การโคลนและลักษณะสมบัติของยีนไคทิเนสที่ได้จากเฮพาโทแพนเครียสของกุ้งกุลาดำ *Penaeus monodon*

นางสาวปณัตดา ยอดแสง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

สาขาวิชาเทคโนโลยีชีวภาพ

คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2548

ISBN 974-14-1942-2

CLONING AND CHARACTERIZATION OF CHITINASE GENE FROM HEPATOPANCREAS FROM BLACK TIGER SHRIMP *Penaeus monodon*

Miss Panutda Youdsang

A Thesis Submitted in Partial Fulfillment of the Requirement
for the Degree of Master of Science Program in Biotechnology
Faculty of Science
Chulalongkorn University
Academic 2005

ISBN 974-14-1942-2

Thesis Title	CLONING AND CHARACTERIZATION OF CHITINASE GENE FROM HEPATOPANCREAS FROM BLACK TIGER SHRIMP *Penaeus monodon*
By	Miss Panutda Youdsang
Field of study	Biotechnology
Thesis Advisor	Rath Pichyangkura, Ph.D.
Thesis Co-advisor	Associate Professor Vichien Rimphanitchayakit, Ph.D.

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

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

THESIS COMMITTEE

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

..... Thesis Advisor
(Rath Pichyangkura, Ph.D.)

..... Thesis Co-advisor
(Associate Professor Vichien Rimphanitchayakit, Ph.D.)

..... Member
(Associate Professor Anchalee Tassanakajon, Ph.D.)

..... Member
(Assistant Professor Alisa Vangnai, Ph.D.)

ปณัตดา ยอดแสง : การโคลนและลักษณะสมบัติของยีนไคทิเนสที่ได้จากเฮพาโทแพนเครียสของกุ้งกุลาดำ *Penaeus monodon* (CLONING AND CHARACTERIZATION OF CHITINASE GENE FROM HEPATOPANCREAS FROM BLACK TIGER SHRIMP *Penaeus monodon* อาจารย์ที่ปรึกษา : อ.ดร. รัฐ พิชญางกูร, อาจารย์ที่ปรึกษาร่วม : รศ.ดร. : วิเชียร ริมพานิชยกิจ 137 หน้า, ISBN 974-14-1942-2

ไคทิเนส (E.C. 3.2.1.14) เป็นเอนไซม์ที่เร่งปฏิกิริยาการย่อยสลายไคติน ไคติเนส สามารถนำมาประยุกต์ใช้ให้เกิดประโยชน์ต่ออุตสาหกรรมในด้านต่างๆ ในการศึกษาครั้งนี้ ไคติเนสได้ถูกนำมาศึกษาลักษณะสมบัติ โดยใช้โปรแกรมทางคอมพิวเตอร์เพื่อค้นหายีน ไคติเนสจากฐานข้อมูลของห้องสมุด cDNA ของกุ้งกุลาดำ (http://pmonodon.biotec.or.th) ทั้งสิ้น 10,100 โคลน พบว่า 3 โคลนมีลำดับเบสเหมือนยีนไคทิเนส โดยได้จากห้องสมุด เม็ดเลือด 2 โคลน และ 1 โคลน จากห้องสมุด hepatopancreas ยีนที่ได้จากห้องสมุด hepatopancreas นี้ได้นำมาศึกษาต่อในการโคลนและแสดงออกของยีนไคทิเนส ไคทิเนสที่โคลนได้ถูกเรียกว่า *ChiHP74* มี open reading frame ขนาด 1995 bp ซึ่งแปลเป็นกรดอะมิโนได้จำนวน 665 กรด อะมิโน มีน้ำหนักโมเลกุล 74 kDa จากการวิเคราะห์ลำดับเบส พบว่า ไคทิเนสชนิดนี้ ประกอบด้วย catalytic domain (CatD) ในกลุ่มของไคทิเนสแฟมิลี่ 18 Tachycitin like domain และโดเมนที่มีกรดอะมิโนโพรลีนจำนวนมากที่ปลายทางด้าน คาร์บอกซิลิก ในศึกษาการแสดงออกของ *ChiHP*74 ใน 2 ระบบ ได้แก่ การแสดงออกในแบคทีเรีย *E. coli* สายพันธุ์ BL21 (DE3) โดยใช้เวกเตอร์ pET-17b และการแสดงออกของยีนในยีสต์ *Pichia pastoris* โดยใช้เวกเตอร์ pPIC9K ผลการทดลองพบว่า *ChiHP74* ไม่มีการแสดงออกในทั้ง 2 ระบบ จากนั้นทำการโคลนและศึกษาการแสดงออกของไคทิเนสเฉพาะบริเวณ catalytic domain (CatD) โดยศึกษาการแสดงออกในระบบของแบคทีเรีย *E. coli* สายพันธุ์ BL21 (DE3) ไคทิเนสที่โคลนได้นี้ถูกเรียกว่า *Chi-CatD* ประกอบไปด้วย open reading frame ขนาด 1278 bp แปลเป็นกรดอะมิโนได้จำนวน 426 กรดอะมิโน มีน้ำหนักโมเลกุล 48 kDa ผลการทดลองพบว่า*Chi-CatD* มี pH ที่เหมาะสมต่อการทำงานอยู่ สองช่วงคือ pH 4 และ pH 7 ส่วนอุณหภูมิที่เหมาะต่อการทำงานคือ 60 องศาเซลเซียส โดย *Chi-CatD* นี้สามารถย่อยเบต้าไคตินได้ดีที่สุดและให้ผลิตภัณฑ์หลักเป็นน้ำตาลโมเลกุลคู่

สาขาวิชา....เทคโนโลยีชีวภาพ... ลายมือชื่อนิสิต.....
ปีการศึกษา.....2548..... ลายมือชื่ออาจารย์ที่ปรึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

##4672320323 : Biotechnology
KEY WORD : CHITINASE / HEPATOPANCREAS

P A N U T D A Y O U D S A N G : G E N E C L O N I N G A N D CHARACTERIZATION OF CHITINASE FROM HEPATOPANCREAS FROM BLACK TIGER SHRIMP *Penaeus monodon* THESIS ADVISOR : RATH PICHAYANGURA, Ph.D. THESIS CO-ADVISOR: ASSISTANT PROFESSOR VICHIEN RIMPHANITCHAYAKIT, Ph.D., 137 pp. ISBN 974-14-1942-2

Chitinase (E.C. 3.2.1.14) is an enzyme that catalyzes the degradation of chitin. Chitinase is useful enzyme for wide applications in several industries. A computer program was used for searching the chitinase gene in the black tiger shrimp (*Penaeus monodon*) database (http://pmonodon.biotec.or.th). A total of 10,100 clones were searched for chitinase gene. Three clones, two clones from a hemocyte library and another clone from hepatopancreas library, were identified as a putative chitinase gene and reconfirmed by BLASTN program. The chitinase gene, *ChiHP74*, found in hepatopancreas cDNA library of black tiger shrimp *P. monodon* has been cloned. The *ChiHP74* contains an open reading frame of 1995 bp. The cDNA encoded for a polypeptide chain of 665 amino acid residues with a predicted molecular weight of 74 kDa. Sequence analysis of the deduced amino acid sequence of chitinase comprised a family 18 catalytic domain (CatD), a tachycitin like domain, and two proline rich domains at the C-terminus. In an expression of this gene, bacterial expression system and yeast expression system were used to express *ChiHP74*. The result showed this chitinase could not be expressed by both systems. Because of no expression of full-length fragment, CatD was chosen to clone and express instead. It was successfully expressed by using pET-17b in *E. coli* strain BL21 (DE3) called *Chi-CatD* which showed an optimum pH in two ranges of 4 and 7. The optimum temperature of this enzyme was 60 ^{o}C. *Chi-CatD* hydrolyzed β-chitin the best and only dimer [$(GlcNAc)_2$] was detected.

Field of study....Biotechnology... Student's signature....Panutda Youdsang..
Academic year.....2548..... Advisor's signature.....
Co-advisor's signature.....

ACKNOWLEDGMENTS

I would like to express my deepest gratitude and appreciation to my advisor, Dr. Rath Pichyangkura for his guidances, supervision, encouragement, and support throughout my study. I am very grateful to my co-advisor, Assistant Professor Dr. Vichien Rimphanitchayakit for his guidances, suggestions and support throughout this thesis.

My gratitude is also extended to Associate Professor Dr. Aran Incharoensakdi, to Associate Professor Dr. Anchalee Tassanakajon, Assistant Professor Dr. Alisa Vangnai for serving as the members of my thesis committee, for their available comments and also useful suggestions.

Thanks are also expressed to all staff members and my friends of the Biotechnology and Biochemistry Department for their help in laboratory. Special thanks are also extended to the members in lab 709 (pee Cell, pee Kung, pee Bum, pee Ben, pee Neng, Pui, Paman), Puen, Pond, Kook, Wiw, Wan, Bow, and Ake for their helps, suggestions, kindness, friendships, and moral.

I wish to acknowledge to the National Science and technology Development Agency and the Graduate school, Chulalongorn University for my financial support.

Finally, I would like to express my deepest gratitude to my parents, my older sisters for their unlimited love, understanding, and encouragement.

Contents

Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

ABBREVIATION

A	Absorbance
bp	base pair
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
^{o}C	Degree celsius
DEPC	diethylpyrocarbonate
IPTG	Isopropyl-b-D-thiogalactopyranoside
mL	Mililiter
mL	Microliter
M	Molar
mM	Milimolar
mM	Micromolar
mg	Microgram
rpm	Revolution per minute
nm	Nanometer
X-gal	5-bromo-4-chloro-3-indoyl-b-D-galactopyranoside
YNB	Yeast Nitrogen Base with Ammonium Sulfate without amino acids

CHAPTER I

INTRODUCTION

1.1 General introduction

1.1.1 Chitin

Chitin, the second most abundant biopolymer in nature, has a chemical structure similar to the cellulose, in which the N-acetylamido groups in chitin have replaced the C2 hydroxyl groups in cellulose (Figure 1.1). Chitin is a common constituent of fungal cell walls, exoskeletons of insects, arachnida, the cuticle of annelids and mollusks (Gooday, 1994), many groups of invertebrates, shell of crustaceans, and extra-cellular polymer of some bacteria. Chitin is composed of the *N*-acetyl-D-glucosamine or 2-acetamido-2-deoxy-D-glucose (GlcNAc) residues, inter-connected through a β-1,4 linkage. The physical structure of chitin is well characterized by using X-ray crystallography and infrared spectroscopy. Comparison of the structure of chitins from different sources has revealed an existence of 3 polymorphic forms in nature, an antiparallel (α-chitin), a parallel (β-chitin) and a mixture of the two forms (γ-chitin). Most chitins, including those from crustaceans, insects, and fungi, are in the α-form. The antiparallel chains of chitins are stacked together and stabilized by the hydrophobic interaction between the surfaces of the glucose rings.

The β-chitin is found in the spines of polychaete *Aphrodite* and the marine diatoms, the pen of the squid *Loligo,* and the tubes of *Pogonoophora*. In contrast to

the α-chitin, the polymer chains in β-chitin are arranged in the same direction. The $–CH_2OH$ groups are all hydrogen bonded between the stacks of the chain.

The γ-chitin has been reportedly found only in the stomach lining of *Loligo*. However, this form is not studied in details as the α and β forms. The truly correct three-dimensional structure has not been established. The model structures of the three chitins are shown in Figure 1.2.

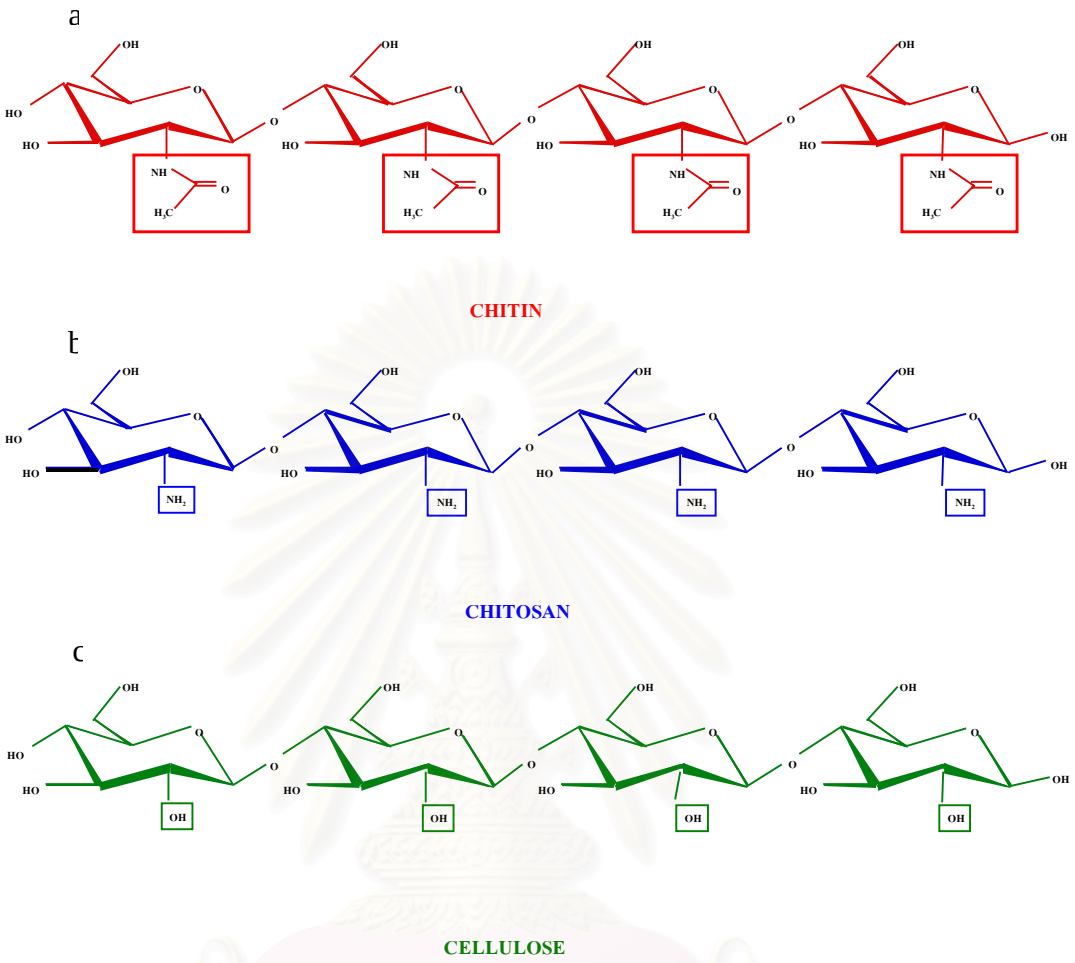


Figure 1.1 The chemical structures of chitin (a), chitosan (b), and cellulose (c).

α-chitin β-chitin γ-chitin

Figure 1.2 The model arrangement of the polymer chains in α-chitin, β-chitin, γ-chitin. Each arrow represents the *N*-acetylglucosamine chain from the nonreducing and to reducing ends.

Table 1.1 The amounts and types of chitin found in the various sources.

Sources of chitin	Amount of chitin (%)	Structural form of chitin
Crustacean		
▪ Crangon (shrimp)	69.1[c]	α-chitin
▪ Alaskan (shrimp)	28.0[d]	
▪ Paralithodes (king crab)	35[b]	
▪ Callinectes (blue crab)	14[a]	
Insect		
▪ May beetle	16.0[c]	α-chitin
▪ Blatella (cockroach)	18.4[c]	
▪ Colcoptera (beetle)	27-35[c]	
▪ Calleria (wax worm)	33.7[c]	
▪ Bombyx (silk worm)	44.2[c]	
▪ Diptera (true fly)	54.8[c]	
▪ Pieris (sulfur butterfly)	64.0[c]	
Mollusca		
▪ Oyster shell	3.6	β-chitin
▪ Clamshell	6.1	
▪ Kill, deproteinized shell	40.2	
▪ Squid ,Octopus	41	
Fungi		
▪ *Pennicilium notatum*	18.5[d]	α-chitin
▪ *Lactarius vaiiereus (mushroom)*	19	
▪ *Mortierella vinacea*	22.0	
▪ *Aspergillus phoenicis*	23.7	
▪ *Histoplasma capsulatum*	25.8	
▪ *Histroplasma farciminosum*	40.0	
▪ *Mucor rouxi*	44.5	
▪ *Aspergillus niger*	42.0[d]	

a. wet body weight
b. dry body weight or total dry weight of cuticle
c. organic weight of cuticle
d. dry weight of cell wall

1.1.2 Chitin and chitosan production

The production of chitin is approximately 10^{10}-10^{11} ton per annum, mostly from the shells of crustaceans much more than the other sources. Generally, the shells of shrimps and crabs consist of 10-30% chitin, protein, mineral and sodium carbonate. Various procedures have been adapted for the isolation and purification of chitin. Traditionally, shell wastes are deproteinized by treating with either sodium hydroxide or potassium hydroxide. The effectiveness of alkali deprotenization depends on the temperature of processing, the concentration of alkali used and the weight ratio of shell against the alkali. Subsequently, demineralization is usually achieved by treating the deproteinized shells with hydrochloric acid. Full demineralization of the shells is possible by using excess amount of acid. Finally, the pigment residues are removed by extraction with organic solvent, such as acetone, chloroform, ethyl acetate or ethanol and ether mixture.

Chitosan is a derivative of chitin and prepared by the deacetylation of chitin. The process is carried out either at elevated temperature or at room temperature using concentrated sodium or potassium hydroxide solution. Importantly, alkali concentration, time and temperature of the process should be controlled because these factors influence the degree of deactylation, the molecular weight and the distribution of deacetylated units along the chain. These three properties govern the usefulness of chitosan for industrial applications.

1.1.3 Solubility of chitin and chitosan

Chitin is highly hydrophobic and insoluble in water and most organic solvents. It is soluble in hexafluoro-isopropanol, hexafluoroacetone, and chloroalcohols in conjugation with aqueous solutions of mineral acids (Madhavan, 1992), and dimethylacetamide containing 5% lithium chloride. Chitosan, the deacetylated product of chitin, is soluble in dilute acids such as acetic acid, and formic acid.

1.1.4 The application of chitin and chitosan

The characteristic features of chitin and chitosan for potentially broad industrial application are that they are 1) natural resources and renewable materials, 2) biodegradable and do not pollute the environment, 3) biocompatible to humans and most animals, 4) almost nontoxic, 5) biological functioning, and 6) changeable in the structure. For these reasons, there are a lot of emphases on devising method of adopting the biomolecules for versatile applications. Chitin and chitosan are applied in many industries such as agriculture, cosmetic, medicine, pharmaceutical, water treatment and biotechnology as shown in Table 1.3.

1.1.4.1 Agriculture application

Agriculture products or seeds are coated with chitin and chitosan for prolonging the viability of the seeds, decreasing decomposition, and protecting from the fungi and insects. For example, soybeans are coated with thin layers of depolymerized chitin, carboxymethyl (CM)-chitin and hydroxyethyl (HE)-chitin.

After the seeds are cultured in the field, it is observed that the seed chitinase, the plant dry weight, and the crop yield increased (Hirano, 1995 and 1999).

1.1.4.2 Biomedical application

Chitin and chitosan are nontoxic and biodegradable polysaccharide that attracts much attention in medicine and pharmaceutics. They possess the gel- and film-forming properties because of their polymeric and cationic characters. Chitosan has been modified for use in controlled release drug delivery system, controlling the rate of drug administration, and prolonging the duration of the drug in the body. The drug is either physically blended or covalently linked to the amino group of chitosan. Generally, it is released from the chitosan matrix by erosion of the polymer after the contact with body fluids (Illum, 1998). Recent finding revealed the antitumerigenic properties of chitin and chitosan oligomers as they inhibited the growth of tumor cells via an immuno-enhancing effect. The researcher showed that the chitin oligomer exhibited growth inhibition effect of Met-A solid tumor transplants into BALB/C mice. The antitumor was assumed to be involved in the increased production of lymphokines, interleukine 1 and 2, sequentially, leading to the manifestation of antitumor affect through proliferation of cytolytic-T lymphocyte. Chitosan has not only the antitumor activity but also an anticancer activity. Therefore, the growth and the invasive procession of cancer cells could selectively be inhibited by chitosan. In other cases, the introduction of chitin and chitosan for human or animal is helpful for healing of different diseases or for the prevention of sickness. Furthermore, chitin-derived products are used for drug and vaccine delivery systems (Felt *et al*., 1998; Illum, 1998), wound and burn treatments (Muzzarelli *et al*., 1999), blood cholesterol lowering agents, anti-clotting agent (Cue, 1999), antimicrobial compounds (Helander

et al., 2001; Bodek, 2002), and enhanced dissolution of some drugs such as ibuprofen (Ilango *et al*., 1999; watanabe *et al*., 1994). Some activities of chitin derivatives are shown in Table 1.2.

1.1.4.3 Food and nutrition applications

The *N*-acetylglucosamine (NAG) moiety, present in human milk, promotes the growth of bifido bacteria, which block the other types of microorganism and generate the lactase required for the digestion of milk lactose. Cow's milk contains only a limited amount of the NAG moiety; hence some infants fed with cow milk may have indigestion. Many animals and some humans, including the elderly, have similar lactose-intolerance (Knorr, 1991). Animal nutritional studies have shown that the utilization of whey may be improved if the diet contains small amounts of chitinous material. This improvement is attributed to the change in the intestinal microflora brought about by the chitinous supplement (Austin *et al*., 1989). Chickens fed with a commercial broiler diet containing 20% dried whey and 2 or 0.5% chitin has significantly improved weight gain compared to the controls (Spreen *et al*., 1984). The feed efficiency ratio shifts from 2.5 to 2.38 due to the incorporation of chitin in the feed (Zikakis *et al*., 1982). Interestingly, chitosan attaches itself to the fat in the stomach before it is digested, thus trapping the fat and preventing its absorption by the digestive tract. Fat, in turn, binds to the chitosan fiber, forming a mass, which can not be absorbed by the body, and is eliminated. Chitosan fiber differs from the other fibres in that it possesses a positive ionic charge, which gives it the ability to bond chemically with the negatively charged lipids, fats and bile acids (Muzzarelli, 1998, 1999).

