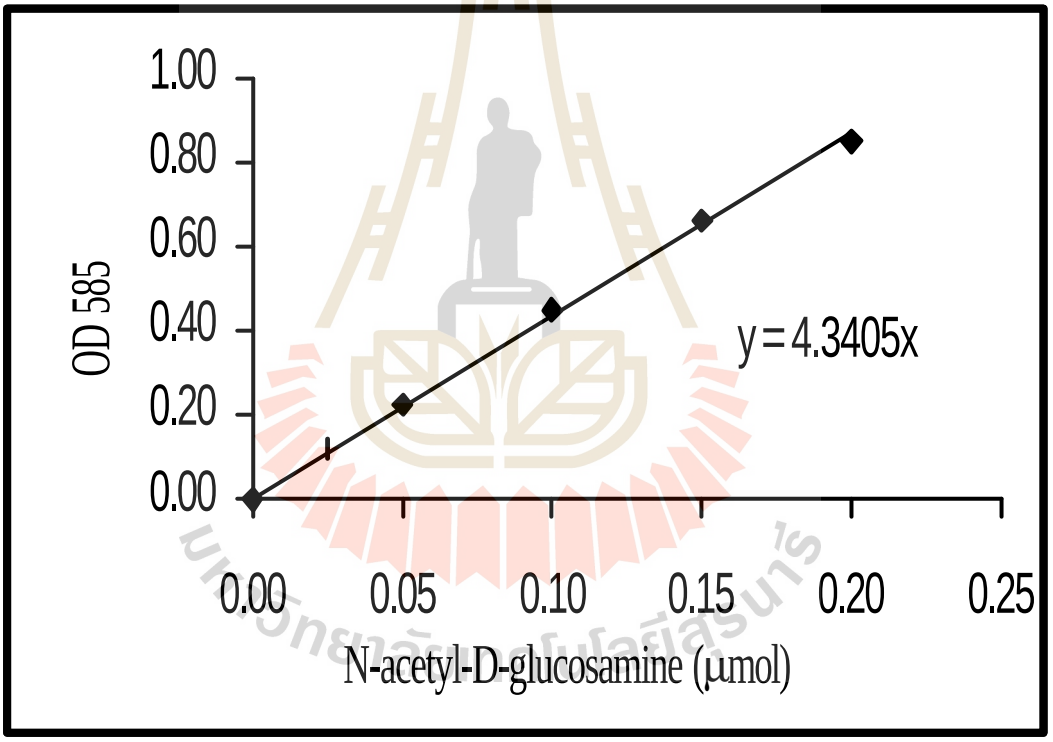
Glycol chitin was obtained by acetylation of glycol chitosan by a modification of the method of Trudel and Asselin (1988). Glycol chitosan (5g) was dissolved in 100 ml of 10% acetic acid by grinding in a mortar. The viscous solution was allowed to stand overnight at 22◦C. Four hundred and fifty milliliters of methanol was slowly added and the solution was vacuum filtered through Whatman No. 4 filter paper. The filtrate was transferred into a beaker and 7.5 ml of acetic anhydride was added with stirring. The resulting gel was allowed to stand for 30 min at room temperature and then cut into small pieces. The liquid extruding from the gel pieces was discarded. Gel pieces were transferred to a waring blender, covered with methanol, and homogenized for 4 min at top speed. The suspension was centrifuged at 27,000 x g for 15 min at 4◦C. The gelatinous pellet was resuspended in 1 volume of methanol, homogenized, and centrifuged as in the proceeding step. The pellet was resuspended in 500 ml distilled water containing 0.02% (w/v) sodium azide and homogenized for 4 min. This was the final 1% (w/v) stock solution of glycol chitin.