Table 1.2 Physiological influences of chitin derivatives in the human body.

Significance	Activities
1. Antimicrobial activity	Reaction with an anionic component of the cell wall, chelation of ions in a metaloenzyme, changing in bacterial adhesion, inhibition of enzyme linking glucans to a chitin
2. Immunostimulation	Activation of macrophage selections and synthesis of interferons and interleukin
3. Chemotactic	Stimulation of a migration of fibroplasts and other stomal cells
4. Action as a source of GlcN and GlcNAc	Rebuilding of an extracellular matrix
5. Enhanced reconstruction of a connective tissue	Healing of an ulcer, meniscal lesions, osteoinduction, and wounds, influence on assemble and an orientation of collagen fibers
6. Dietary significance	Anticholeaterolemic and antiulcer activity lowering of a body overweight
7. Growth stimulation	Molecular recognition and entrapment of growth factor, stimulation of lectin type activity

1.1.4.4 Biotechnology application

The consumer demand for food without harmful chemical additive has focused the efforts in the discovery of novel natural antimicrobial additives. Because of the positive charge on the C-2 of the GlcN monomers at low pH, chitosan is more soluble, and has a better antimicrobial activity than chitin. Antimicrobial activity is made possible by the formation of chitosan complexes with anionic components of the cell walls (Hirano *et al*., 1995) such as *N*-acetylmuramic, sialic acid, and neuraminic acid, as well as an inactivation of microbial metaloenzymes through a chelation of metal ions. The minimal inhibitory concentrations of a chitosan and its derivatives vary significantly for different bacterial cultures. These variations are suggested to be due to the existing differences in the molecular weight and degree of acetylation of chitosan. Chitosan and its derivatives are also used as added components of cosmetic, toothpaste, and moisturizer. Chitosan has moisturizing effect on the skin. The effect is dependent on the molecular weight and the degree of deacetylation. Chitosan also offers protection against mechanical hair damage, and exhibits an antielectrostatic effect on hair. High-molecular weight chitosan increases the water resistance of emulsions protecting against irradiation. A cosmetic cream, supplemented with chitosan, increases a bioactive activity of lipophilic ingredients, such as vitamins, which better penetrate the outer layer of skin. This benefit is also caused by the activation of fibroblasts, and improvement of collagen deposition (Horner, 1997). Furthermore, dental fluids and toothpaste are added chitosan, which can seal dentinal tubules, and protect against microbial infection but maintain diffusion of ions and water. Moreover, the addition of chitin to the coating of waterproof textile causes a large increase in its water vapor permeability. The

finishing of wool fibers with chitin derivatives improves their dye-ability and colorfastness.

1.1.4.5 Environment and pollution control applications

Chitin, chitosan, and chitosan derivative containing cell walls are used as dye-removing agent such as methyl orange, phenolic dyes, polycyclic dyes, acid dyes, and azo dyes by hydrophobic interaction or sorption via their backbone or amino group. Chitin and chitosan have been reported to adsorb cadmium (Jha *et al*., 1988) and lead (McKay *et al*., 1989) on its surface from seawater, and used for the removal of a hexavalent chromium. Interestingly, highly porous gelled chitosan beads cross-linked with glutaraldehyde are effective for the removal of cadmium ions from the wastewater. Moreover, chitosan was used for the sorption of uranium from the nuclear effluents. It is indicated that the carboxylic and amino groups of chitosan act directly as ion exchange to bind uranyl ions.

Table 1.3 The currently practical uses of chitin, chitosan, and their derivatives.

Medical; • Bandages, Sponges • Artificial Blood Vessels • Blood Cholesterol Control • Tumor Inhibition • Membranes • Dental/Plaque Inhibition • Skin Burns/Artificial Skin • Eye Humor Fluid • Contact Lens • Controlled Release of Drugs • Bone Disease Treatment **Pulp and Paper;** • Surface Treatment • Photographic Paper • Carbonless Copy Paper **Membranes;** • Reverse Osmosis • Permeability Control • Solvent Separation **Water Treatment;** • Removal of Metal Ions, Dyes, Pesticides and Phenols • Flocculant/Coagulant: • Proteins, Amino Acids • Filtration	**Food Indutries;** • Removal of Dyes, Solids, Acids • Preservatives • Color Stabilization • Animal Feed Additive • Emulsifying Agent • Dietary Fibre **Agriculture;** • Seed Coating • Leaf Coating • Hydroponics/Fertilizer • Controlled Agrochemical Release **Cosmetics and Toiletries;** • Make-up Powder • Nail Polish • Moisturizers • Bath Lotion • Face, Hand and Body Creams • Toothpaste • Foam Enhancing **Biotechnology;** • Enzyme Immobilization • Protein Separation • Chromatography • Cell Recovery • Cell Immobilization • Ultrafiltration Membranes

1.1.5 Chitinase

Chitinase (EC 3.2.1.14) are glycosyl hydrolase that catalyze the hydrolysis of β(1,4)–linked *N*-acetyl-glucosamine (GlcNAc) polysaccharide (Figure 1.3). Chitinase have been detected in a wide range of organisms such as fungi, crustaceans (Watanabe *et al*., 1998), and insects (Ahmad *et al*, 2003) as well as in organisms that do not contain chitin such as bacteria, viruses, higher plants (Lam and Ng, 2000), animals and human. The roles of chitinase in these organisms are diverse. In invertebrates, chitinase acts by degrading the exoskeleton chitin in the cuticle or shell of an ecdysis (Kramer and Koga, 1986). Fungi produce chitinase to modify chitin, which are used as the important cell wall components. In bacteria, chitinase is used to digest chitin and utilize it as the carbon and energy sources. In higher plants, the chitinase is produced in the process of defense mechanism against fungal pathogens.

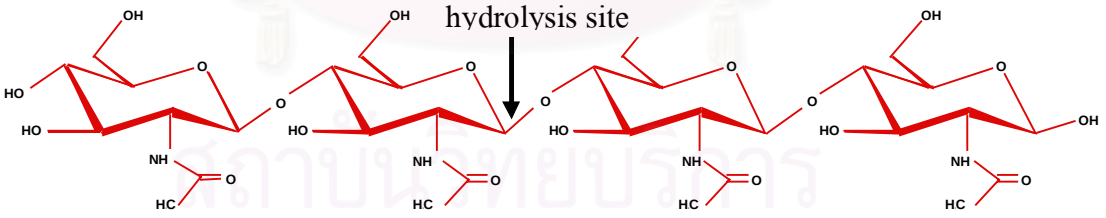


Figure 1.3 A hydrolysis reaction occurring within the GlcNAc chain. An arrow marks the hydrolysis site of chitin, the β(1,4)–linked *N*-acetyl-glucosamine (GlcNAc) polysaccharide substrate of chitinase.

1.1.6 Classification of chitinase according to their mode of action

Chitinases are classified as endochitinase and exochitinase. The endochitinase activity is defined as the random cleavage at the internal points in the chitin chain with the release of *N,N'*–diacetyl-D-glucosamine [chitobiose, $(ClcNAc)_2$] as a major product, the triacetylchitotriose, and a mixture of oligomers. The exochitinase activity is defined as the progressive hydrolysis starting at the non-reducing ends of chitin with the release continuously of $(GlcNAc)_2$ or GlcNAC.

1.1.7 Classification of chitinase based on amino acid sequences

Chitinases are found primarily in two families of glycohydrolases, family 18 and 19. Chitinases in family 18 are found in a wide range of organisms including bacteria, fungi, viruses, and animals, including the chitinases from class III and V of higher plants. Chitinases in Family 18, represented by the enzymes of *Coccidioides immitis*, *Trihoderma harzianum, Bacillus circulans,* and *Serratia marcessens*, contain several conserved signature amino acids, as shown in Figure 1.4a. These signature sequences are found in the active site including the Glu residue (E), which is crucial to the catalytic mechanism. Chitinases in family 19 include almost exclusively chitinases from plants in class I, II (Ponath *et al*., 2000), IV. It includes also the bacterial chitinases, *Streptomyces griseus* HUT 6307 chitinase C. The conserved amino acid sequence of chitinases in this family, represented by barley (*Hoedeum*

valgare), Potato (*Solanum tuberosum*), pea (*pisum sativum*), and *Arabidopsis thaliana*, is shown in Figure 1.4b. Interestingly, there are two glutamic acid residues which are important in the mechanism of action of the enzyme.

Figure 1.4 The amino acid sequence alignment of chitinase in family 18 (a) from *Coccidioides immitis, Trihoderma harzianum*, *Bacillus circulans,* and *Serratia marcessens* and family 19 (b) from *Hoedeum valgare*, *Solanum tuberosum*, *pisum sativum* and *Arabidopsis thaliana*.

1.1.8 Structure and function of chitinase

Chitin degradation of numerous chitinases involves the synergistic action of multiple proteins. These proteins typically include conserved modules that function as a catalytic domain and a chitin binding domain, and may contain a fibronectin type III like domain in which the function is unknown. An arrangement of these domains is different among the different chitinases (Figure 1.5).

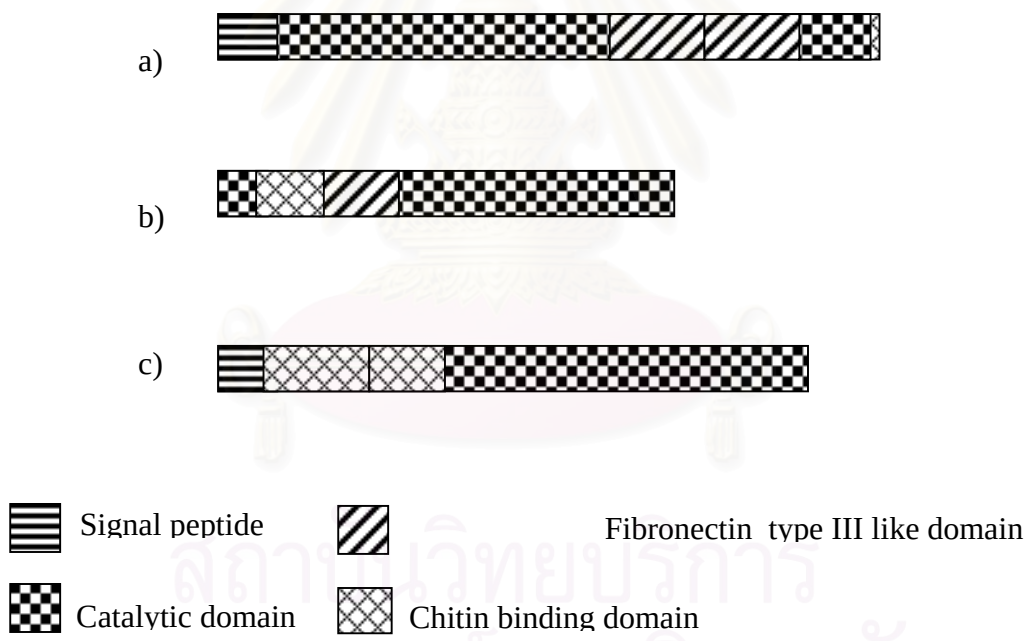


Figure 1.5 The domain arrangement of chitinases from various microorganisms.
a) Chitinase A1 from *B. circulans* wl-12
b) Chitinase C from *Alteromonas* sp.O-7
c) Chitinase A from *Microbulbifer degradan* 2-4

1.1.8.1 Catalytic domains

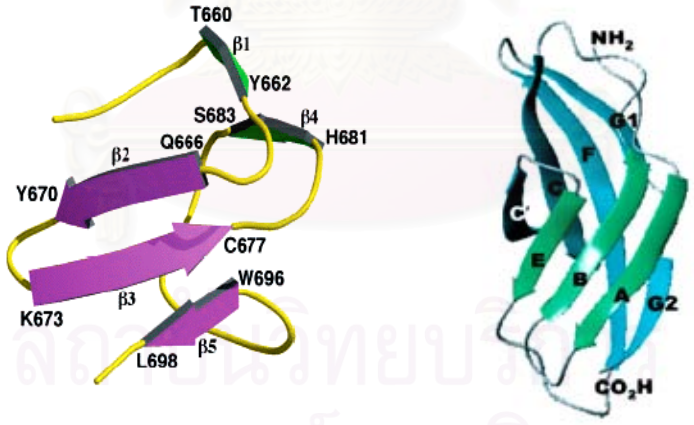
The three-dimensional (3D) structures of several family 18 chitinases have been solved, including those of *Serratia marcescens* chiA and the plant enzyme hevamine. The 3D structure reveals that they share a similar $(\beta/\alpha)_8$ barrel (Figure 1.6a,b). There are eight strands of parallel β sheet laid down with a α helix as the return stroke. The eight strands of the sheet bend into a barrel structure with the helices forming a ring toward the outside. However, the structure of only one member of family 19 chitinase, having been solved is *Hoedeum valgare* (barley). The 3D structure of the protein reveals a mixture of secondary structure, including ten α-helical segments, and one three-stranded β sheet as shown in Figure 1.6c.

a) b) c)

Figure 1.6 The 3D structure of some members of family 18 and 19 chitinase
a) Chitinase A from *Serratia marcescens* (family 18)
b) Chitinase from hevamine (family 18)
c) Chitinase from barley (family 19)

1.1.8.2 Chitin binding domain (chBD)

There are three families of chitin binding domains (chBD). The chBD family 1 is found in the chitinases from *Manduca sexta*, and *Penaeus japonicus*. The chBD family 2 is found in the chitinases from *Arabidopsis thaliana*, *Nicotiana tabacum*, and *Oryza sativa*. The chBD family 3 is found in the chitinases from *Bacillus licheniformis*, *Closidium paraputrificum*, and *Serratia marcescens*. Chitinase A1 from *Bacillus circulans* WL-12, which has no chitin-binding domain, has significant lower hydrolyzing activity for the insoluble substrate, indicating that the function of chitin binding domain of the chitinase A1 is to bind with the insoluble substrate.



a) b)

Figure 1.7 The three-dimensional structure of chitin-binding domain (a) and fibronectin type III-like domain (b) from *Bacillus circulans* WL-12

1.1.8.3 Fibronectin type III like domain (FnIIID)

FnIIID has been identified in a number of bacterial depolymerases and an animal fibronectin. It is likely that the bacterial module was initially acquired from an animal source and was then spread further among distantly related bacteria by the horizontal transfers. In several instances, these FnIIID occur as tandem repeats. The 3D structure of FnIIID of chitinase A1 WL-12, solved by NMR technique, is a β-sandwich fold with two antiparallel β-sheets that are packed face to face. One sheet is composed of three β-strands and the other of four β -strands. There are three loops in each N-terminal and C-terminal directions connecting to the seven β-strands (Figure 1.7b). The function of this module is unknown. Watanabe *et al* (1994) have shown that the deletion of the FnIIID of *B. circulans* chitinase A1 significantly reduces the activity of chitinase on chitin, but not on soluble substrates.

1.1.9 Mechanism of chitinases

There are two general mechanisms for acid-catalyzed glycoside hydrolysis which result in either (i) retention of the stereochemistry of the anomeric oxygen at C1' relative to the initial configuration or (ii) inversion of the stereochemistry

Extensive studies of the mechanism of a hen egg white lysozyme (HEWL) show that glycoside hydrolysis requires two acidic residues (Glu36 and Asp52), one of which is protonated. The consensus view of the mechanism (Figure 1.8a) involves the protonation of the β-(1,4)-glycosidic oxygen atom, leading to an oxocarbenium ion intermediate, which is stabilized by the secondary carboxylate group (either through covalent or electrostatic interactions) and then attacked by a solvent

molecule, which replaces the leaving sugar group. Distortion of the D sugar by interaction between the enzyme and substrate is through to play a major role in the transition state stabilization. Nucleophilic attack by water yields the hydrolysis product, which necessarily retains the initial anomeric configuration. This is commonly referred to as the double displacement mechanism of hydrolysis.

The X-ray crystal structure of a family 19 chitinase isolated from barley shows structural similarities with HEWL, suggesting an analogous double displacement mechanism. However, subsequent analysis of the anomeric products for the two family 19 chitinases shows that an inversion of the anomeric configuration accompanies these reactions. This observation rules out the double displacement mechanism of HEWL. A possible mechanism explaining inversion is a concerted single displacement reaction in which a bound water molecule acts as the nucleophile. Although, this water molecule was not observed in the crystal structure, the second catalytic carboxylate group is at a sufficient distance to allow coordination of a water molecule, consistent with a single displacement mechanism.

The substrate binding cleft of barley chitinase is an extensive one, and it has been hypothesized to contain at least six sugar binding subsites, labeled A-F, from the non-reducing end. The hydrolytic profile for hexasaccharides by barley chitinase suggests that the preferred binding of substrates may be at sites B-G that is, hexasaccharides are cleaved into two trisaccharides. This binding mode is together with the catalytic residues. Two carboxylates are hypothesized to be responsible for the catalysis, Glu67 as the catalytic acid and Glu89 as a base. Hydrolysis would occur between sugars in sites D and E, a convention developed for hen. The importance of these two residues to catalysis has since been confirmed by site-directed mutagenesis.

The conversion of these acids to the corresponding amide eliminates the measurable activity. The mechanism is hypothesized to be an inverting one, because the space between the “second carboxylate”, Glu89, and the susceptible glycosidic bond demands that the attacking water is interposed. This inverting mechanism is confirmed by using nuclear magnetic resonance (NMR) to follow the anomeric hand of the sugar product, which is an α– form. This result is consistent with a similar work showing that the chitinase from *Dioscorea opposite* (yam) proceeds with an inversion of a product. It is reasonable to assume that the family 19 chitinases work in this way (Figure 1.8b). The inverting mechanism proceeds through a positively charged oxocarbonium intermediate which has a distorted geometry; it is assumed a roughly half-chair configuration compared with the chair conformation of the other sugars. The single displacement mechanism involves Glu89 acting as a base to polarize the attacking water molecule, whereas Glu67 acts as an acid to protonate O4 of the leaving sugar.

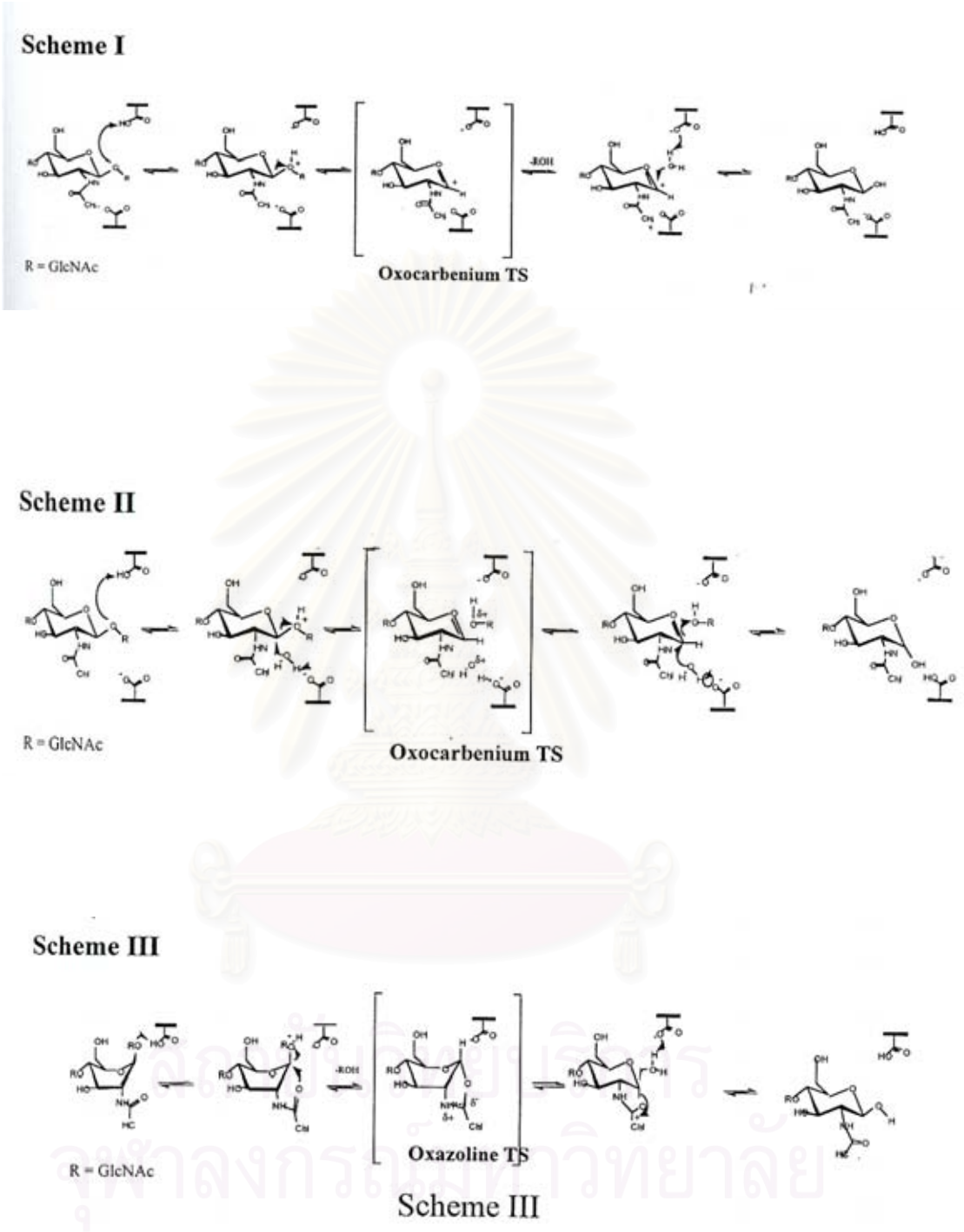


Figure 1.8 Mechanism of. catalysis of lysozyme (a), family 19 chitinase (b), and family 18 chitinase (c)

Family 18 chitinases have not been studied extensively as those from family 19. It has been suggested that the retaining mechanism of family 18 chitinases may involve in substrate assistance. That is, the *N*- acetyl group at position 2 of the scissile sugar may itself facilitate the reaction via the formation of transient oxazolinium intermediates as shown in Figure 1.8c. This view is supported by the crystal structure of a complex between the chitinase called hevamine and the inhibitor allosamidin which contains as oxazoline moiety which mimics the transition of the reaction. Allosamidin strongly inhibits family 18 chitinases, but does not inhibit the hen egg white lysozyme and human lysozyme. It consists of β-1,4-linked *N*-acetylallosamine residues, oxazoline derivative, and allosamizoline.

1.1.10 Applications of chitinase

1.1.10.1 Chitinases in biological control

The public concern over the harmful effects of chemical pesticides on the environment and human health has enhanced the search for safer, environmentally friendly control alternatives. Control of plant pests by the application of biological agents holds great promise as an alternative to the use of chemical substances. It is generally recognized that biological control agents are safer and more environmentally sound than is reliance on the use of high volumes of pesticides. Due to the importance of chitinolytic enzymes in insect, nematode, and fungal growth and development, they are receiving attention in regard to their development as bio-pesticides or chemical defense proteins in transgenic plants and microbial bio-control agents. In this sense, biological control of some soil-borne fungal diseases has been

correlated with chitinase production. Fungi and bacteria producing chitinases exhibit antagonism against fungi, and inhibition of fugal growth by plant chitinases has been demonstrated. Insect pathogenic fungi have a considerable potential for the biological control of insect pests. Entomopathogenic fungi apparently overcome physical barriers of the host by producing multiple extracellular enzymes including chitinolytic enzymes, which help to penetrate the cuticle and facilitate infection.

1.1.10.2 Aggressive and defensive roles for chitinase

Chitinases are produced by a wide variety of pathogenic and parasitic microbes and invertebrates during their attack on chitin-containing organisms. The chitinase of insect and algal viruses, yeast killer toxin plasmids, bacterial and fungal pathogens of fungi and insects, and parasitic protozoa play roles in dissolving the cell walls of fungi, exoskeletons and peritrophic membranes of arthropods. The salivas of some invertebrate predators have chitinolytic activity which may be involved in their attack on their prey. Chitinases play a major defensive role in all plants against attack by fungi, and perhaps also against attack by insect pests. The plant chitinases form a very large and diverse assemblage of enzymes from the two families of glycosyl hydolases. At least, some vertebrates, including fish and humans, also may utilize chitinases in their defense against pathogenic fungi and some parasites.

1.1.10.3 Production of chitooligosaccharides

A chitinase from *Vibrio alginnolyticus* is used to prepare chitopentaose and chititriose, which show an anti-tumor activity from colloidal chitin. Indeed, specific combination of chitinolytic enzymes with high levels of endo-chitinases and a low activity of *N*-acetylglucosaminidase and exo-chitinases would be necessary to

obtain a desired chain length of the oligomers. To obtain the GlcNAc, the higher proportion of exo-chitinases and *N*-acetylglucosaminidase is necessary. Alternatively, transglycosylation activity of variety of endo-chitinases will also be useful to generate desired chitooligomers and sometimes oligomers with changed glycosidic linkages. A chitinase from *Trichoderma reesei* also exhibits a similar type of an efficient transglycosylation reaction with the tetramer, giving the hexamer and a dimer as the major products. The purified *N*-acetylglucosaminidase of *Norcardia orientials* also exhibits transglycosylation activity. The β-1,6-linked disaccharide of GlcNAc and trisaccharide was synthesized during the hydrolysis of chitobiose.

1.2 Chitinase in Arthropods

1.2.1 Chitinase in insects

In insects, the cuticle and peritrophic matrix contain chitin that fulfill the role of providing tensile strength and impermeability to the exoskeleton, but unlike the endoskeleton lacks growth capability (Kramer *et al*., 1986). To grow, the insect has to undergo repeated cycles of moulting which are brought about chitinases and β-*N*-acetylglucosaminidase. Chitinase expression therefore coincides with the moulting process and is developmentally regulated in insects. Chitinase expressions are under hormonal control and are induced stage- and tissue dependently to degrade the chitin in the exoskeleton and peritrophic membrane (Kramer *et al*., 1993; Kramer and Muthukrishnan, 1997). Since chitin is a structural component in insects, chitin synthesis and hydrolysis are considered to be potential targets for pest management. A detailed understanding of chitin metabolism in target pests is necessary for the

development of chitinase-based insecticides. The chitinase genes have been cloned from various insects. In lepidopteron insects, chitinase cDNAs were cloned from *Manduca sexta* (Krameret *et al*., 1993), *Bombyx mori* (Kim *et al*., 1998), *Hyphantria cunea* (Kim *et al*., 1998), *Spodoptera litura* (Shinoda *et al*., 2001), and *Choristoneura fumiferana* (Zheng *et al*., 2002). These lepidopteron chitinases exhibit extensive similarity in both their amino acid sequences (70–80% identities to each other) and domain structures. The overall structures of chitinase genes have been determined in those from *M. sexta* (Choi *et al*., 1997) and *B. mori* (Mikitani *et al*., 2000), and their genomic organization is almost identical (Abdel-Banat and Koga, 2001). The copy number of the chitinase gene of *B. mori* has been estimated as one (Mikitani *et al*., 2000; Abdel-Banat and Koga, 2001), while there are at least four chitinase genes in dipteran insects (de la Vega *et al*., 1998).

1.2.2 Chitinase in crustaceans

Chitinases are involved in nutrient digestion, the partial breakdown of the exoskeleton prior to molting, and the regulation of physical properties of the peritrophic membrane. Chitinases are present in hepatopancreas of crustaceans and are thought to be involved in the digestion of chitin-containing food. The enzyme activity in this tissue is detected at significant levels throughout the molting cycle (Spindler-Barth *et al.*, 1990, Watanabe, *et al*., 1996). The crustaceans grow by periodic ecdysis (shedding of old exoskeleton) and subsequent reconstruction of a new one. Before molting, two types of chitinolytic enzymes, chitinase and β-*N*-acetylhexosaminidase, are induced in the integument to degrade a chitin. Chitin is

degraded to soluble disaccharides by the chitinase and subsequently hydrolyzed to *N*-acetylglucosamine by β-*N*-acetylhexosaminidase. When chitin is degraded, the products of hydrolysis are ultimately recycled for the synthesis of a new cuticle. Three chitinase genes have been isolated from the kuruma shrimp *Penaeus japonicus*. Two are expressed in the hepatopancreas (Watanabe *et al*., 1996, 1998) and the other is expressed in the cuticular tissues. These three genes are expressed in the intermolt and premolt stages either in the hepatopancreas or in the cuticle.

1.3 *Penaeus monodon*

Penaeus monodon, black tiger shrimp, belongs to the Arthropoda, the largest phylum in the animal kingdom. The taxonomic definition of *Penaeus monodon* is as follow (Baily-Brook and Moss, 1992)

Phylum Arthopoda

Subphylum crustacean

Class Malacostraca

Subclass Eumalacostraca

Order Decapoda

Suborder Natantia

Infraorder Penaeidea

Subfamily Penaeoidea

Family Penaeidae Rafinesque, 1985

Genus Penaeus Fabricius, 1798

Subgenus *Penaeus*

Specie *monodon*

Scientific name: *Penaeus monodon* Fabricius, 1798

Common name: Jumbo tiger prawn, Giant tiger prawn, Blue tiger prawn, panda prawn (Australia), Ushiebi (Japan).

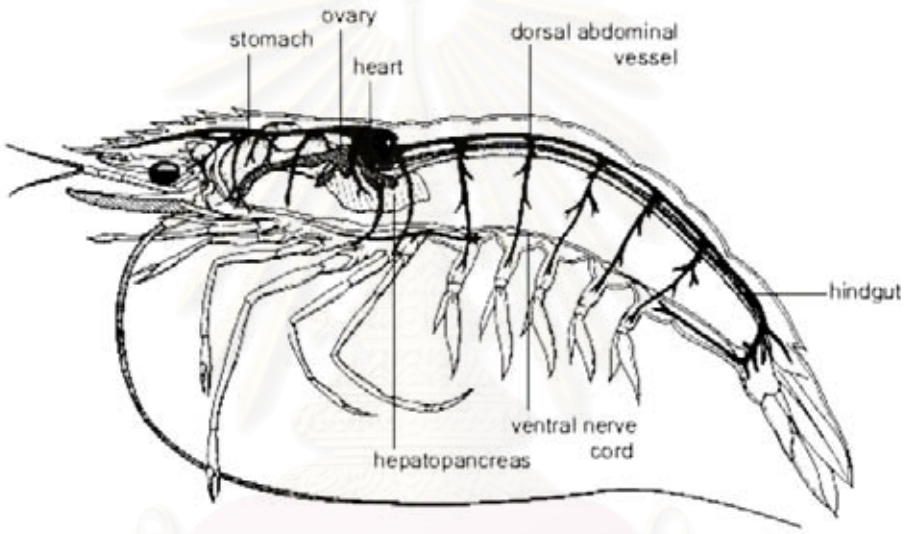


Figure 1.9 Lateral view of the internal anatomy of a female *Penaeus monodon* (Primavera, 1990)

A chitinase from the black tiger shrimp, *Penaeus monodon,* encodes a 621-amino acid protein possessing the functional domains of the chitinase protein family. The *Penaeus monodon* chitinase1 (*PmChi*-1) gene product is 81.8% identical to a chitinase1 protein expressed in the hepatopancreas of *Penaeus japonicus.* Analysis by reverse transcription-polymerase chain reaction (RT-PCR) indicates that *PmChi*-1 messenger RNA is detectable in the hepatopancreas and the gut. *PmChi*-1 activity

increases during molting cycle, suggesting that hepatopancrease is involved in the degradation of endogenous chitin in the gut peritrophic membrane prior to molting.

1.3 Antimicrobial peptide

Antimicrobial peptides are widespread in the living kingdom, and a large number of these molecules have been isolated from vertebrates and invertebrates (Hetru *et al*., 1994) as well as from plants. Despite the diversity in their structures, most of these antimicrobial substances are small (less than 10 kDa), cationic and amphipathic peptides. For the time being and for convenience, these antimicrobial peptides are tentatively classified into four distinct groups based on their amino acid sequences, secondary structures, and functional similarities. These are i) linear basic peptides forming amphipathic α-helices, which are devoid of cysteine residues, such as the cecropins, the first antimicrobial peptide isolated from the insect hemolymph; ii) peptides with one to six intra-molecular disulfide bridges, such as the arthropod defensins, cysteine-rich antimicrobial peptides isolated from a scorpion and a bivalve mollusk, and the hemocytes of horseshoe crabs containing various cystein-rich cationic peptides (tachyplesin, polyphemusin, tachycitin, tachystatin, and big defensin) with inhibitory effects on the growth of Gram-positive and Gram-negative bacteria and fungi; iii) one or two amino acids-rich peptides, such as arginine-, tryptophan-, histidine-, glycine-, and proline-rich peptides, for example, glycine-rich peptides; attacin and scarcotoxin, proline-rich peptides; drosocin from *Drosophila* hemolymp; iv) peptides derived from the partial hydrolysis of large precursor proteins, histone, and hemocyanin (Bulet *et. al*., 2004). The mode of action, the broad

activity, the molecular diversity, and the noncytotoxicity of all these circulating antimicrobial peptides make them very attractive as therapeutic agents for pharmaceutical or agricultural applications. The feature requiring for antimicrobial peptides are selective toxicity on microbial cells, short bacterial killing time, broad antimicrobial spectra and no bacterial resistance development (Matsuzaki, 2001).

A preliminary study of genes expressed in the black tiger shrimp, *Penaeus monodon*, comprised the construction of several cDNA libraries. One of this is the cDNA library from the hepatopancreas. The cDNA clone was sequenced and compared with those in the GenBank. A shrimp chitinase gene was isolated. In this study, the information obtained from the chitinase clone was used for the cloning of the complete chitinase gene from hepatopancreas of the black tiger shrimp. Attempt was made to express and characterize this chitinase.

CHAPTER II

MATERIALS & METHODES

Equipments

Autoclave, Model MLS-3020, Sunyo, Japan

Autopipette, Pipetman, Gilson, France.

Centrifuge, Model 5804R, Eppendorf.

Electrophoresis unit, Model Mini-protein II cell, BioRad, USA.

High performance liquid chromatography, Shimadzu, Japan.

Incubator, Model OB-28L Fisher Scientific Inc., USA.

Magnetic stirrer and heater, Model IKAMA®GRH, Janke & Kunel Gmbh & Co.KG, Japan.

Microfuge tubes, Bioactive, Thailand.

Orbital shaker, Gallenkamp, Germany.

pH meter, Model PHM95, Radiometer Copenhegen, Denmark.

Power supply, Model EC 139-90, E-C Apparatus Corporation.

Spectrophotometer, Jenway 6400, England.

Transformation apparatus, Bio-RAD, USA.

Vortex, Model K 550-GE, Scientific Inc., USA.

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

Chemicals

Acetonitrile (HPLC grade), Merck, Germany.

Acrylamide, Merck, Germany.

Agarose, SEAKEM LE, FMC Bioproducts, USA.

Ammonium hydroxide, Merck, Germany.

Ammonium persulfate, Sigma, USA.

Ammonium sulfate, Sigma, USA.

Ampicillin, Biobasic Inc., Thailand.

Boric acid, Merck, Germany.

Bromophenol blue, Merck, Germany.

β-mercaptoethanol, fluka, Swithzerland.

5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal), Sigma, USA.

Calcium chloride, Merck, Germany.

Chloroform, Sigma, USA.

Citric acid, Sigma, USA.

Coomassie® brilliant blue G 250, Fluka, Switzerland.

Dipotassium hydrogen phosphate anhydrous, Carlo Erba Reagenti, Italy.

Disodium hydrogen phosphate, Fluka, Switzerland.

Ethidium bromide, Sigma, USA.

Ethylenediamine tetraacetic acid (EDTA), Fluka, Switzerland.

Ethyl alcohol absolute, Carlo Erba Reagenti, Italy.

Ethylene glycol chitin, Seikagrku Coporation, Japan.

Formaldehyde, Sigma, USA.

Glacial acetic acid, BDH, England.

Glucose, Sigma, USA.

Glycerol, Scharlau, Spain.

Glycine, Sigma, USA.

ImProm-II™ Reverse Transcription System, Promega, USA.

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

Isoamyl alcohol, Merck, Germany.

Magnesium sulfate 7-hydrate, BDH, England.

Methanol, Scharlau, Spain.

N-acetyl-D-glucosamine, Sigma, USA.

N,*N*'-dimethylformamide, Merck, Germany.

N,*N*'-methyl-bis-acrylamide, Sigma, USA.

N,*N*,*N*',*N*'-tetramethyl-1,2-diaminoethane, Carlo Erba Reagenti, Italy.

Phenol, BDH, England.

Potassium acetate, Merck, Germany.

Potassium chloride, Sigma, USA.

Potassium ferricyanide, BDH, England.

Potassium phosphate monobasic, Carlo Erba Reagenti, Italy.

Qiaquick Gel Extraction Kit, Qiagen, Germany.

Qiaquick Miniprep Kit, Qiagen, Germany.

Qiaquick PCR Purification Kit, Qiagen, Germany.

Sodium azide, BDH, England.

Sodium carbonate, BDH, England.

Sodium chloride, Univar, Australia.

Sodium dihydrogen orthophosphate, Carlo Erba Reagenti, Italy.

Sodium dodecyl sulfate, Sigma, USA.

Sodium hydroxide, Carlo Erba Reagenti, Italy.

Tris-base, USB, USA.

Trisodium citrate dihydrate , Carlo Erba Reagenti, Italy.

Triton X, Merck, Germany.

Tryptone, Scharlau, Spain.

Xylene cyanol FF, Sigma, USA.

Yeast extract, Scharlau, Spain.

Yeast nitrogen base with ammonium sulfate without amino acid, Difco, USA.

Bacterial strains

Escherichia coli BL21(DE3) (F^- *ompT hsdS(r_Bm_B) gal dcm*(DE3)) was used for *chiHP74* and *CatD* expression.

Escherichia coli BL21(DE3)pLysS (F^- *ompT hsdS(r_Bm_B) gal dcm*(DE3) pLysS (Cm^R)) was used for *chiHP74* and *CatD* expression.

Escherichia coli Rosetta(DE3)pLysS (F^- *ompT hsdS(r_Bm_B) gal dcm lacY*1 (DE3) pLysSRARE(Cm^R)), which provided the rare codon tRNAs, was used for *chiHP74* and *CatD* expression.

Escherichia coli JM109 (F^- traD36 $proA^+$ *$proB^+$ $lacI^q$ lacZ ΔM15/recA1 endA1gyrA96 thi hsdR17 supE44 Δ(lac-proAB,mcr)*) was used for DNA manipulation.

Escherichia coli Top10 (F^- *mcr*A, Δ*(mrr-hsdRMS-mcrBC, ϕ80lacZΔM15* Δ *lacX74 deoR rec1 araD139* Δ*(ara-leu)7696galU galK rpsL (Str^R) endA1 nupG)* was used for DNA manipulation.

Escherichia coli XL10 Gold ultracompetent cells (Tet^R Δ(*mcr*A)183 *Δ(mcr*CB-*hsd*SMR-*mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F' *proAB lacl^q* ZΔ*M15 Tn10*(Tet^R)Amy Cam^R]^a

Yeast strain

Pichia pastoris KM71 His^+ Mut^S

Plasmid vectors

- The pET17b was used as an overexpression vector for chitinase gene in the bacterial expression system.
- The pGEM T-easy was used as an alternative vector for TA cloning.
- The pPIC9K was used as an overexpression vector for chitinase gene in the yeast expression system (Invitrogen).

Enzymes

- Restriction endonucleases, *BamH*I, *Bgl*II, *Eco*RI, *Eco*RV, *Mfe*I, *Nde*I, *Not*I, *Pst*I, and *Sma*I were purchased from New England Biolabs Inc.
- Lysozyme was purchased from Sigma.
- Lyticase was purchased from Sigma.
- Pfu polymerase was purchased from Sigma.
- proteinase K was purchased from GIBCO-BRL.
- RNase A was purchased from Sigma.
- T4 DNA ligase was purchased from Promega.

Media preparation

Media for bacteria

Luria-Bertani broth (LB medium)

LB broth consists of 1% (w/v) Bacto tryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl. For a solid medium, 1.5% (w/v) agar is added. Supplementation of ampicillin to the final concentration of 100 μg/ml is optional.

LB-colloidal chitin agar (LB-CC agar)

LB-CC agar consists of 1% (w/v) Bacto tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 1.5% (w/v) agar and 0.05% (w/v) colloidal chitin. Ampicillin is supplemented when needed.

Colloidal chitin minimum medium (CCMM)

CCMM consists of 0.02% (w/v) colloidal chitin, 0.05% (w/v) yeast extract, 0.1% (w/v) $(NH_4)_2SO_4$, 0.03% (w/v) $MgSO_4.7H_2O$, 0.6% (w/v) KH_2PO_4 and 1% (w/v) K_2HPO_4, pH 7.5. For a solid medium, 1.5% (w/v) agar was added. Ampicillin is supplemented when needed.

Media for yeast

Yeast extract peptone dextrose medium (YPD)

YPD consists of 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) dextrose (glucose). For a solid medium, 1.5% (w/v) agar was added. Geneticin is supplemented to the final concentrations of 0.25, 0.5, 0.75, 1.0, 1.5, and 1.75 mg/ml from the stock solution of 100 mg/ml.

Minimal dextrose medium (MD)

MD consists of 1.34% (w/v) YNB, 4×10^{-5} % biotin, and 2% (w/v) dextrose. For a solid medium, 1.5% (w/v) agar was added.

Buffered glycerol-complex medium (BMGY), and buffered methanol-complex medium (BMMY)

BMGY consists of 1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% (w/v) YNB, 100 mM potassium phosphate, pH 6, 4×10^{-5} % biotin, and 1% glycerol. For BMMY, 0.5% methanol is added instead of glycerol into the medium.

Preparation of cDNA

Hepatopancreas total RNA preparation

Hepatopancreas, collected from the shrimp, was added 1 ml of TRI reagent and briefly homogenized. The homogenate was stored at room temperature for 5 minutes to permit complete dissociation of nucleoprotein complexes. Consequentially, 200 µl of chloroform was added and vigorously shaken for 15 seconds. The mixture was incubated at room temperature for 2-5 minutes and centrifuged at 12,000×g for 15 minutes at 4 ºC. The colorless upper aqueous phase was transferred to a fresh microcentrifuge tube. RNA was precipitated by the addition of 500 µl of isopropanol. The mixture was left at room temperature for 5-10 minutes and centrifuged at 12,000×g for 10 minutes at 4 ºC. After centrifugation, the pellet was washed with 500 ml of 75% (v/v) cold ethanol. Then, the pellet was collected by centrifugation at 12,000×g for 10 minutes at 4 ºC and briefly air-dried for 5-10 minutes. The total RNA was dissolved with 50 µl of diethylpyrocarbonate (DEPC)-treated water, determined and estimated the concentration by measuring the optical density at 260 nm, and calculating in µg/ml unit, using the following equation:

$$[\text{RNA}] = OD_{260} \times \text{dilution factor} \times 40^{*}$$

* The optical density (OD) at 260 nm of 1.0 corresponds to the RNA of approximately 40 µg/ml (Sambrook et al., 1989).

Formaldehyde-agarose gel electrophoresis

A 1% (w/v) formaldehyde agarose gel was prepared using 1× MOPS buffer (0.2 mM MOPS, 50 mM sodium acetate and 10 mM EDTA, pH 7.0). The gel slurry was boiled until complete solubilization, and allowed to cool to 60 ºC. Formaldehyde (0.66 M) and ethidium bromide (0.2 µg) were added to the gel and poured into a chamber set.

Ten micrograms of the total RNA in 3.5 µl of DEPC-treated water, 5 µl of formamide, 1.5 µl of 10× MOPS and 2 µl of formaldehyde were mixed and

incubated at 65 ºC for 15 minutes. The mixture was immediately placed on ice. One-forth volume of the gel-loading buffer (50% v/v glycerol, 1 mM EDTA, pH 8.0 and 0.5% w/v bromphenol blue) was added to each sample. The sample was loaded into the 1% agarose gel containing formaldehyde and ethidium bromide. The RNA marker was used as a standard RNA marker. Electrophoresis was carried out in 1× MOPS buffer at 50 volts, until bromphenol blue was migrated approximately ¾ of the gel length. The total RNA was visualized as fluorescent bands using a UV transilluminator (UVP).