20. Preparation of Swollen chitin

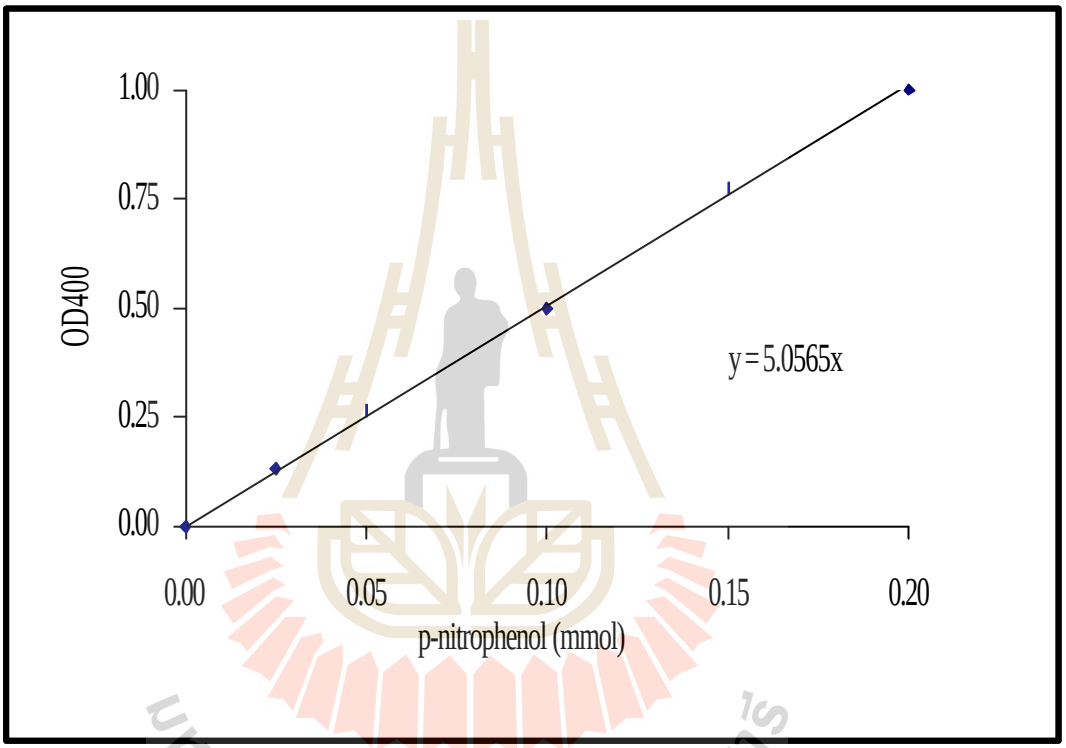
Swollen chitin was prepared according to the method of Monreal and Reese (1968). Chitin flakes, 10 g, were added into 100 ml of 85% (v/v) phosphoric acid. The suspension was left stirred constantly overnight at 4°C. An excess volume of distilled water was added into the suspension to precipitate the acid-treated chitin. The precipitate was harvested by centrifugation at 10,000 x *g* at 4°C for 30 min. The precipitate was washed thoroughly until the pH of the suspension was nearly neutral. The swollen chitin was at 4°C until used.

Appendix D

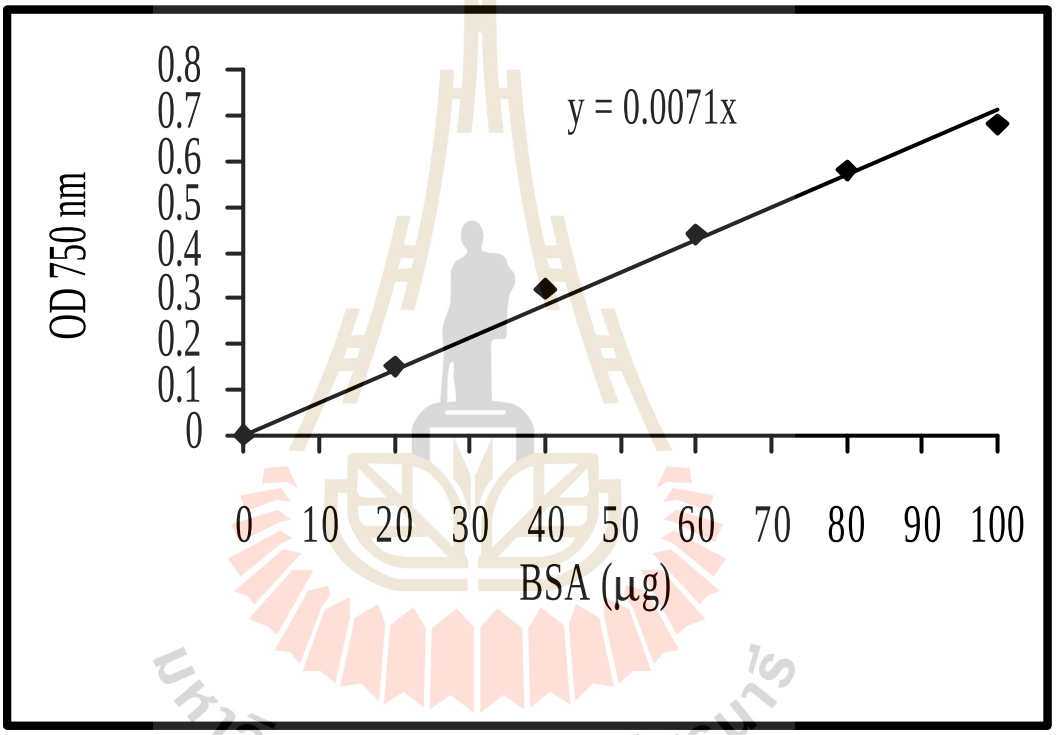
1. Standard curve of ***N***-acetyl-D-glucosamine