First-stranded cDNA synthesis

The first-strand cDNA was generated using the method according to ImProm-II™ Reverse Transcription System. Three micrograms of the total RNA sample and 0.1 µM of oligo(dT) primers were mixed. Sterile water was added and adjusted the final volume to 5 µl. The reaction was incubated at 70 ˚C for 15 minutes and immediately placed on ice for more than 5 minutes. Subsequently, 2 µl of 5× reaction buffer, 2 µl of the dNTP mix (5 mM of each dATP, dCTP, dGTP, and dTTP), 1µl of reverse transcriptase (200 units/µl), and 0.5 µl of recombinant RNasin® ribonuclease inhibitor (20U) were added and gently mixed. The reaction was incubated at 42 ºC for 1 hour. Consequently, the reaction was incubated at 75 ºC for 10 minutes to terminate the reverse transcription activity. The cDNA was diluted with nuclease free water to 1:10 dilutions, used as template for PCR reaction, and stored at -20 ºC until used.

Amplification of chitinase gene

The cDNA from the hepatopancreas of *P. monodon* was used as template for PCR amplification of the chitinase gene with a pair of degenerate oligonucleotide primers. The two primers were designed based on the ORF of chitinase 1 gene from *Penaeus monodon* (*PmChi-1*). The cloning sites were incorporated into the forward and reverse primers. Their sequences were as follows:

1. Forward primer with the *Nde*I site

   HpforI: 5’ GGAATTCCATATGGTGAGCGGCCGAGTT 3’

2. Reverse primers
   - Reverse primer with *Bam*HI site for shorter chitinase gene

     HprevII: 5’ CGGGATCCAGGCTCTGACTTGGAAAG 3’

   - Reverse primer with *Not*I site for longer chitinase gene

NHPyRewII: 5’AAGGAAAAAAGCGGCCGCTCAATGGTACACTTTAGTTC 3’

   - Reverse primer with *Bam*HI site for the catalytic domain (CatD) of chitinase

     RCatD: 5’ CGGGATCCCTAAAGAGTGGGAGC3’

PCR amplification was performed as follows: pre-denaturation at 94 ºC for 5 minutes, 25 cycles of denaturation at 94 ºC for 30 seconds, annealing at 55 ºC for 30 seconds, extension at 72 ºC for 4 minutes. The final extension step was performed at 72 ºC for 10 minutes. A prominent PCR product size of full-length and CatD were approximately 1.95, and 1.2 kb, respectively.

A chitinase gene was purified by phenol-chloroform precipitation method (see Appendices A). After precipitation, the DNA pellet was dissolved in 20 µl of TE buffer. The gene was tailed with an adenine nucleotide and ligated to the pGEM-T Easy vector, called a recombinant plasmid (see Appendix B). The recombinant plasmid was transformed into an *E. coli* strain Top10 using electroporation. The transformants were selected using the blue white screening on the ampicillin agar plates. A single white colony of *E. coli* harboring a recombinant plasmid was grown in 1.5 ml LB broth containing 100 µg/ml of ampicillin and incubated at 37 ºC for an overnight. The recombinant plasmid was prepared by a minipreparation method as described by Sambrook and Russell (2001) and digested with *Nde*I and *Bam*HI at 37 º C for an overnight. The digested plasmid was analyzed by 0.8% agarose gel electrophoresis. The sizes of full-length and CatD PCR product were approximately 1.95 and 1.2 kb in length, respectively. Each DNA fragment was purified by using the QIAquick Gel Extraction Kit.

Recombinant expression of chitinase gene in the *Escherichia coli* expression system

Preparation of the expression vector pET-17b

The expression vector used in this experiment was pET-17b. The restriction sites used to clone the chitinase gene into vector were *Nde*I and *Bam*HI. The pET-17b was prepared according to midipreparation method as described by Sambrook and Russell (2001).

A single-colony of *E. coli* TOP 10, containing the pET-17b, was inoculated into 25 ml of LB medium supplemented with 100 μg/ml ampicillin. The culture was incubated overnight at 37 ºC, transferred into a 15-ml tube and harvested by centrifugation at 5,000 rpm, 4 ºC for 3 minutes. The pellet was resuspended in 1 ml of solution I (25 mM Tris-HCl, pH 8, 10 mM Na_2EDTA, 50 mM glucose and 5 mg lysozyme), gently mixed and kept on ice for 5 minutes. A 2 ml of freshly prepared solution II (1% SDS, 0.2 N NAOH) was added, mixed by inversion and kept on ice for 10 minutes. The mixture was neutralized by adding 1.5 ml of 7.5 M ammonium acetate, mixed and left on ice for 20 minutes. After centrifugation at 5,000 rpm, 4 ºC for 15 minutes, the aqueous phase was transferred to a new tube. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and gently mixed. The mixture was centrifuged at 5,000 rpm, 4 ºC for 15 minutes. Consequently, two volumes of absolute ethanol were added, mixed and stored at -20 ºC for 30 minutes. The plasmid was collected by centrifugation at 5,000 rpm, 4 ºC for 15 minutes and air dried for 10 minutes.

The pellet was dissolved in TE-buffer containing 20 μg/ml of DNase-free RNaseA, incubated at 37 ºC for 10 minutes. The plasmid was then extracted with phenol:chloroform:isoamyl. Then, 50 μl of 3M sodium acetate and one volume of phenol:chloroform:isoamyl were added, extracted and centrifuged at 5,000 rpm, 4 ºC for 15 minutes. Two volumes of absolute ethanol were added, mixed and stored at -20 ºC for 30 minutes. The plasmid was collected by centrifugation at 5,000 rpm, 4 ºC for 15 minutes, and air-dried for 10 minutes. The pellet was resuspended in TE-buffer and stored at -20 ºC until used.

Restriction enzyme digestion

Since the expression vector pET-17b contains *Nde*I and *Bam*HI restriction sites, these two restriction sites were then incorporated into the primer used for the amplification of chitinase gene. The vector and clone of the amplified chitinase gene were digested with *Nde*I and *Bam*HI. Approximately 20 μg of DNA was digested in 50 μl reaction volume, containing 1× *Bam*HI buffer (150 mM KCl, 10 mM Tris-HCl, 10 mM Mg_2Cl, 1 mM dithiothreitol), 100 μg/ml BSA, and 10 units of *Bam*HI and *Nde*I. The reaction was incubated at 37 ºC for an overnight.

After digestion with *Nde*I and *BamH*I, the pET-17b was extracted with a phenol:chloroform solution and ethanol precipitated. The DNA pellet was dissolved in 20 μl of TE buffer. The DNA concentration was estimated using 0.8% agarose gel electrophoresis under UV transilluminator by comparing with the λ/*Hin*dIII marker. The digested pET-17b was used for the cloning of chitinase gene.

Ligation

A suitable molecular ratio between vector and inserted DNA in a mixture of cohesive-end ligation is usually 1:3. To calculate the appropriate amount of PCR product (insert) used in ligation reaction, the following equation was used:

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

The 10 μl ligation reaction was composed of 1 μl of 10× T4 DNA ligase buffer, 1 unit of T4 DNA ligase, 10 ng of *Nde*I *and Bam*HI digested pET-17b and 4 ng of *Nde*I *and Bam*HI digested amplified chitinase gene. The reaction was incubated at 12 ºC for an overnight. One microlitre of the ligation mixture was transformed into the competent *E. coli* Top10.

Electrotransformation

The competent cells were gently thawed on ice (see Appendix C). Forty microlitre of cell suspension were mixed well with 1 μl of the ligation reaction,

and placed on ice for 1 minute. The cells were transformed by setting the GENE pulser apparatus (Bio-RAD) as follows: 25 μF, 200 Ω of the pulse controller unit, and 2.50 kV. After one pulse was applied, the cells were immediately resuspended with 1 ml of LB broth. The cell suspension was incubated at 37 ºC with shaking at 250 rpm for 45 minutes. The cell suspension was spread on the LB agar plate containing 100 μ g/ml ampicillin and incubated at 37 ºC for an overnight. After incubation, colonies were randomly selected for plasmid isolation.

Isolation of the recombinant plasmid

The recombinant plasmid containing chitinase gene was selected by restriction enzyme digestion using *Nde*I *and Bam*HI. The digested plasmid was analyzed by 0.8% agarose gel electrophoresis. The clone containing the correct DNA fragment of approximately 1.95 and 1.2 kb for full-length and CatD, respectively, were selected. DNA sequencing was performed to confirm the correct junction of the vector and the inserted DNA as well as the sequence of the chitinase gene.

Expression of recombinant chitinase gene

After the correct expression clone was identified, a recombinant chitinase gene was used for transformation into the expression host. The expression hosts used in this experiment were *E. coli* strains BL21(DE3), BL21(DE3)pLysS, and Rosetta(DE3)pLysS. A single colony was picked from the spread plate and inoculated in 10 ml LB containing the appropriate antibiotic (100 μg/ml of ampicillin for BL21 (DE3), 100 μg/ml of ampicillin and 34 μg/ml of chloramphenicol for BL21(DE3) pLysS and Rosetta(DE3)pLysS in 125 ml Erlenmeyer flask. The culture was incubated with shaking at 37 ºC and 30 ºC until the OD_{600} reached 0.8. Consequently, cell culture was inoculated in the 500 ml of LB medium containing appropriate antibiotic. When the optical density at 600 nm (OD_{600}) reached 0.6-0.8 (approximately 2-3 hours), IPTG was added to the final concentration of 0.25 mM and then incubated overnight. One milliliter of culture medium was collected after induction at 0, 1, 2, 3, 4 hours, and overnight. Both the pellet and medium fractions were collected by centrifugation at 5,000 rpm for 10 minutes at 4 ºC, and analyzed by

SDS-PAGE. The chitinase was followed by activity staining and chitinase activity assay. The pellet was resuspended in 100 µl of 2X loading dye. This sample was heated for 20 minutes in boiling water. Five microlitre of the denatured sample was aliquot and 5 µl of 2X loading dye was added together to obtain 10 µl of sample for running in PAGE. This new sample was reheated for 10 minutes in boiling water before loading. The medium fraction was added 1/10 volume of 100% TCA (w/v), gently vortexed for 15 seconds and placed on ice for a minimum of 15 minutes. Consequently, the mixture was centrifuged at 12,000 rpm for 10 minutes. The pellet was washed twice with 100 µl of acetone. The pellet was dried thoroughly and resuspened in 50 µl of 1 N NaOH. Ten microliters of sample was mixed with 1× sample buffer and heated for 10 minutes in boiling water. Finally, the mixture was stored at -20 ºC until used.

Expression of chitinase in the *Pichia pastoris* expression system

Screening of the recombinant plasmid

The *Pichia* muti-copy expression vector, pPIC9K, was selected for chitinase expression. It carries kanamysin resistance to G418 in *Pichia.* Spontaneous generation of multiple insertion events, which occurs in *Pichia* at a frequency of 1-10%, can be identified by the level of resistance to G418.

Pichia transformants were selected on histidine deficient medium and screened for their level of resistance to G418. An increasing level of resistance to G418 indicates multiple copies of the kanamycin resistance gene and of the interested gene. Increasing copy number of the interested gene in a recombinant *Pichia* strain may increase protein expression levels.

Amplification of cDNA encoding chitinase

For the construction of an expression cassette, the forward and reverse primers were designed. In addition to the convenience of cloning, *Mfe*I site was added

to the 5'-end of the forward PCR primers. Besides, a *Not*I site was added to the 3'-end of the reverse primer. Primer sequences were:

NHPyfor: 5' GCGCAATTGATGGTGAGCGGCCGAGTT 3'
NHPyRewII: 5' AAGGAAAAAAGCGGCCGCTCAATGGTACACTTTAGTTC 3'

These primers were used to amplify the chitinase gene, which was then cloned in-frame into the *Pichia* expression vector, pPIC9K, and transform to *E. coli* XL-10 GOLD cells.

The PCR amplification was performed as follows: denaturation at 94 ºC for 5 minutes, 25 cycles of denaturation at 94 ºC for 30 seconds, annealing at 55 ºC for 30 seconds and extension at 72 ºC for 4 minutes, and final elongation step at 72 ºC for 10 minutes. A prominent PCR product of approximate 2 kb was cloned and sequenced.

Construction of the recombinant plasmid

A PCR product was purified by Qiaquick PCR Purification Kit, cloned into the pGEM-T easy vector and transformed the *E. coli* Top10. The recombinant colonies were selected by blue-white colony screening. The plasmid containing chitinase was subcloned into the pPIC9K vector. The recombinant plasmid and the pPIC9K vector were digested with *Mfe*I/*Not*I and *ECo*RI/*Not*I, respectively. After digestion, the reaction was loaded and separated on 0.8% agarose gel. The target band of each reaction was eluted and purified from gel through the QIAquick Gel Extraction Kit. These two fragments was ligated together and transformed into *E. coli* XL-10 GOLD. The transformants were screened on a LB/ampicillin plate. The recombinant colonies were selected for plasmid extraction and restriction enzyme analysis. The plasmid containing the chitinase gene was sequenced.

Electrotransformation

The recombinant plasmid was prepared by midipreparation. The extracted plasmid was linearized by digested with restriction enzyme *Sac*I.

The competent cells were gently thawed at room temperature and then immediately placed on ice. Eighty microlitres of the cell suspension was mixed with 5-20 μg of the *Sac*I linearized chitinase-containing plasmid and placed on ice for approximately 5 minutes. The mixture of cell and DNA was transformed by electroporation in a cold 0.2 cm cuvette with the apparatus setting as 25 μF of the Gene pulser; 200 Ω of the pulse controller unit, and 1.50 kV of the Gene pulser apparatus (Bio- RAD). After one pulse was applied, the cells were immediately resuspended with 1 ml of ice cold steriled 1 M sorbital and transferred to a test tube. The 250 μl cell suspension was spread onto the YPD agar plates and incubated at 30 º C until colonies appear. After incubation, colonies were subsequently screened for G418 resistance at a final concentration of 0.25, 0.5, 0.75, 1, and 1.5 mg/ml G418. Colony PCR was used to confirm the presence of the target gene in the chromosome of recombinant yeast before it was used in protein expression.

Detection of the desired recombinant plasmid by colony PCR

The PCR reaction was used to confirm the presence of chitinase gene in the yeast chromosome. The yeast genomic DNA was used as a template and amplified with the 5'AOX and α-factor primers. The yeast genomic DNA was prepared by picking a single colony and resuspending in 10 μl of water. Then, 5 μl of a 5 U/μl solution of lyticase was added. The mixture was incubated at 30 ºC for 10 minutes, and frozen at -80 ºC for 10 minutes or immersed in the liquid nitrogen for 1 minute. The amplification reaction was prepared in a 50 μl reaction volume containing 5 μl of cell lysate, 25 mM of each dNTP (dATP, dCTP, dTTP, dGTP), 1× PCR buffer, 2.5 mM $MgCl_2$ and 10 pmole/μl of each primers. Sterile distilled water was added to make the final volume to 49.6 μl. The amplification was started with initial denaturation at 95 ºC for 5 minutes. The *Taq* DNA polymerase of 0.8 units was added. The reaction was followed by 30 cycles of 95 ºC for 1 minute, 54 ºC for 1 minute and 72 ºC for 1 minute. The final extension was 72 ºC for 7 minutes. The resulting PCR reaction was analyzed on the 1% agarose gel to determine whether the DNA fragment was successfully amplified.

Expression of chitinase in yeasts

A single colony of selected clone was inoculated in 5 ml of YPD in a 50-ml conical flask, cultured at 30 ºC with shaking for an overnight, and used as the starter. The 0.2 ml of the starter was then inoculated into 100 ml of BMGY medium in a 1-liter flask. The culture was grown overnight to an optical density at 600 nm (OD_{600}) of 2-6 (approximately 16-18 hours). The cells were harvested by centrifugation at 3,000g for 5 minutes at room temperature. To induce the recombinant protein expression, decant the supernatant and resuspend cell pellet in 10 ml BMMY medium. Place suspension in a 100 ml baffled flask and return to incubator to continue to grow. Add 100% methanol to a final concentration of 0.5% (v/v) every 24 hours to maintain induction. At every 24 hours for 6 days transfer 1 ml of the expression culture to a 1.5 ml microcentrifuge tube. Centrifuge at 10000 rpm in a microcentrifuge for 2-3 minutes at room temperature. Transfer the supernatant to a separate tube and store the supernatant at -80 ^{o}C until ready to assay. Analyze the cell pellets for protein expression by SDS-Polyacrylamide Gel Electrophoresis. These samples are used to analyze expression levels and determine the optimal time post-induction to harvest.

Characterization of chitinase

Preparation of chitinase

The overnight cell culture was collected and centrifuged at 5,000 rpm for 10 minutes at 4 ºC. The supernatant was removed and stored at 4 ºC, whilst the pellet was resuspended in 0.25% cold lysis buffer (3 mM EDTA, 20 mM Tris-HCl buffer, pH 7.5, 0.5% triton-X 100) and sonicated using the Bendelin Sonoplus uW 2200 (Bandelin Electronic) with the KE76 probe (6 mm diameter) for 2 minutes, 20% of amplitude. The sample was stored at -20 ºC.

Chitinase activity assay

Chitinase activity was assayed using a modified method of Schales (Imoto, 1971). The assay was based on the increase of reducing sugar. The reaction volume of 1.5 ml consisted of an appropriate amount of enzyme solution, 1.0 mg/ml β-chitin, 0.15 ml of 1.0 M phosphate buffer, pH 7.0, and 200 μl of chitinase. Reaction mixture was incubated at 37 ºC for 1 hour. Two microliters of color reagent (0.5 g potassium ferricyanide in 1 liter of 0.5 M sodium carbonate) was added to the reaction mixture to stop the reaction. The solution was heated for 15 minutes, and rapidly cooled. Excess β-chitin was removed by centrifugation at 5,000 rpm for 15 minutes. The supernatant (A_1) was measured for the optical density at 420 nm with a spectrophotometer. A bank value (A_0) was obtained by using pre-heat enzyme (enzyme was heated in boiling water for 20 minutes). The enzyme activity was determined from the different values of A_0-A_1. One unit (U) of enzyme activity was defined as the amount of the enzyme capable to liberate 1 μmole of either *N*-acetylglucosamine or D-glucosamine per hour (Appendix E).

Optimum pH of chitinase

The optimum pH of chitinase was determined by assaying the activity of the enzyme at various values of pH. A universal buffer was used in the range of 2.6-10. The chitinase activity was assayed according the method described above.

Optimum temperature of chitinase

The optimum temperature of chitinase was determined by assaying the chitinase activity at temperatures in the range of 30-80 ºC. The assay was done using a method described above.

Substrate specificity

The crude enzyme was incubated with each of the following substrates; 0.1 mg/ml partially *N*-acetyl chitin (PNAC), 1.0 mg/ml colloidal chitin, 1.0 mg/ml regenerated chitin, 1.0 mg/ml β-chitin, 0.1 mg/ml 75% colloidal chitosan, and 0.1

mg/ml 100% colloidal chitosan at 37 ºC for 1 hour. The activity was assayed by using a method described above.

Determination of hydrolytic products

The crude chitinase was incubated under optimum conditions for an overnight. The completely hydrolyzed sample was centrifuged at 12,000 rpm for 10 minutes. The products in the supernatant liquid were analyzed by HPLC and TLC methods. For HPLC method, 0.3 ml of the supernatant liquid was mixed with 0.7 ml of acetronitrile, and filtered through a 0.45-micron filter. The hydrolytic products were analyzed by HPLC through the Shode Asahipak NH_2P-50 column using a mobile solvent of 30:70 water:acetronitrile and a flow rate 1.0 ml/min. The reaction mixture of 20 µl was injected into the HPLC column and the optical density at 210 nm was measured with a UV detector.

For the TLC method, the supernatant liquid was dried by vacuum centrifugation. Subsequently, the pellet was resuspended with 10 µl of deionized water and spotted on the silica gel plate for thin layer chromatography (TLC). A mixture of *n*-propanal:ethanol:water (5:2:1, v/v) was used as the developing solvent, and the amino sugars were detected by spraying the plate with aniline-diphenylamine reagent and baking at 120 ºC for 30 minutes.

CHAPTER III

RESULTS

Searching and identifying the chitinase genes from the database

A total of 10,100 EST sequences in *Penaeus monodon* EST database (http://pmonodon.biotec.or.th) were searched for putative chitinase genes by using a searching program. A complete search shows that the EST database contained three putative chitinase genes: two from a hemocyte library and another from a hepatopancreas library. Sequences of these putative genes were reconfirmed by alignment with BLASTN program. The results show all sequences are similar to chitinase gene. One clone found in the hepatopancreas library, named *HPa0029,* was selected for study in all experiments. A homology searching of this putative chitinase gene using the BLASTN program demonstrate has 88% homology to the *Penaeus japonicus chitinase* 1, named *PjChi*-1 (Watanabe, T., *et al* 1996) and 87% homology to the *Penaeus monodon chitinase* 1, named *PmChi*-1 (Tan *et al*., 2000) as shown in Figure 3.1

Recombinant expression of chitinase gene in the *Escherichia coli* expression system

Hepatopancreas collection and total RNA preparation

From the BLASTN result, the full-length of this gene was investigated from an expressed gene, total RNA, by using specific primer in order to amplify the

expressed fragment from start codon to the site of 2027 and 2008 *Pjchi*-1 and *PmChi*-1, respectively, after stop codon (Figure 3.1). On the reverse primer, this primer was designed at the location that more conserves on both *Pjchi*-1 and *PmChi*-1. However, reverse primer sequences were based on *PmChi*-1 which was more similar to the chitinase gene found in *P. monodon* than *Pjchi*-1 did.

To obtain the mRNA, a hepatopancreas of normal shrimp was collected and was ground in liquid nitrogen using chilled motars and pestles. The TRIreagent (Molecular biology grade) was added for extracting a total RNA from hepatopancreas as described in Materials and Methods. The A_{260}/A_{280} ratio of the total RNA sample was 1.64, which is the expected ratio for acceptable quality of the total RNA. The extracted total RNA was examined by electrophoresis on a denaturing formaldehyde-agarose gel as shown in Figure 3.2. The result shows the total RNA extracted from hepatopancreas.

```
Pjchi1          CCGAGTGACCGTCGCTATCAGCTCCTCCGAGGACACTTCGCTGACCG---GCGGCTCTTG 57
Pmchi1          ----------------------GCACGAGGAGACTCGACGCACTGCAGACCGTTCTCT 36
HP0029          ------------------------------------------------------------

Pjchi1          CGCTCATTCGCTGCGACTCCGCCAAGATGCTGAGCGGACGCGTTTTAGCAGCTCTTGTAG 117
Pmchi1          TGGTCGTCCGTTGAGACTCCACCAAGATGCTGAGCGGCCGAGTTTTGGCAGTCCTAGTGA 96
HP0029          ------------------------------------------------------------

Pjchi1          CTGTCGCAGCGTTGGCAACACTGGTGTTGGCTGACCCGAGAATCGAGCGGGAAGGAGCGC 177
Pmchi1          CGCTTGCAGCATTGGCAACACTAGTGTTGGCTGACCCGAGATTCGAGCAAGAAGGAGCCC 156
HP0029          ------------------------------------------------------------

Pjchi1          AGCGCAAGTGGGTTCGCCCCGAGGGTCAGGCGCGGCGCGTGTGTTACTACGAGGCGTGGG 237
Pmchi1          AGCGGCGGTGGGTGCGGCCCGAGGGCCAGGCGCGCCGCGTGTGCTACTACGAGGCGTGGG 216
HP0029          ------------------------------------------------------------

Pjchi1          CGATCTACCGGCCCGGCGACGGCTTCTACGACATCGAGGACATTCCCGCCGGCCTGTGCA 297
Pmchi1          CCATCTACCGGCCCGGCGACGGCTTCTACGACATCGAGGACATCCCCGCCAACCTGTGCA 276
HP0029          ------------------------------------------------------------

Pjchi1          CGGACCTCATCTACTCGTTCATCGGCCTTTCCAATGTGACCTGGGAAGTGCTC-TTCTTG 356
Pmchi1          CGGACCTCATCTACTCGTTCATTGGCCTCTCCAACGTCACGTGGGAAGTGCTCATTCTCG 336
HP0029          ------------------------------------------------------------

Pjchi1          ACCCTGAGTACGACATCAACATGAATGGCTTCCGGCGGTTCGTGGCGCTGAAGGAGAAGT 416
Pmchi1          ACCCTGAGTACGACATCAACCTTGACGGTTTCCGCCGGTTCGTGGCGCTGAAGGACAAGT 396
HP0029          ------------------------------------------------------------

Pjchi1          ATCCCGACATGAAGACGAACATTGCCGTCGGGGGCTGGGCCGAGGGCGGCAGGAAGTACT 476
Pmchi1          ACCCTGACATGAAGACAAACATCGCCGTGGGCGGCTGGGCCGAGGGCGGAAGGAAGTACT 456
HP0029          ------------------------------------------------------------

Pjchi1          CGCAGATGGTGATGGTCGCCGAGAGGAGGGCGTCCTTCATCAGAAGCGTCGTCCAGCTGC 536
Pmchi1          CGCAGATGGTGATGGTGCCCGAAAGGAGGGCGTCTTTCATCAGGAGCGTCGTCCAGCTGC 516
HP0029          ------------------------------------------------------------

Pjchi1          TCACTGACTACGGCTTCGACGGCCTGGACTTGGACTGGGAATACCCTGGCGCCACAGACC 596
Pmchi1          TCACCGACTACGGCTTCGACGGGTTGGACTTGGACTGGGAATACCCTGGCGCCACAGACC 576
HP0029          ------------------------------------------------------------

Pjchi1          GCGGAGGCCAGTATGCCGACAAGGACAACTTCCTTAAGTTCGTGCAGGACGTGCGCGAGG 656
Pmchi1          GAGGAGGCCAATATGCCGATAAGGACAACTTCCTTAAACTGGTGCAGGAGCTGCGCGAAG 636
HP0029          ------------------------------------------------------------


Pjchi1          CCTTCGACACGGTAGGGCTCGGCTGGGAGATCACCTGCGCCGTGCCTGTGGCCAAGTTCC 716
Pmchi1          CCTTCGACACCGTGGGGCTGGGCTGGGAAATCACGTGCGCCGTGCCCGTGGCCAAGTTCC 696
HP0029          -------------GGAAAAGGATGGGAACTGACGTGTGCTGTCCCTGTGGCCAAGTTCA 46
                             **    ** *****  * ** ** ** ** ** ************
```

```
Pjchi1          GCCTGCAGGAGGGTTACCACGTGCCTCAGCTCTGCAGTCTGCTGGACGCCATCCACCTGA 776
Pmchi1          GCCTGCAGGAGGGCTACCATGTGCCTCAGCTCTGCAGCCTGCTGGACGCCATCCACCTGA 756
HP0029          GGCTGCAGGAAGGGTACCACGTCCCGGAGCTCTGCAGTCTGCTGGACGCGATTCATCTTA 106
                * ******** ** ***** ** **  ********** ********** ** ** ** *

Pjchi1          TG-CGTACGACCTGAGGGGCAACTGGGTCGGCTTCGCGGACGTCCACTCCATGCTGTACA 835
Pmchi1          TGACGTACGACCTGCGGGGCAACTGGGTCGGCTTCGCGGACGTGCACTCCATGCTGTACA 816
HP0029          TGACGTATGATCTGCGTGGGAACTGGGTTGGCTTTGCGGACGTCCACTCCATGTTGTATC 166
                ** **** ** *** * ** ******** ***** ******** ********* ****

Pjchi1          GGCGGCCCGGGCTGGACGAGTGGGCTTACGAGAAGCTCAACGTGAACGACGGCGCTTTAC 895
Pmchi1          CTCGGCCCGGACTGGACGAGTGGGCCTACGAGAAGCTGAACGTGAACGACGGCGCTCTCC 876
HP0029          AGCGACCGGGTCTCGATCAGTGGTCCTACGAGAAGCTCAATGTGAACGACGGCGCCCTTC 226
                  ** ** ** ** **  ***** * *********** ** **************  * *

Pjchi1          TGTGGGTGGAATTCGGTTGTCCCCGCGATAAGCTGGTGGTCGGGACGCCATTCTACGGGC 955
Pmchi1          TGTGGGTGGAATTCGGGTGTCCGCGTGATAAGTTGGTGGTCGGGACGCCATTCTACGGGC 936
HP0029          TGTGGGAGGAATTCGGGTGCCCTCGCGACAAGCTGGTGGTGGGGACGCCCTTCTACGGGA 286
                ****** ********* ** ** ** ** *** ******* ******** *********

Pjchi1          GCACCTACACGCTCGGCGACCCCAACAACAACGACCTTCACGCGCCCATCAAGAAGTGGG 1015
Pmchi1          GCACCTACACGCTGGGTGACCCCACCAACAACGGCCTGCACGCGCCCATCAAGAAGTGGG 996
HP0029          GGACCTACACGCTGGGGGACCCTAACAACAACGACCTCCACGCACCCATCAAAGCGTGGT 346
                * *********** ** ***** * ******** *** ***** ********   ****

Pjchi1          AAGGAGGAGGCAAGCCCGGCCCTTACACCAACGCCACGGGCACCATGGCTTACTTCGAGA 1075
Pmchi1          AGGGAGGCGGCAAGCCCGGCCCTTATACCAACGCCACCGGCACTATGGCTTACTTCGAGA 1056
HP0029          TGGGAGGAGGTAACCCAGGGCCCTATACCAACGCCACGGGAACTCTCGCTTACTTCGAGA 406
                  ***** ** ** ** ** ** ** *********** ** **  * *************

Pjchi1          TCTGCCTCATGATGCAGGAGGACTCAGAGTGGGTCGATCGCTACGATGACGTCGGCCTCG 1135
Pmchi1          TCTGCCTCATGATGAAGGAGGACTCCGAGTGGGTCGATCGCTACGATGACGTCGGCCTCG 1116
HP0029          TCTGCAGCATGATGGTCCAGGACTCGGCTTGGGTGGATCGCTACGATGACGTCGGCCTCG 466
                *****  *******    ******* *  ***** *************************

Pjchi1          TCCCCTTTACGCACAAAGGCGACCAGTGGGTGGGCTACGAAGACCCTGACAGCCTCAAGA 1195
Pmchi1          TCCCATTCACGCACAAAGGCGACCAGTGGGTGGGCTACGAGGACCCTGACAGTCTCAAGA 1176
HP0029          TTCCCTTCACGCATAATGGTGACCAGTGGGTGGGCTACGAGGACCCGGCCAGCCTCAAGA 526
                * ** ** ***** ** ** ******************** ***** * *** *******

Pjchi1          TCAAGATGGACTTCATCCGCGAGCAGGGCTACCTGGGCGCCATGACCTGGGCCATCGACC 1255
Pmchi1          TCAAGATGGACTTCATCCGCGAGCAGGGCTACCTCGGCGCCATGACCTGGGCCATCGACC 1236
HP0029          TCAAGATGGACTACATCCGGGACATGGGTTTCCTCGGGGCCATGACGTGGGCCATCGACC 586
                ************ ****** **   *** * *** ** ******** **************

Pjchi1          AGGACGACTTCCGGAGCTGGTGTGGAAGAGGGAAGAACCCGATGATGAACACTATATACG 1315
Pmchi1          AGGACGACTTCCGGAACTGGTGTGGAAGGGGGAACGACCCGATGATGAACACCATTTACG 1296
HP0029          AGGACGACTACAAG---------------------------------------------- 600
                ********* *  *
```

```
Pjchi1          AAGGCATGAAGGATTACATAGTGCCTGTCGCTCCCACTCTTCCCCCAACTACAACAACTC 1375
Pmchi1          ATGGCATGAAGGACTACGTAGTGC-TGTTGCTCCCACTCTTCCTCCAACCACAACAAGCC 1355
HP0029          ------------------------------------------------------------

Pjchi1          CCCATTGGACGCCACCAACCACCACTACCACGACACGGGACCCCAGCATCACCACGACTA 1435
Pmchi1          CCTGGTGGACCCCACCAACTACTACTACCACAACACGGGACCCCAGCATCACCACGACCA 1415
HP0029          ------------------------------------------------------------

Pjchi1          CGAGGGACCCCAATTTGCCGACCACGACTATGGGACCTATAGACTGTACTGTGCAAGAAT 1495
Pmchi1          CGAGAGATCCCAACTTGCCGACCACAACTATGGGGCCTATTGACTGTACTGTGCAAGAAT 1475
HP0029          ------------------------------------------------------------

Pjchi1          ACTGGCCGCATCCGGACTGTGACAAGTACTACTGGTGCTTCGAGGGCGAGCCGCACCTGG 1555
Pmchi1          ACTGGCCGCATCCGGACTGTGACAAGTACTACTGGTGCTTCGAAGGCATACCCCACCTGG 1535
HP0029          ------------------------------------------------------------

Pjchi1          AGTACTGCCCCGCCGGTACTGTGTGGAACCAGGCCATCAAGGCGTGTGACTGGCCGGCCA 1615
Pmchi1          AGTACTGCCCCGCTGGCACCGTGTGGAACCAGGCTATCAAGGCGTGCGACTGGCCGGCCA 1595
HP0029          ------------------------------------------------------------

Pjchi1          ACGTGGACACTTCCGGCTGCAACATGCCCTCCCTCTCGAAGGG-GTCGGCCAGCCGCTGC 1674
Pmchi1          ACGTGGACACCTCCGGCTGCAACATGCCCTCGCTCTCGAAAGACGCCAGCCAGCGG---C 1652
HP0029          ------------------------------------------------------------

Pjchi1          CGCTCCACAACAGCATTCCGCTCAACGTCCGGCCCAAAGGGCAC-GCCGTCAAACCACCT 1733
Pmchi1          CCCTCCACAACACTATCCCTCTCAACGCCGAACCAAGGGGACCCCGCACTCGGGCAAGGC 1712
HP0029          ------------------------------------------------------------

Pjchi1          TCCAAGAAGATTGCGTCACCCAAGTCTCTGTCCAACAAGCCAGCACACCCTAAGCCTTTA 1793
Pmchi1          GCC--GAAGGTT---CCTCTTAACTTGATTTCCAAGAAGCCAGCCCCGGCGAAGTCTTTA 1767
HP0029          ------------------------------------------------------------

Pjchi1          CCTGTGAAATCAGCAAAAGCTAAACCAGTTCACAAAAAGCCAGCACCTGCTAAACTAGCA 1853
Pmchi1          CCTACGAAGTCAGTTGATGCTAAATTAGTTCACAATAATGCACCACCTGCTAAACCAGCA 1827
HP0029          ------------------------------------------------------------

Pjchi1          CCTGTTAAGCTACCACTTGTTCAGCCACAACAGGTTAAACCAGTTGATGGAAAGCCAGTC 1913
Pmchi1          CATGCTAAGCCTCTTCATGCTAAACCAGTTCGTGCTAAGCCAGCACCGGTAAAGCCACTT 1887
HP0029          ------------------------------------------------------------

Pjchi1          C--------TAGTTCATGCTAAAGCCCCGACGGGTAACGCAAATCCTGCCAAGCCA-GAA 1964
Pmchi1          CATACTAAGCAATTCATGCTAT-GCCAGCACAGGTAA-GTCAGCTATACTAAACCACAAA 1945
HP0029          ------------------------------------------------------------

Pjchi1          CCTACTAAGTCAGAAGTTGGTAATCCAGAACCTGCCAACCCAAAACTTGCTAAGCCAGAA 2024
Pmchi1          GCGGGTAACCTAGAACCTTCCACACCAGAACCGTCGAACCTAAAACTTTCCAAGTCAGAG 2005
HP0029          ------------------------------------------------------------
```

```
Pjchi1          CCTGTCAAGTCATCTCCCCCATTGATGATGAAGATGTTAAAGCATTAATGTC-------- 2076
Pmchi1          CCTGTTAAGTCAGTACCCCCCCTGTTAATGAAGATGGTAAAGAACTAAAGTGTACCATTG 2065
HP0029          ------------------------------------------------------------

Pjchi1          ------------------------------------------------------------
Pmchi1          AGAATAAGCCGCCAAAAGGAGGTCTCCAAATCCTTCATTTGTGAATGAATACAAAAGTAT 2125
HP0029          ------------------------------------------------------------

Pjchi1          ------------------------------------------------------------
Pmchi1          CATTAATGATAAAAAAGAGAACATCATACAAATGGTGTGGCAAGAAAAGGTAAGGAAGTA 2185
HP0029          ------------------------------------------------------------

Pjchi1          ------------------------------------------------------------
Pmchi1          TCATCTAGAAGTAAATAAATAAATCACTGAGTCAGAGTAGAAAGTAATATCATTTTTGAG 2245
HP0029          ------------------------------------------------------------

Pjchi1          ------------------------------------------------------------
Pmchi1          AAGACAACAATAATAATAACTATAACATTAATAATAACTAATAGAGACAAAAATAATTAA 2305
HP0029          ------------------------------------------------------------

Pjchi1          ------------------------------------------------------------
Pmchi1          CATTATAGCTGGATAAATCAACGGAAATATAAGTTGACAACATTAAAGAAAACCTTGTGT 2365
HP0029          ------------------------------------------------------------

Pjchi1          ------------------------------------------------------------
Pmchi1          TAGTCATTTTCTGTGCGAATTTTTGCATAACAAAATTTGCTAGTTTATGAAATAAAAAAT 2425
HP0029          ------------------------------------------------------------

Pjchi1          ------------------------------
Pmchi1          GTTTGAAACAGTAAAAAAAAAAAAAAAAA 2455
HP0029          ------------------------------
```

Figure 3.1 The nucleotide alignment of the *HPa0029* with the *chitinase*1 gene of *P. monodon* (*PmChi*-1) and *chitinase*1 gene of *P. japonicus* (*PjChi*-1). The start codon of *PmChi*-1 and *PjChi*-1 had shown in box. The blue letter is stop codon of *PmChi*-1. The green letter is stop codon of *PjChi*-1. The underline letter is the oligonucleotide primers.

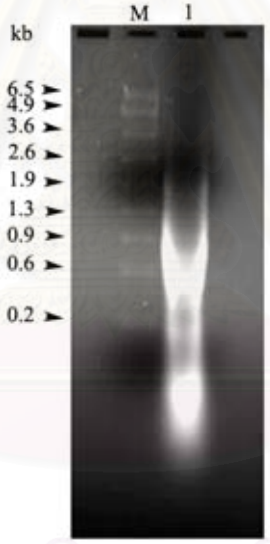


Figure 3.2 The total RNA extracted from the hepatopancreas of *P. monodon* electrophoresed on a 1% formaldehyde agarose gel. Lane M is the RNA maker, and Lane 1 is the total RNA.

Amplification of the cDNA encoding chitinase

The total RNA was used in reverse transcription. Reverse transcription was primed by oligo $(dT)_{15}$ primers to obtain a cDNA. A chitinase gene cDNA sample was amplified, using a specific of primers, designed from the sequence of a previous cloned chitinase gene, *PmChi-1* (Tan et al., 2000). The forward and reverse primers for the amplification were shown below:

HpforI	5’ GGAATTCCATATGGTGAGCGGCCGAGTT 3’
HPRewII	5’ CGGGATCCAGGCTCTGACTTGGAAAG 3’

The forward primer was designed from a site of +1 of *PmChi-1* added with *NdeI* restriction site and the reverse primer was designed from the site of +1950 of *PmChi-1* added with *BamHI* restriction site. Between these sites, the PCR amplified from these primers was the full-length gene based on *PmChi-1*.

PCR amplification was performed by pre-denaturation at 94 ºC for 5 minutes, 25 cycles of denaturation at 94 ºC for 30 seconds, annealing at 55 ºC for 30 seconds, and extension at 72 ºC for 4 minutes, and the final extension at 72 ºC for 10 minutes. The PCR product was electrophoretically separated on a 0.8% agarose gel. A prominent PCR product of chitinase gene with the approximated size of 1.95 kb was observed as shown in Figure 3.3.

Figure 3.3 Agarose gel electrophoresis of the amplified chitinase gene. The PCR product was separated on a 0.8% agarose gel and visualized by ethidium bromide staining. Lane M is a DNA marker, and lane 1 is the PCR product.

Construction of a pGEM-T easy recombinant plasmid containing the chitinase gene

The PCR product from the previous step was purified and digested for overnight with *NdeI* and *BamHI* restriction enzymes. A pGEM-T easy vector was also digested for overnight with *NdeI* and *BamHI* restriction enzymes. Both reactions were separated on 0.8% agarose gel. The target band was eluted from gel and purified by using Qiaquick Gel Extraction Kit (Qiagen). The digested fragments were ligated together by ligase for overnight. The ligation reaction was transformed into the *E. coli* Top10 cells. The recombinant clone was selected on LB agar plate containing ampicillin and X-Gal. The white colonies were randomly selected and cultured in LB broth containing ampicillin overnight. The cultures were subjected to plasmid extraction and digestion with *Nde*I and *Bam*HI restriction enzymes to prove the correction of recombinant plasmid. Subsequently, these reactions were analyzed by 0.8% agarose gel electrophoresis. The result showed that the recombinant plasmid contained a chitinase gene of an approximated size of 1.95 kb. The inserted DNA was purified from the agarose gel for further ligation into the pET-17b.

Construction of the pET-17b recombinant plasmid containing the chitinase gene

After the DNA fragment of chitinase gene was eluted from the 0.8% agarose gel by using Qiaquick Gel Extraction Kit (Qiagen), the inserted DNA was then subcloned into the pET-17b expression vector at the *Nde*I and *Bam*HI sites. The pET-17b expression vector was also digested for overnight with *Nde*I and *Bam*HI restriction enzymes and then purified by using Qiaquick Gel Extraction Kit (Qiagen). The ligation mixture was transformed into the *E. coli* Top10 cells. The transformants was randomly selected for a plasmid extraction. After an extraction, the plasmids from each clone were digested with *Nde*I and *BamH*I restriction enzymes and then analyzed by 0.8% agarose gel electrophoresis (Figure 3.4) to confirm the presence of inserted fragment. The analysis revealed that the recombinant plasmid indeed contained the chitinase gene in pET-17b since the size of 1.95 kb was observed. This plasmid was named pChi-HP.

Sequence analysis

To confirm whether the inserted fragment was chitinase gene, the pChi-HP was subjected to DNA sequencing. After the pChi-HP was sequenced, the DNA sequence was compared to the *PmChi-1* gene by using the clustalW program. The comparison result revealed that the *Chi-HP* was a homologue to *PmChi*-1 with 98% homology. Interestingly, the *Chi-HP* contained one adenine base insertion at a position of 1835, causing a frame shift on downstream of this site, and resulting in change of stop codon to the position of 1995, as shown in Figure 3.5. Moreover, the *Chi-HP* gene was electronically translated to deduce amino acid. This amino acid sequence was subjected to BLASTP program to determine domain(s) containing within the gene. The result showed that the *Chi-HP* gene contained four domains, namely, signal peptide, suggesting that it is the precursor of a secretary protein, catalytic domain, tachycitin like domain (chitin binding domain), and proline rich domain (Figure 3.6 and Figure 3.7).

The signal peptide was predicted by SignalP 3.0 Server program. The prediction result shown the signal peptide was spanning 22 residues from residue 1 to 22 and the cleavage site between residue 22 and 23 of *Chi-HP*.

The catalytic domain of *Chi-HP* was spanning 426 residues from residue 23 to 425 of *Chi-HP*. Since the *Chi-HP* could not be predicted the structure; therefore the catalytic domain of *Chi-HP* was chosen to predict the structure instead. The homology modeling of the structural amino acid of catalytic domain of *Chi-HP* was accomplished by SWISS Model Protein Modeling, which was shown in Figure 3.8. The *Chi-HP* was classified into family 18 glycosylhydrolase, which was shown a $(\alpha/\beta)_8$ barrel. Furthermore, the amino acid alignment of chitinases from *Chi-HP*, *Bombyx mori (ChiM), Helicoverpa armigera (ChiH), and Bacillus circulans* WL21 (*ChiA*1) were shown the conserved region, (F/L)DGxDxDxE, where x was any amino acid. This result confirms that *Chi-HP* was classified into family 18 glycosylhydrolase because this conserved region was found in only family 18 (Figure 3.9).