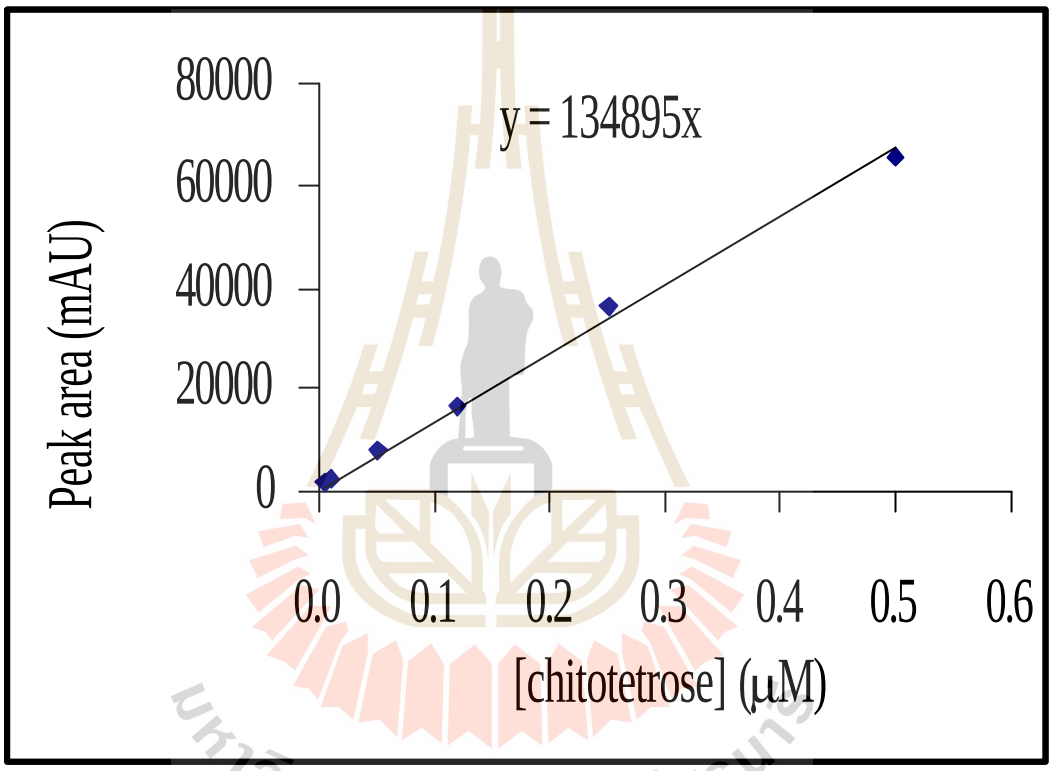
2. Standard curve of ***p***-nitrophenol



3. Standard curve of BSA by Lowry method



4. Standard curve of ***N,N¢,N¢¢,N¢ ¢ ¢*** -tetraacetylchitotetraose



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