Thirdly domain, the amino acid sequence of *Chi-HP* was aligned by BLASTP program which can detect putative conserved domains. The result indicated that the residues from 467 to 518 of *Chi-HP* were chitin binding domain and has 20 %

homology to the tachycitin of horseshoe crab, *Tachypleus tridentatus* (Suetake T., 2002). Therefore, this chitin binding domain was called tachycitin like domain. Moreover, the amino acid alignment of tachycitin like domain of *Chi-HP* with tachycitin has shown the conserved cysteine 6 residues as shown in Figure 3.10. This result indicated that tachycitin like domain consists of 3 disulfide bridges.

For the last domain, the proline rich domain, there are 2 regions of this domain. The first region was spanning 40 residues from residue 426 to 466 of *Chi-HP*. The second region was estimated spanning 131 residues from residue 519 to 650 of *Chi-HP*. This second region was not determined because *Chi-HP* has no the stop codon. Most proline rich domain contained antimicrobial activity, so, the proline rich domain of *Chi-HP* was aligned with antimicrobial peptide database by using computer program. The result has shown that the proline rich domain of *Chi-HP* has the homology of 26.22% to Penaeidins from *Penaeus vannamei* (Atlantic white shrimp) (Yang, Y. *et al*. 2003) and 25.92% to Apidaecins from honeybee (Casteela-Josson K. et al., 1993) (Figure 3.11, 3.12).

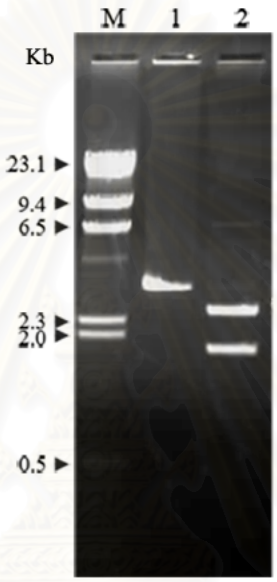


Figure 3.4 Agarose gel electrophoresis of the recombinant chitinase gene in pET-17b (pET-17b-Chi-HP). The DNA was separated on a 0.8% agarose gel and then visualized by ethidium bromide staining. Lane M is a DNA marker, lane 1 is the undigested pET-17b-Chi-HP, and lane 2 is the pET-17b-Chi-HP digested with *Nde*I and *Bam*HI.

```
chi-hp          ATGGTGAGCGGCCGAGTTTTGGCAGTCCTAGTGACGCTTGCAGCATTGGCAACACTAGTG 60
Pmchi-1         ATGGTGAGCGGCCGAGTTTTGGCAGTCCTAGTGACGCTTGCAGCATTGGCAACACTAGTG 60
                ************************************************************

chi-hp          TTGGCTGACCCGAGATTCGAGCAAGAAGGAGCCCAGCGGCGGTGGGTGCGGCCCGAGGGC 120
Pmchi-1         TTGGCTGACCCGAGATTCGAGCAAGAAGGAGCCCAGCGGCGGTGGGTGCGGCCCGAGGGC 120
                ************************************************************

chi-hp          CAGGCGCGCCGCGTGTGCTACTACGAGGCGTGGGCCATCTACCGGCCCGGCGACGGCTTC 180
Pmchi-1         CAGGCGCGCCGCGTGTGCTACTACGAGGCGTGGGCCATCTACCGGCCCGGCGACGGCTTC 180
                ************************************************************

chi-hp          TACGACATCGAGGACATCCCCGCCAACCTGTGCACGGACCTCATCTACTCGTTCATTGGC 240
Pmchi-1         TACGACATCGAGGACATCCCCGCCAACCTGTGCACGGACCTCATCTACTCGTTCATTGGC 240
                ************************************************************

chi-hp          CTCTCCAACGTCACGTGGGAAGTGCTCATTCTCGACCCTGAGTACGACATCAACCTGAAC 300
Pmchi-1         CTCTCCAACGTCACGTGGGAAGTGCTCATTCTCGACCCTGAGTACGACATCAACCTTGAC 300
                ********************************************************  **

chi-hp          GGTTTCCGGCGGTTCGTGGCGCTGAAGGACAAGTACCCTGACATGAAGACAAACATCGCC 360
Pmchi-1         GGTTTCCGCCGGTTCGTGGCGCTGAAGGACAAGTACCCTGACATGAAGACAAACATCGCC 360
                ******** ***************************************************

chi-hp          GTGGGCGGCTGGGCCGAGGGCGGAAGGAAGTACTCGCAGATGGTGATGGTGCCCGAAAGG 420
Pmchi-1         GTGGGCGGCTGGGCCGAGGGCGGAAGGAAGTACTCGCAGATGGTGATGGTGCCCGAAAGG 420
                ************************************************************

chi-hp          AGGGCGTCTTTCATCAGGAGCGTCGTCCAGCTGCTCACCGACTACGGCTTCGACGGGTTG 480
Pmchi-1         AGGGCGTCTTTCATCAGGAGCGTCGTCCAGCTGCTCACCGACTACGGCTTCGACGGGTTG 480
                ************************************************************

chi-hp          GACTTGGACTGGGAATACCCTGGCGCCACAGACCGAGGAGGCCAATATGCCGATAAGGAC 540
Pmchi-1         GACTTGGACTGGGAATACCCTGGCGCCACAGACCGAGGAGGCCAATATGCCGATAAGGAC 540
                ************************************************************

chi-hp          AACTTCCTTAAACTGGTGCAGGAGCTGCGCGAAGCCTTCGACACCGTGGGGCTGGGCTGG 600
Pmchi-1         AACTTCCTTAAACTGGTGCAGGAGCTGCGCGAAGCCTTCGACACCGTGGGGCTGGGCTGG 600
                ************************************************************

chi-hp          GAAATCACGTGCGCCGTGCCCGTGGCCAAGTTCCGCCTGCAGGAGGGCTACCATGTGCCT 660
Pmchi-1         GAAATCACGTGCGCCGTGCCCGTGGCCAAGTTCCGCCTGCAGGAGGGCTACCATGTGCCT 660
                ************************************************************

chi-hp          CAGCTCTGCAGCCTGCTGGACGCCATCCACCTGATGACGTACGACCTGCGGGGCAACTGG 720
Pmchi-1         CAGCTCTGCAGCCTGCTGGACGCCATCCACCTGATGACGTACGACCTGCGGGGCAACTGG 720
                ************************************************************
```

```
chi-hp          GTCGGCTTCGCGGACGTGCACTCCATGCTGTACACTCGGCCCGGACTGGACGAGTGGGCC 780
Pmchi-1         GTCGGCTTCGCGGACGTGCACTCCATGCTGTACACTCGGCCCGGACTGGACGAGTGGGCC 780
                ************************************************************

chi-hp          TACGAGAAGCTGAACGTGAACGACGGCGCTCTCCTGTGGGTGGAATTCGGGTGTCCGCGT 840
Pmchi-1         TACGAGAAGCTGAACGTGAACGACGGCGCTCTCCTGTGGGTGGAATTCGGGTGTCCGCGT 840
                ************************************************************

chi-hp          GATAAGTTGGTGGTCGGGACGCCATTCTACGGGCGCACCTACACGCTGGGTGACCCCACC 900
Pmchi-1         GATAAGTTGGTGGTCGGGACGCCATTCTACGGGCGCACCTACACGCTGGGTGACCCCACC 900
                ************************************************************

chi-hp          AACAACGGCCTGCACGCGCCCATCAAGAAGTGGGAGGGAGGCGGCAAGCCCGGCCCTTAT 960
Pmchi-1         AACAACGGCCTGCACGCGCCCATCAAGAAGTGGGAGGGAGGCGGCAAGCCCGGCCCTTAT 960
                ************************************************************

chi-hp          ACCAACGCCACCGGCACTATGGCTTACTTCGAGATCTGCCTCATGATGAAGGAGGACTCC 1020
Pmchi-1         ACCAACGCCACCGGCACTATGGCTTACTTCGAGATCTGCCTCATGATGAAGGAGGACTCC 1020
                ************************************************************

chi-hp          GAGTGGGTCGATCGCTACGATGACGTCGGCCTCGTCCCATTCACGCACAAAGGCGACCAG 1080
Pmchi-1         GAGTGGGTCGATCGCTACGATGACGTCGGCCTCGTCCCATTCACGCACAAAGGCGACCAG 1080
                ************************************************************

chi-hp          TGGGTGGGCTACGAGGACCCTGACAGTCTCAAGATCAAGATGGACTTCATCCGCGAGCAG 1140
Pmchi-1         TGGGTGGGCTACGAGGACCCTGACAGTCTCAAGATCAAGATGGACTTCATCCGCGAGCAG 1140
                ************************************************************

chi-hp          GGCTACCTCGGCGCTATGACCTGGGCCATCGACCAGGACGACTTCCGGAACTGGTGTGGA 1200
Pmchi-1         GGCTACCTCGGCGCCATGACCTGGGCCATCGACCAGGACGACTTCCGGAACTGGTGTGGA 1200
                ************** ********************************************

chi-hp          AGGGGACAGAACCCGATGATGAACACCATTTACGATGGCATGAAGGACTACGTAGTGCCT 1260
Pmchi-1         AGGGGGAACGACCCGATGATGAACACCATTTACGATGGCATGAAGGACTACGTAGTGC-T 1259
                *****  *  ************************************************ *

chi-hp          GTTGCTCCCACTCTTCCTCCAACCACAACAAGCCCCTGGTGGACCCCACCAAATAATACT 1320
Pmchi-1         GTTGCTCCCACTCTTCCTCCAACCACAACAAGCCCCTGGTGGACCCCACCAACTACTACT 1319
                ************************************************** ** ****

chi-hp          ACCACAACACGGGACCCCAGCATCACCACGACCACGAGAGATCCCAACTTGCCGACCACA 1380
Pmchi-1         ACCACAACACGGGACCCCAGCATCACCACGACCACGAGAGATCCCAACTTGCCGACCACA 1379
                ************************************************************

chi-hp          ACTATGGGGCCTATTGACTGTACTGTGCAAGAATACTGGCCGCATCCGGACTGTGACAAG 1440
Pmchi-1         ACTATGGGGCCTATTGACTGTACTGTGCAAGAATACTGGCCGCATCCGGACTGTGACAAG 1439
                ************************************************************
```

```
chi-hp          TACTACTGGTGCTTCGAAGGCATACCCCACCTGGAGTACTGCCCCGCTGGCACCGTGTGG 1500
Pmchi-1         TACTACTGGTGCTTCGAAGGCATACCCCACCTGGAGTACTGCCCCGCTGGCACCGTGTGG 1499
                ************************************************************

chi-hp          AACCAGGCTATCAAGGCGTGCGACTGGCCGGCCAACGTGGACACCTCCGGCTGCAACATG 1560
Pmchi-1         AACCAGGCTATCAAGGCGTGCGACTGGCCGGCCAACGTGGACACCTCCGGCTGCAACATG 1559
                ************************************************************

chi-hp          CCCTCGCTCTCGAAAGACGCCAGCCAGCGGCCCCTCCACAACACTATCCCTCTCAACGTC 1620
Pmchi-1         CCCTCGCTCTCGAAAGACGCCAGCCAGCGGCCCCTCCACAACACTATCCCTCTCAACG-C 1618
                ************************************************************ *

chi-hp          CGAACCAAGGGGACCCCGCACTCGGGCAAGGCGCCAAAGGTTCCTCTTAACTTGATTTCC 1680
Pmchi-1         CGAACCAAGGGGACCCCGCACTCGGGCAAGGCGCCGAAGGTTCCTCTTAACTTGATTTCC 1678
                ********************************** ***********************

chi-hp          AAGAAGGCAGCCCCGGCGAAGTCTTTACCTGCGAAGTCAGTTGATGCTAAATTAGTTCAC 1740
Pmchi-1         AAGAAGCCAGCCCCGGCGAAGTCTTTACCTACGAAGTCAGTTGATGCTAAATTAGTTCAC 1738
                ****** ********************** ****************************

chi-hp          AATAATGCACCGCCTGCTAAACCAGCACATGCTAAGCCTCTTCATGCTAAACCAGTTCGT 1800
Pmchi-1         AATAATGCACCACCTGCTAAACCAGCACATGCTAAGCCTCTTCATGCTAAACCAGTTCGT 1798
                *********** ************************************************

chi-hp          GCTAAGCCAGCACCGGTAAAGCCACTTCATACTAAAGCAATTCATGCTATGCCAGCACAG 1860
Pmchi-1         GCTAAGCCAGCACCGGTAAAGCCACTTCATACTAA-GCAATTCATGCTATGCCAGCACAG 1857
                ********************************** ***********************

chi-hp          GTAAAGTCAGCTCATACTAAACCACAA-GCGGCTAACCTAGAATCTTCCAAACCAGAACC 1919
Pmchi-1         GTAA-GTCAGCT-ATACTAAACCACAAAGCGGGTAACCTAGAACCTTCCACACCAGAACC 1915
                **** ******* ************** **** ********** ****** *********

chi-hp          GTCGAACCCAAAACTTTCCAAGTCAGAGCCT---------------------------- 1979
Pmchi-1         GTCGAACCTAAAACTTTCCAAGTCAGAGCCTGTTAAGTCAGTACCCCCCCTGTTAATGAA 1975
                ******** ********************

chi-hp          ------------------------------------------------------------ 2012
Pmchi-1         GATGGTAAAGAACTAAAGTGTACCATTGAGAATAAGCCGCCAAAAGGAGGTCTCCAAATC 2035

chi-hp          ------------------------------------------------------------
Pmchi-1         CTTCATTTGTGAATGAATACAAAAGTATCATTAATGATAAAAAAGAGAACATCATACAAA 2095

chi-hp          ------------------------------------------------------------
Pmchi-1         TGGTGTGGCAAGAAAAGGTAAGGAAGTATCATCTAGAAGTAAATAAATAAATCACTGAGT 2155
```

```
chi-hp          ------------------------------------------------------------
Pmchi-1         CAGAGTAGAAAGTAATATCATTTTTGAGAAGACAACAATAATAATAACTATAACATTAAT 2215

chi-hp          ------------------------------------------------------------
Pmchi-1         AATAACTAATAGAGACAAAAATAATTAACATTATAGCTGGATAAATCAACGGAAATATAA 2275

chi-hp          ------------------------------------------------------------
Pmchi-1         GTTGACAACATTAAAGAAAACCTTGTGTTAGTCATTTTCTGTGCGAATTTTTGCATAACA 2335

chi-hp          ----------------------------------------------------------
Pmchi-1         AAATTTGCTAGTTTATGAAATAAAAAATGTTTGAAACAGTAAAAAAAAAAAAAAAAAA 2393
```

Figure 3.5 The nucleotide alignment of *Chi-HP* with the *PmChi-1*. *Chi-HP* contained one adenine base insertion at position of 1835 is shown in bold letter. The underline letter at position of 1861 is shown the old stop codon. The new stop codon is shown in box at position of 1995.

```
   10         20         30         40         50         60
GTGATAACGACGGCCAGTGAATGTAATCGACTCACTATAGGGCGAATTGGGCCCGACGTC

         70         80         90        100        110        120
GCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATCGATTGGAATTCCATATGGTGAGCGG
                                             M  V  S  G

        130        140        150        160        170        180
CCGAGTTTTGGCAGTCCTAGTGACGCTTGCAGCATTGGCAACACTAGTGTTGGCTGACCC
 R  V  L  A  V  L  V  T  L  A  A  L  A  T  L  V  L  A  D  P

        190        200        210        220        230        240
GAGATTCGAGCAAGAAGGAGCCCAGCGGCGGTGGGTGCGGCCCGAGGGCCAGGCGCGCCG
 R  F  E  Q  E  G  A  Q  R  R  W  V  R  P  E  G  Q  A  R  R

        250        260        270        280        290        300
CGTGTGCTACTACGAGGCGTGGGCCATCTACCGGCCCGGCGACGGCTTCTACGACATCGA
 V  C  Y  Y  E  A  W  A  I  Y  R  P  G  D  G  F  Y  D  I  E

        310        320        330        340        350        360
GGACATCCCCGCCAACCTGTGCACGGACCTCATCTACTCGTTCATTGGCCTCTCCAACGT
 D  I  P  A  N  L  C  T  D  L  I  Y  S  F  I  G  L  S  N  V

        370        380        390        400        410        420
CACGTGGGAAGTGCTCATTCTCGACCCTGAGTACGACATCAACCTGAACGGTTTCCGGCG
 T  W  E  V  L  I  L  D  P  E  Y  D  I  N  L  N  G  F  R  R

        430        440        450        460        470        480
GTTCGTGGCGCTGAAGGACAAGTACCCTGACATGAAGACAAACATCGCCGTGGGCGGCTG
 F  V  A  L  K  D  K  Y  P  D  M  K  T  N  I  A  V  G  G  W

        490        500        510        520        530        540
GGCCGAGGGCGGAAGGAAGTACTCGCAGATGGTGATGGTGCCCGAAAGGAGGGCGTCTTT
 A  E  G  G  R  K  Y  S  Q  M  V  M  V  P  E  R  R  A  S  F

        550        560        570        580        590        600
CATCAGGAGCGTCGTCCAGCTGCTCACCGACTACGGCTTCGACGGGTTGGACTTGGACTG
 I  R  S  V  V  Q  L  L  T  D  Y  G  F  D  G  L  D  L  D  W

        610        620        630        640        650        660
GGAATACCCTGGCGCCACAGACCGAGGAGGCCAATATGCCGATAAGGACAACTTCCTTAA
 E  Y  P  G  A  T  D  R  G  G  Q  Y  A  D  K  D  N  F  L  K

        670        680        690        700        710        720
ACTGGTGCAGGAGCTGCGCGAAGCCTTCGACACCGTGGGGCTGGGCTGGGAAATCACGTG
 L  V  Q  E  L  R  E  A  F  D  T  V  G  L  G  W  E  I  T  C
```

```
       730       740       750       760       770       780
CGCCGTGCCCGTGGCCAAGTTCCGCCTGCAGGAGGGCTACCATGTGCCTCAGCTCTGCAG
 A  V  P  V  A  K  F  R  L  Q  E  G  Y  H  V  P  Q  L  C  S

       790       800       810       820       830       840
CCTGCTGGACGCCATCCACCTGATGACGTACGACCTGCGGGGCAACTGGGTCGGCTTCGC
 L  L  D  A  I  H  L  M  T  Y  D  L  R  G  N  W  V  G  F  A

       850       860       870       880       890       900
GGACGTGCACTCCATGCTGTACACTCGGCCCGGACTGGACGAGTGGGCCTACGAGAAGCT
 D  V  H  S  M  L  Y  T  R  P  G  L  D  E  W  A  Y  E  K  L

       910       920       930       940       950       960
GAACGTGAACGACGGCGCTCTCCTGTGGGTGGAATTCGGGTGTCCGCGTGATAAGTTGGT
 N  V  N  D  G  A  L  L  W  V  E  F  G  C  P  R  D  K  L  V

       970       980       990      1000      1010      1020
GGTCGGGACGCCATTCTACGGGCGCACCTACACGCTGGGTGACCCCACCAACAACGGCCT
 V  G  T  P  F  Y  G  R  T  Y  T  L  G  D  P  T  N  N  G  L

      1030      1040      1050      1060      1070      1080
GCACGCGCCCATCAAGAAGTGGGAGGGAGGCGGCAAGCCCGGCCCTTATACCAACGCCAC
 H  A  P  I  K  K  W  E  G  G  G  K  P  G  P  Y  T  N  A  T

      1090      1100      1110      1120      1130      1140
CGGCACTATGGCTTACTTCGAGATCTGCCTCATGATGAAGGAGGACTCCGAGTGGGTCGA
 G  T  M  A  Y  F  E  I  C  L  M  M  K  E  D  S  E  W  V  D

      1150      1160      1170      1180      1190      1200
TCGCTACGATGACGTCGGCCTCGTCCCATTCACGCACAAAGGCGACCAGTGGGTGGGCTA
 R  Y  D  D  V  G  L  V  P  F  T  H  K  G  D  Q  W  V  G  Y

      1210      1220      1230      1240      1250      1260
CGAGGACCCTGACAGTCTCAAGATCAAGATGGACTTCATCCGCGAGCAGGGCTACCTCGG
 E  D  P  D  S  L  K  I  K  M  D  F  I  R  E  Q  G  Y  L  G

      1270      1280      1290      1300      1310      1320
CGCTATGACCTGGGCCATCGACCAGGACGACTTCCGGAACTGGTGTGGAAGGGGACAGAA
 A  M  T  W  A  I  D  Q  D  D  F  R  N  W  C  G  R  G  Q  N

      1330      1340      1350      1360      1370      1380
CCCGATGATGAACACCATTTACGATGGCATGAAGGACTACGTAGTGCCTGTTGCTCCCAC
 P  M  M  N  T  I  Y  D  G  M  K  D  Y  V  V  P  V  A  P  T
```

```
      1390      1400      1410      1420      1430      1440
TCTTCCTCCAACCACAACAAGCCCCTGGTGGACCCCACCAAATAATACTACCACAACACG
 L  P  P  T  T  T  S  P  W  W  T  P  P  N  N  T  T  T  T  R

      1450      1460      1470      1480      1490      1500
GGACCCCAGCATCACCACGACCACGAGAGATCCCAACTTGCCGACCACAACTATGGGGCC
 D  P  S  I  T  T  T  T  R  D  P  N  L  P  T  T  T  M  G  P

      1510      1520      1530      1540      1550      1560
TATTGACTGTACTGTGCAAGAATACTGGCCGCATCCGGACTGTGACAAGTACTACTGGTG
 I  D  C  T  V  Q  E  Y  W  P  H  P  D  C  D  K  Y  Y  W  C

      1570      1580      1590      1600      1610      1620
CTTCGAAGGCATACCCCACCTGGAGTACTGCCCCGCTGGCACCGTGTGGAACCAGGCTAT
 F  E  G  I  P  H  L  E  Y  C  P  A  G  T  V  W  N  Q  A  I

      1630      1640      1650      1660      1670      1680
CAAGGCGTGCGACTGGCCGGCCAACGTGGACACCTCCGGCTGCAACATGCCCTCGCTCTC
 K  A  C  D  W  P  A  N  V  D  T  S  G  C  N  M  P  S  L  S

      1690      1700      1710      1720      1730      1740
GAAAGACGCCAGCCAGCGGCCCCTCCACAACACTATCCCTCTCAACGTCCGAACCAAGGG
 K  D  A  S  Q  R  P  L  H  N  T  I  P  L  N  V  R  T  K  G

      1750      1760      1770      1780      1790      1800
GACCCCGCACTCGGGCAAGGCGCCAAAGGTTCCTCTTAACTTGATTTCCAAGAAGGCAGC
 T  P  H  S  G  K  A  P  K  V  P  L  N  L  I  S  K  K  A  A

      1810      1820      1830      1840      1850      1860
CCCGGCGAAGTCTTTACCTGCGAAGTCAGTTGATGCTAAATTAGTTCACAATAATGCACC
 P  A  K  S  L  P  A  K  S  V  D  A  K  L  V  H  N  N  A  P

      1870      1880      1890      1900      1910      1920
GCCTGCTAAACCAGCACATGCTAAGCCTCTTCATGCTAAACCAGTTCGTGCTAAGCCAGC
 P  A  K  P  A  H  A  K  P  L  H  A  K  P  V  R  A  K  P  A

      1930      1940      1950      1960      1970      1980
ACCGGTAAAGCCACTTCATACTAAAGCAATTCATGCTATGCCAGCACAGGTAAAGTCAGC
 P  V  K  P  L  H  T  K  A  I  H  A  M  P  A  Q  V  K  S  A

      1990      2000      2010      2020      2030      2040
TCATACTAAACCACAAGCGGCTAACCTAGAATCTTCCAAACCAGAACCGTCGAACCCAAA
 H  T  K  P  Q  A  A  N  L  E  S  S  K  P  E  P  S  N  P  K
```

```
      2050       2060       2070       2080       2090       2100
ACTTTCCAAGTCAGAGCCTGGATCCCGAATCACTAGTGATTCGCGGCCGCCTGCAGGTCG
 L   S   K   S   E   P

      2110       2120       2130       2140       2150       2160
ACCATATGGGAGAGCTCCCAGCGCGGTGAGTGCATAGCTTGAGTATTCTATAGGTCACCT
```

Figure 3.6 The nucleotide and deduced amino acid sequences of chitinase gene (*Chi-HP*) isolated from the hepatopancreas of *P. monodon*. *Chi-HP* contains the hydrophobic signal peptide (red box), catalytic domain (blue alphabets), tachycitin like domain or chitin-binding domain (pink alphabets), and proline rich domain (green alphabets). The six conserved cystein residues are shown in orange boxed. The underline letters are shown the oligonucleotide primers.

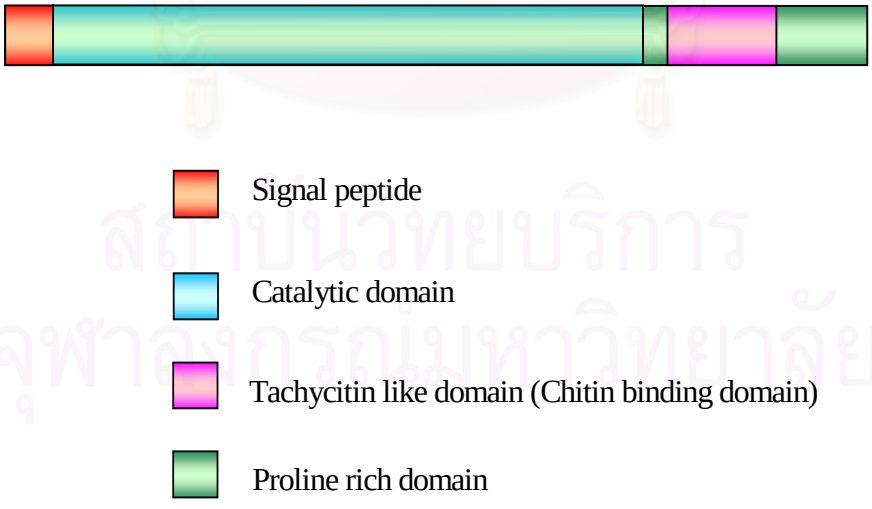


Figure 3.7 A model of the putative *Chi-HP* gene consisting of several domains.

Figure 3.8 Structure of Catalytic domain of Chi-HP. Theoretical structure of catalytic domain of Chi-HP was accomplished by SWISS Model Protein Modeling (SWISS-MODEL version 36.0002)

```
Chi-HP          -------MVSGRVLAVLVTLAALATLVLADPRFEQ------------------------ 28
ChiM            ---------MRAIFATLAVLASCAALVQSDS---------------------------- 22
ChiH            ---MWPPRLLRWAVTVLLIVAVITPASDSTSTVRRRLRKPSKVSTSVTSSVSRSSDQIIS 57
chiA1           MINLNKHTAFKKTAKFFLGLSLLLSVIVPSFALQP------------------------ 35
                              :  ::   .   .

Chi-HP          ----------------------EGAQRRWVRPEGQARRVCYYEAWAIYRPGDGFYDIE 64
ChiM            ----------------------------------RARIVCYFSNWAVYRPGVGRYGIE 46
ChiH            ASVNRPKIRGRPNIASRKSSAAIDNSVSTDHHKDKDGYKIVCYYTNWSQYRTKIGKFTPE 117
chiA1           ----------------------------ATAEAADSYKIVGYYPSWAAYGRN---YNVA 63
                                                : * *:  *: *      :

Chi-HP          DIPANLCTDLIYSFIGLSNV---------------------------TWEVLILDPEY 95
ChiM            DIPVDLCTHLIYSFIGVTEK---------------------------SSEVLIIDPEL 77
ChiH            DIQPDLCTHIIFAFGWLKKG---------------------------KLSSFESNDET 148
chiA1           DIDPTKVTHINYAFADICWNGIHGNPDPSGPNPVTWTCQNEKSQTINVPNGTIVLGDPWI 123
                **     *.: ::*  :                                .   .  :

Chi-HP          DINL-----------NGFRR-FVALKDKYPDMKTNIAVGGWAEGGRKYSQMVMVPERRAS 143
ChiM            DVDK-----------SGFRN-FTSLRSKHPDVKFMVAVGGWAEGGSKYSHMVAQKSTRMS 125
ChiH            KDGK-----------TGLYDRINALKKANPKLKTLLAIGGWSFGTQKFKEMSATRYARQT 197
chiA1           DTGKTFAGDTWDQPIAGNINQLNKLKQTNPNLKTIISVGGWTWSN-RFSDVAATAATREV 182
                . .              *    :  *:.  *.:*  :::***: .  ::..:      *

Chi-HP          FIRSVVQLLTDYGFDGLDLDWEYPGATDRGGQY---ADKDNFLKLVQELREAFDTVG--- 197
ChiM            FIRSVVDFLKKYDFDGLDLDWEYPGAADRGGSF---SDKDKFLYFVQELKRAFIRAG--- 179
ChiH            FIYSAIPYLRDRNFDGLDVDWEYP----KGG-----EDKKNYVLLLKELREAFEAEAQEV 248
chiA1           FANSAVDFLRKYNFDGVDLDWEYPVSGGLDGNSKRPEDKQNYTLLLSKIREKLDAAG--A 240
                *  *.:  * . .***:*:*****     .*      **.::  ::.:::. :   .

Chi-HP          --LGWEITCAVPVAKFRLQEGYHVPQLCSLLDAIHLMTYDLRGNWVGFADVHSMLYTRPG 255
ChiM            --RGWELTAAVPLANFRLMEGYHVPELCQELDAIHVMSYDLRGNWAGFADVHSPLYKRP- 236
ChiH            KKPRLLLTAAVPVGPDNIKGGYDVPAVASYLDFINVMAYDFHGKWERETGHNAPLYSPSS 308
chiA1           VDGKKYLLTIASGASATYAANTELAKIAAIVDWINIMTYDFNGAWQKISAHNAPLNYDPA 300
                          : .. .   . .:: :. :* *::*:**:.* *  :  :: *   .

Chi-HP          LDEWAY---EKLNVNDGALLWVEFGCPRDKLVVGTPFYGRTYTLGDPTNN-GLHAPIKKW 311
ChiM            HDQWAY---EKLNVNDGLNLWEEKGCPTNKLVVGIPFYGRSFTLSAGNNNYGLGTYINKE 293
ChiH            DSEWR----KQLSVDHAAHLWVKLGAPKEKLIIGMPTYGRTFTLSNPNNF----KVNSPA 360
chiA1           ASAAGVPDANTFNVAAGAQGHLDAGVPAAKLVLGVPFYGRGWDGCAQAGN----GQYQTC 356
                 .      : :.*  .     . * *  **::* * *** :      .       .

Chi-HP          EGGGKPGPYTNATGTMAYFEICLMMKED-SEWVDRYDDVGLVPFTHKGD--QWVGYEDPD 368
ChiM            AGGGDPAPYTNATGFWAYYEICTEVDADGSGWTKKWDEFGKCPYAYKGT--QWVGYEDPR 351
ChiH            SGGGKAGEYTKEGGFLAYYEVCDILRNG---GTYVWDDEMKVPYAINGD--QWVGFDDEK 415
chiA1           TGGSSVG--TWEAGSFDFYDLEANYINK-NGYTRYWNDTAKVPYLYNASNKRFISYDDAE 413
                 **.. .  *   *   ::::           .  :::    *:  :.   :::.::*
```

```
Chi-HP          SLKIKMDFIREQGYLGAMTWAIDQDDFRNWCGRGQNPMMNTIYDGMKDYVVPVAP----- 423
ChiM            SVEIKMNWIKEKGYLGAMTWAIDMDDFKGLCGE-ENPLIKLLHKHMSNYTVPPAR----- 405
ChiH            SIRNKMRWIKDNGFGGAMVWTVDMDDFSGSV---------------------------- 446
chiA1           SVGYKTAYIKSKGLGGAMFWELSGDRNKTLQNKLKADLPTGGTVPPVDTTAPSVPGNARS 473
                *:  *  :*:.:*  *** * :. *

Chi-HP          TLPPTTTSPWWAPPTTTTTTRDPSITTTTRDPNLPTTTMGPIDCTVQEYWPHPDCDKYYW 483
ChiM            TGHTTPTPEWARPPSTPS---DP----SEGDP-IPTTTTTTVKPTTTRTTARP------- 450
ChiH            ------------------------------------------------------------
chiA1           TGVTANSVTLAWNASTDNVGVTGYNVYNGANLATSVTGTTATISGLTAGTSYTFTIKAKD 533


Chi-HP          CFEGIPHLEYCPAGTVWNQAIKACDWPANVDTSGCNMPSLSKDASQRPLHNTIPLNVRTE 543
ChiM            ----------TTTTTKVPHGTTEEDFDINVR------PEVEELPTENEVDNADVCNSEDD 494
ChiH            ------------------------------------------------------------
chiA1           AAGNLSAASNAVTVSTTAQPGGDTQAPTAPTNLASTAQTTSSITLSWTASTDNVGVTGYD 593


Chi-HP          GTPHSGKAPKVPLNLISKKAAPAKSLPAKSVDAKLVHNNAPPAKPAHAKPLHAKPVRAKP 603
ChiM            YVPDKKECSKY-WRCVNGEGVQFSCQPGTIFNVKLNVCDWP------------------- 534
ChiH            ------------------------------------------------------------
chiA1           VYNGTALATTVTGTTATISGLAADTSYTFTVKAKDAAGNVSAASN------------AV 640


Chi-HP          APVKPLHTKAIHAMPAQVKSAHTKPQAANLEPSKPEPSNPKLSKSEP------------- 663
ChiM            ---ENTDRPELLAMCERRGSAVLVSTGDNLQRET------------------------- 565
ChiH            ------------------------------------------------------------
chiA1           SVKTAAETTNPGVSAWQVNTAYTAGQLVTYNGKTYKCLQPHTSLAGWEPSNVPALWQLQ- 699


Chi-HP          N 664
ChiM            -
ChiH            -
chiA1           -
```


Figure 3.9 The amino acid sequence alignment of chitinase in family 18 from *Chi-HP*, *Bombyx mori (ChiM), Helicoverpa armigera (ChiH), and Bacillus circulans* WL21 (*ChiA*1). The conserved region of chitinase family 18 is shown in box.

```
tachycitin      ------------------------------------------------------------
Chi-hp          MVSGRVLAVLVTLAALATLVLADPRFEQEGAQRRWVRPEGQARRVCYYEAWAIYRPGDGF 60

tachycitin      ------------------------------------------------------------
Chi-hp          YDIEDIPANLCTDLIYSFIGLSNVTWEVLILDPEYDINLNGFRRFVALKDKYPDMKTNIA 120

tachycitin      ------------------------------------------------------------
Chi-hp          VGGWAEGGRKYSQMVMVPERRASFIRSVVQLLTDYGFDGLDLDWEYPGATDRGGQYADKD 180

tachycitin      ------------------------------------------------------------
Chi-hp          NFLKLVQELREAFDTVGLGWEITCAVPVAKFRLQEGYHVPQLCSLLDAIHLMTYDLRGNW 240

tachycitin      ------------------------------------------------------------
Chi-hp          VGFADVHSMLYTRPGLDEWAYEKLNVNDGALLWVEFGCPRDKLVVGTPFYGRTYTLGDPT 300

tachycitin      ------------------------------------------------------------
Chi-hp          NNGLHAPIKKWEGGGKPGPYTNATGTMAYFEICLMMKEDSEWVDRYDDVGLVPFTHKGDQ 360

tachycitin      ----------------------YL----------AFR--CGR-YSPCLD---DG------- 16
Chi-hp          WVGYEDPDSLKIKMDFIREQGYLGAMTWAIDQDDFRNWCGRGQNPMMNTIYDGMKDYVVP 420
                                       **          **  ***  .* ::   **

tachycitin      --PNVN------LYS-------------------------------C----------CS- 26
Chi-hp          VAPTLPPTTTSPWWTPPNNTTTTRDPSITTTTRDPNLPTTTMGPIDCTVQEYWPHPDCDK 480
                  *.:       ::                               *          *.

tachycitin      FYNCHKCLARLENCPKGLHYNAYLKVCDWPS---KAGC--TSVNKECH---LWKTX---- 74
Chi-hp          YYWCFEGIPHLEYCPAGTVWNQAIKACDWPANVDTSGCNMPSLSKDASQRPLHNTIPLNV 540
                :* *.: :.:** ** *  :*  :*.****:   .:**  .*:.*:.    * :*

tachycitin      ------------------------------------------------------------
Chi-hp          RTKGTPHSGKAPKVPLNLISKKAAPAKSLPAKSVDAKLVHNNAPPAKPAHAKPLHAKPVR 600

tachycitin      ------------------------------------------------------------
Chi-hp          AKPAPVKPLHTKAIHAMPAQVKSAHTKPQAANLESSKPEPSNPKLSKSEPVKSVPPLLMK 660

tachycitin      ----
Chi-hp          MVKN 664
```

Figure 3.10 An amino acid alignment of *Chi-Hp* with tachycitin. Cystein residues were labeled in red letter.

```
Pen-2           ------------------------------------------------------------
Pen2-4          ------------------------------------------------------------
pen-3a          ------------------------------------------------------------
chihp74         MKDYVVPVAPTLPPTTTSPWWAPPTTTTTTRDPSITTTTRDPNLPTTTMGPIDCTVQEYW 60


Pen-2           -----MRLVVCLVFLASFALVCQGGAH---------------RG---------------- 24
Pen2-4          -----MRLVVCLVFLASFALVCQGEAY---------------RG---------------- 24
pen-3a          -----MRLVVCLVFLASFALVCQGQVY---------------KG---------------- 24
chihp74         PHPDCDKYYWCFEGIPHLEYCPAGTVWNQAIKACDWPANVDTSGCNMPSLSKDASQRPLH 120
                      :   *:  :. :     * .                 *


Pen-2           ----------G--FTGPIPRPP---------------------------PHGRP----P 40
Pen2-4          ----------G--YTGPIPRPP---------------------------PIGRP----P 40
pen-3a          ----------G--YTRPIPRPP---------------------------PFVRPLPGGP 44
chihp74         NTIPLNVRTEGTPHSGKAPKVPLNLISKKAAPAKSLPAKSVDAKLVHNNAPPAKPAHAKP 180
                         *  .:   *: *                                *  :*    *


Pen-2           LG--PI--CNACYR-LSFSDVRICCNFLG------KCCHLVKG----------------- 72
Pen2-4          LR--PV--CNACYR-LSVSDARNCCIRFG------SCCHLVKG----------------- 72
pen-3a          IG--PYNGCPVSCRGISFSQARSCCSRLG------RCCHVGKGYSG-------------- 82
chihp74         LHAKPVRAKPAPVKPLHTKAIHAMPAQVKSAHTKPQAANLEPSKPEPSNPKLSKSEPAKS 240
                :   *     .  : :  .  :     .       ..::  .


Pen-2           -----------
Pen2-4          -----------
pen-3a          -----------
chihp74         VPPLLMKMVKN 251
```

Figure 3.11 An amino acid alignment of proline rich domain of *Chi-Hp* with Penaeidins.

```
Apid22          -------------------------MKNFALAILVVT----------FVVAVFGNTN--- 22
Apid14          -------------------------MKNFALAILVVT----------FVVAVFGNTN--- 22
Apid73          RPVYIPQPRPPHPRLRREAEPEAEPGNNRPVYIPQPRPPHPRLRREAELEAEPGNNRPVY 60
chihp74         -MKDYVVPVAPTLPPTTTSPWWAPPTTTTTTRDPSITTTTRDPNLPTTTMGPIDCTVQEY 59
                                          .. .                   .  . .

Apid22          LDPPTRP-TRLRREAEPEAEPGNNRPVYIPQPRP------------------------- 55
Apid14          LDPPTRP-ARLRREAKPEAEPGNNRPIYIPQPRP------------------------- 55
Apid73          ISQPRPPHPRLRREAEPEAEPGNNRPVYIPQPRPPHPRLRREAELEAEPGNNRPVYISQP 120
chihp74         WPHPDCDKYYWCFEGIPHLEYCPAGTVWNQAIKACDWPANVDTSGCNMPSLSKDAS---Q 116
                   *         *. *. *     .::    :.

Apid22          --PHPRLRREAEPEAEPGNNRPVYIPQ------PRPPHPRLRREAEPEAEPGNNRPVYIP 107
Apid14          --PHPRLRREAEPKAEPGNNRPIYIPQ------PRPPHPRLRREAESEAEPGNNRPVYIP 107
Apid73          RPPHPRLRREAEPEAEPGNNRPVYIPQ------PRPPHPRLRREAEPEAEPGNNRPVYIP 174
chihp74         RPLHNTIPLNVRTEGTPHSGKAPKVPLNLISKKAAPAKSLPAKSVDAKLVHNNAPPAKPA 176
                   *  :  :...:. * ..:.  :*       . *.:.   :..:.:   .*  *.  .

Apid22          QPRPPHPRLRR----EAEP-------------------------EAEPGNNRP-VYIPQ 136
Apid14          QPRPPHPRLRRE--PEAEPGNNRPVYIP--QPRPPHPRLR--REPEAEPGNNRP-VYIPQ 160
Apid73          QPRPPHPRLRREAEPEAEPGNNRPVYIP--QPRPPHPRLRREAKPEAKPGNNRP-VYIPQ 231
chihp74         HAKPLHAKPVRAKPAPVKPLHTKAIHAMPAQVKSAHTKPQAANLEPSKPEPSNPKLSKSE 236
                :.:* *.:  *     .:*                          ::*  ..* :  .:

Apid22          PRPPHPRI------- 144
Apid14          PRPPHPRI------- 168
Apid73          PRPPHPRI------- 239
chihp74         PAKSVPPLLMKMVKN 251
                 •   . * :
```

Figure 3.12 An amino acid alignment of *Chi-Hp* with Apidaecins.

Expression of the recombinant protein

Recombinant expression of chitinase gene in the *Escherichia coli* expression system

To express the chitinase isolated from hepatopancreas, after the correction of this gene was confirmed in several ways, the recombinant plasmid, pET-17b-Chi-HP, was transformed into the *E. coli* expression host cells, both Rosetta (DE3)pLysS and BL21. Each transformant was cultured in LB medium containing 100 μg/ml ampicillin. IPTG was used as an inducer for activating the protein expression. After induction with 1 mM IPTG for an overnight, the culture was collected followed by centrifugation to separate the supernatant liquid out of cell pellet. The cell pellet was solubilized in the 2X SDS-PAGE loading dye, whereas the soluble proteins in the supernatant liquid were precipitated in a 10% final concentration of trichloroacetic acid as described in Materials and Methods. The precipitated proteins were collected by centrifugation. Proteins pellet was solubilized in 2X loading dye. Both the solubilized cells and the soluble proteins were analyzed by 10% SDS-PAGE. The result illustrated that neither Rosetta(DE3)pLysS nor BL21 (DE3) host cells showed the expression of chitinase (Figure 3.13 and Figure 3.14).

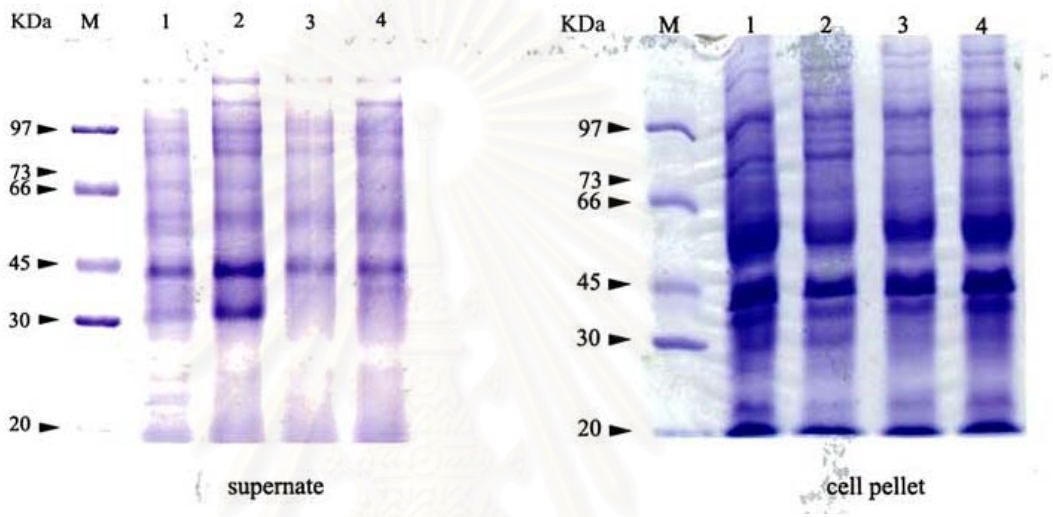


Figure 3.13 SDS-PAGE of the crude enzyme preparation of the pChi-HP-transformed *E. coli* Rosetta(DE3)pLysS. Lane M is the protein marker, Lane 1 is the un-induced pET-17b transformant, Lane 2 is the induced pET-17b transformant, Lane 3 is the un-induced pET-17b-Chi-HP transformant, and Lane 4 is the induced pET-17b-Chi-HP transformant.

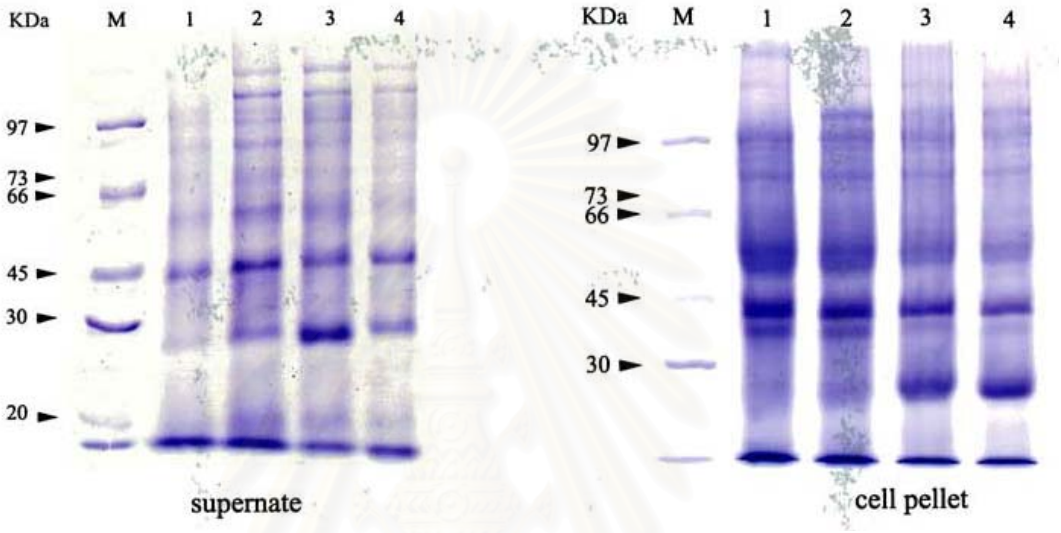


Figure 3.14 SDS-PAGE of the crude enzyme preparation of the pChi-HP-transformed *E. coli* BL21(DE3). Lane M is the protein marker, Lane 1 is the un-induced pET-17b transformant, Lane 2 is the induced pET-17b transformant, Lane 3 is the un-induced pET-17b-Chi-HP transformant, and Lane 4 is the induced pET-17b-Chi-HP transformant.

Typically, the expected band should be visualized at the size of 72.69 KDa based on deduced amino acid sequence. From the result of SDS-PAGE, there was no different intensity of the target band between un-induced and induced pET-17b-Chi-HP transformant. This was concordant with the result from chitinase activity staining (data not shown). Therefore the sequence of *Chi-HP* was rechecked. From the sequencing result, *Chi-HP* has one added adenine base causing frame shift at downstream of gene resulting in change of stop codon which differed from *PmChi*-1 sequence. The new reverse primer was designed again for covering the exact stop codon of *Chi-HP* gene at 3' terminus. The new reverse primer sequence was illustrated as follows

NHPyRewII: 5'-AAGGAAAAAAGCGGCCGCTCAATGGTACACTTTAGTTC-3'. This new reverse primer added with *NotI* restriction site at 5'-end was primed at the site of +2008 which the exact stop codon for this gene was located at the site of +1995. Subsequently, the cDNA chitinase gene was reamplified by the old forward and the new reverse primers. The PCR reaction was separated on 0.8% agarose gel and the prominent PCR product which approximates size of 2 kb was purified as the same method. The purified fragment was digested with *NotI* and *NdeI* restriction enzymes, and then was separated and eluted from 0.8% agarose gel. The digested fragment was ligated to the pET-17b digested with the same restriction enzymes. The recombinant plasmid, pET-17b-ChiHP74, was transformed, sequenced, and expressed, respectively, in both BL21(DE3) and Rosetta(DE3)pLysS expression host cells.

After this recombinant plasmid was sequenced, the sequencing result of the new sequence shows this chitinase gene, *ChiHP*74, contains the open reading frame (ORF) of 1995 bp encoding a polypeptide of 665 amino acid residues with a predicted molecular mass of 74 kDa (Figure 3.15).

```
        10        20        30        40        50        60
CGGCGAGTCCTGCTCCGGCCGCCTGGCGGCCGCGGGAATTCGATTGGAATTCATATGGTG
                                                    M  V

        70        80        90       100       110       120
AGCGGCCGAGTTTTGGCAGTCCTAGTGACGCTTGCAGCATTGGCAACACTGGTGTTGGCT
S  G  R  V  L  A  V  L  V  T  L  A  A  L  A  T  L  V  L  A

       130       140       150       160       170       180
GACCCGAGATTCGAGCAAGAAGGAGCCCAGCGGCGGTGGGTGCGGCCCGAGGGCCAGGCG
D  P  R  F  E  Q  E  G  A  Q  R  R  W  V  R  P  E  G  Q  A

       190       200       210       220       230       240
CGCCGCGTGTGCTACTACGAGGCGTGGGCCATCTACCGGCCCGGCGACGGCTTCTACGAC
R  R  V  C  Y  Y  E  A  W  A  I  Y  R  P  G  D  G  F  Y  D

       250       260       270       280       290       300
ATCGAGGACATCCCCGCCAACCTGTGCACGGACCTCATCTACTCGTTCATTGGCCTCTCC
I  E  D  I  P  A  N  L  C  T  D  L  I  Y  S  F  I  G  L  S

       310       320       330       340       350       360
AACGTCACGTGGGAAGTGCTCATTCTCGACCCTGAGTACGACATCAACCTGAACGGTTTC
N  V  T  W  E  V  L  I  L  D  P  E  Y  D  I  N  L  N  G  F

       370       380       390       400       410       420
CGGCGGTTCGTGGCGCTGAAGGACAAGTACCCTGACATGAAGACAAACATCGCCGTGGGC
R  R  F  V  A  L  K  D  K  Y  P  D  M  K  T  N  I  A  V  G

       430       440       450       460       470       480
GGCTGGGCCGAGGGCGGAAGGAAGTACTCGCAGATGGTGATGGTGCCCGAAAGGAGGGCG
G  W  A  E  G  G  R  K  Y  S  Q  M  V  M  V  P  E  R  R  A

       490       500       510       520       530       540
TCTTTCATCAGGAGCGTCGTCCAGCTGCTCACCGACTACGGCTTCGACGGGTTGGACTTG
S  F  I  R  S  V  V  Q  L  L  T  D  Y  G  F  D  G  L  D  L

       550       560       570       580       590       600
GACTGGGAATACCCTGGCGCCACAGACCGAGGAGGCCAATATGCCGATAAGGACAACTTC
D  W  E  Y  P  G  A  T  D  R  G  G  Q  Y  A  D  K  D  N  F

       610       620       630       640       650       660
CTTAAACTGGTGCAGGAGCTGCGCGAAGCCTTCGACACCGTGGGGCTGGGCTGGGAAATC
L  K  L  V  Q  E  L  R  E  A  F  D  T  V  G  L  G  W  E  I
```

```
       670       680       690       700       710       720
ACGTGCGCCGTGCCCGTGGCCAAGTTCCGCCTGCAGGAGGGCTACCATGTGCCTCAGCTC
T  C  A  V  P  V  A  K  F  R  L  Q  E  G  Y  H  V  P  Q  L

       730       740       750       760       770       780
TGCAGCCTGCTGGACGCCATCCACCTGATGACGTACGACCTGCGGGGCAACTGGGTCGGC
C  S  L  L  D  A  I  H  L  M  T  Y  D  L  R  G  N  W  V  G

       790       800       810       820       830       840
TTCGCGGACGTGCACTCCATGCTGTACACTCGGCCCGGACTGGACGAGTGGGCCTACGAG
F  A  D  V  H  S  M  L  Y  T  R  P  G  L  D  E  W  A  Y  E

       850       860       870       880       890       900
AAGCTGAACGTGAACGACGGCGCTCTCCTGTGGGTGGAATTCGGGTGTCCGCGTGATAAG
K  L  N  V  N  D  G  A  L  L  W  V  E  F  G  C  P  R  D  K

       910       920       930       940       950       960
TTGGTGGTCGGGACGCCATTCTACGGGCGCACCTACACGCTGGGTGACCCCACCAACAAC
L  V  V  G  T  P  F  Y  G  R  T  Y  T  L  G  D  P  T  N  N

       970       980       990      1000      1010      1020
GGCCTGCACGCGCCCATCAAGAAGTGGGAGGGAGGCGGCAAGCCCGGCCCTTATACCAAC
G  L  H  A  P  I  K  K  W  E  G  G  G  K  P  G  P  Y  T  N

      1030      1040      1050      1060      1070      1080
GCCACCGGCACTATGGCTTACTTCGAGATCTGCCTCATGATGAAGGAGGACTCCGAGTGG
A  T  G  T  M  A  Y  F  E  I  C  L  M  M  K  E  D  S  E  W

      1090      1100      1110      1120      1130      1140
GTCGATCGCTACGATGACGTCGGCCTCGTCCCATTCACGCACAAAGGCGACCAGTGGGTG
V  D  R  Y  D  D  V  G  L  V  P  F  T  H  K  G  D  Q  W  V

      1150      1160      1170      1180      1190      1200
GGCTACGAGGACCCTGACAGTCTCAAGATCAAGATGGACTTCATCCGCGAGCAGGGCTAC
G  Y  E  D  P  D  S  L  K  I  K  M  D  F  I  R  E  Q  G  Y

      1210      1220      1230      1240      1250      1260
CTCGGCGCCATGACCTGGGCCATCGACCAGGACGACTTCCGGAACTGGTGTGGAAGGGGA
L  G  A  M  T  W  A  I  D  Q  D  D  F  R  N  W  C  G  R  G

      1270      1280      1290      1300      1310      1320
CAGAACCCGATGATGAACACCATTTACGATGGCATGAAGGACTACGTAGTGCCTGTTGCT
Q  N  P  M  M  N  T  I  Y  D  G  M  K  D  Y  V  V  P  V  A
```

```
      1330      1340      1350      1360      1370      1380
CCCACTCTTCCTCCAACCACAACAAGCCCCTGGTGGGCCCCACCAACTACTACTACCACA
P  T  L  P  P  T  T  T  S  P  W  W  A  P  P  T  T  T  T  T

      1390      1400      1410      1420      1430      1440
ACACGGGACCCCAGCATCACCACGACCACGAGAGATCCCAACTTGCCGACCACAACTATG
T  R  D  P  S  I  T  T  T  T  R  D  P  N  L  P  T  T  T  M

      1450      1460      1470      1480      1490      1500
GGGCCTATTGACTGTACTGTGCAAGAATACTGGCCGCATCCGGACTGTGACAAGTACTAC
G  P  I  D  C  T  V  Q  E  Y  W  P  H  P  D  C  D  K  Y  Y

      1510      1520      1530      1540      1550      1560
TGGTGCTTCGAAGGCATACCCCACCTGGAGTACTGCCCCGCTGGCACCGTGTGGAACCAG
W  C  F  E  G  I  P  H  L  E  Y  C  P  A  G  T  V  W  N  Q

      1570      1580      1590      1600      1610      1620
GCTATCAAGGCGTGCGACTGGCCGGCCAACGTGGACACCTCCGGCTGCAACATGCCCTCG
A  I  K  A  C  D  W  P  A  N  V  D  T  S  G  C  N  M  P  S

      1630      1640      1650      1660      1670      1680
CTCTCGAAAGACGCCAGCCAGCGGCCCCTCCACAACACTATCCCTCTCAACGTCCGAACC
L  S  K  D  A  S  Q  R  P  L  H  N  T  I  P  L  N  V  R  T

      1690      1700      1710      1720      1730      1740
GAGGGGACCCCGCACTCGGGCAAGGCGCCAAAGGTTCCTCTTAACTTGATTTCCAAGAAG
E  G  T  P  H  S  G  K  A  P  K  V  P  L  N  L  I  S  K  K

      1750      1760      1770      1780      1790      1800
GCAGCCCCGGCGAAGTCTTTACCTGCGAAGTCAGTTGATGCTAAATTAGTTCACAATAAT
A  A  P  A  K  S  L  P  A  K  S  V  D  A  K  L  V  H  N  N

      1810      1820      1830      1840      1850      1860
GCACCGCCTGCTAAACCAGCACATGCTAAGCCTCTTCATGCTAAACCAGTTCGTGCTAAG
A  P  P  A  K  P  A  H  A  K  P  L  H  A  K  P  V  R  A  K

      1870      1880      1890      1900      1910      1920
CCAGCACCGGTAAAGCCACTTCATACTAAAGCAATTCATGCTATGCCAGCACAGGTAAAG
P  A  P  V  K  P  L  H  T  K  A  I  H  A  M  P  A  Q  V  K

      1930      1940      1950      1960      1970      1980
TCAGCTCATACTAAACCACAAGCGGCTAACCTAGAACCTTCCAAACCAGAACCGTCGAAC
S  A  H  T  K  P  Q  A  A  N  L  E  P  S  K  P  E  P  S  N
```

```
      1990      2000      2010      2020      2030      2040
CCAAAACTTTCCAAGTCAGAGCCTGCTAAGTCAGTACCACCACTGCTAATGAAGATGGTA
P  K  L  S  K  S  E  P  A  K  S  V  P  P  L  L  M  K  M  V

      2050      2060      2070      2080      2090      2100
AAGAACTAAAGTGTACCATTGAGCGGCCGCTTTTTTCCTTAATCACTAGTGAATTCGCGG
K  N  *

      2110      2120      2130      2140      2150
CCGCCTGCAGGTCGACCATATGGAGAGCTCCCAANCGCGTGATCAATGGGG
```

Figure 3.15 Nucleotide sequence and deduced amino acid sequence of Chitinase (*ChiHp74*) isolated from hepatopancreas mRNA of *Penaeus monodon* and deduced amino acid sequence. The open reading frame (ORF) of 1995 bp encodes a polypeptide of 665 amino acid residues. The underline letters are shown nucleotide primers. The asterisk represented a stop codon.

To express this new recombinant plasmid, the pET-17b-*ChiHP*74 was transformed into *E.coli* both Rosetta(DE3)pLysS and BL21 strains by the same methods of *Chi-HP* expression. The result showed that both Rosetta(DE3)pLysS and BL21 host cells shown no expression (Figure 3.16 and Figure 3.17).

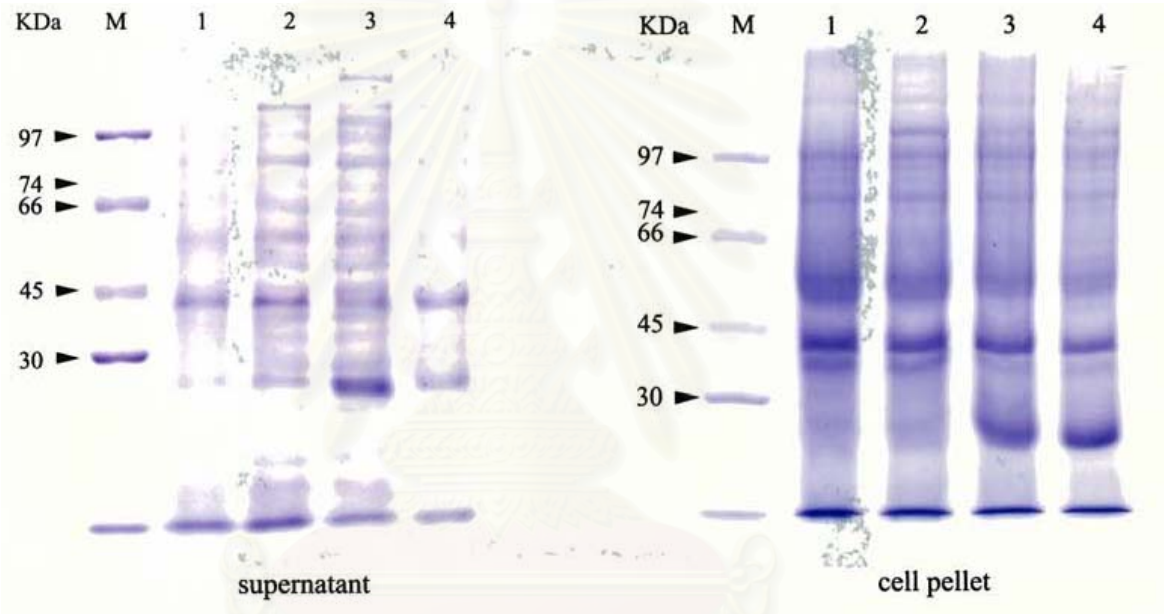


Figure 3.16 SDS-PAGE of the crude enzyme preparation of the pET-ChiHP74-transformed *E. coli* Rosetta(DE3)pLysS. Lane M is the protein marker, Lane 1 is the un-induced pET-17b transformant, Lane 2 is the induced pET-17b transformant, Lane 3 is the un-induced pET-17b-Chi-HP transformant, and Lane 4 is the induced pET-17b-Chi-HP transformant.

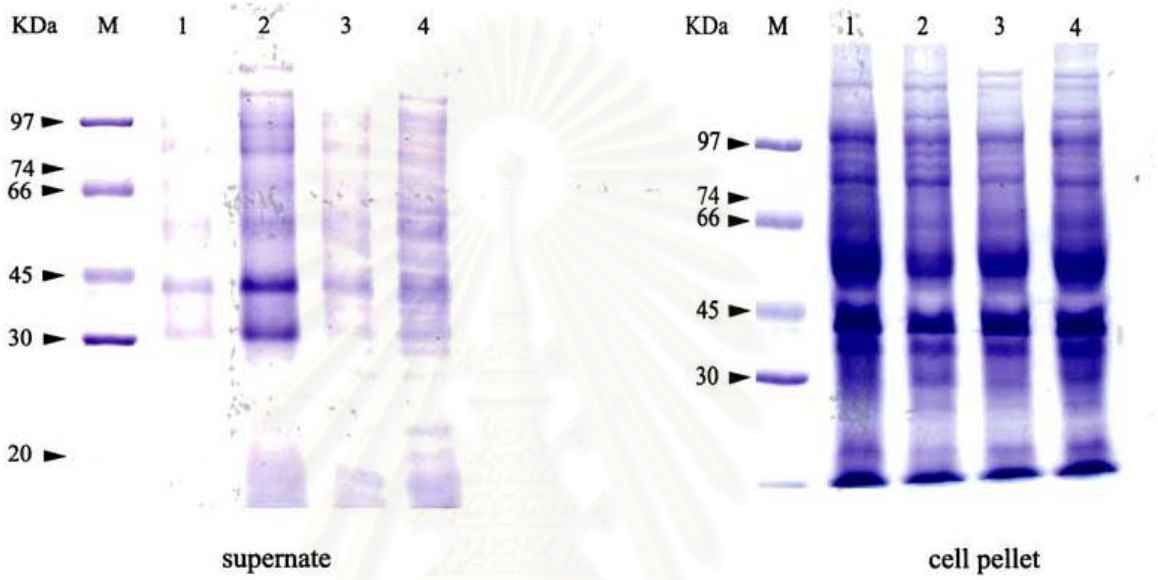


Figure 3.17 SDS-PAGE of the crude enzyme preparation of the pET-ChiHP74-transformed *E. coli* BL21(DE3). Lane M is the protein marker, Lane 1 is the un-induced pET-17b transformant, Lane 2 is the induced pET-17b transformant, Lane 3 is the un-induced pET-17b-Chi-HP transformant, and Lane 4 is the induced pET-17b-Chi-HP transformant.

Also, the expected band should be visualized at the size of 74.26 KDa based on deduced amino acid sequence. From the result of SDS-PAGE, there was no different intensity of the target band between un-induced and induced pET-17b-ChiHP74 transformant. This was concordant with the result from chitinase activity staining (data not shown).

From the undetectable level of expression results of both *Chi-HP* and *ChiHP74* full-lengths, several domains were considered to be separately cloned for monitoring the activity of chitinase. The catalytic domain was one chosen to be cloned into pET-17b. To obtain the catalytic-domain fragment, firstly, a new reverse primer was redesigned to prime within the catalytic domain region. This new primer named RCatD added with stop codon and *BamHI* restriction site at the 5' end was primed at the site of +1284 based on *PmChi1* DNA sequence. The RCatD primer has a sequence as

RCatD: 5' CGGGATCCCTAAAGAGTGGGAGC3'

To generate the catalytic-domain fragment, the forward primer, HpforI, and the reverse primer, RCatD, were used in amplification of cDNA. The amplification reaction was separated on 0.8% agarose gel; the prominent PCR product size of 1.27 kb was observed (Figure 3.18). This band was eluted and purified from the gel as the same method. The purified fragment was digested with *NdeI* and *BamHI* restriction enzymes for overnight. The digestion reaction was separated on 0.8% agarose gel, and eluted and purified from the gel. The digested fragment was subjected to ligate with pET-17b vector digested with *NdeI* and *BamHI* restriction enzymes. When the recombinant plasmid was transformed into E. coli JM109, cells were grown for overnight, which were used in plasmid extraction. This plasmid was subjected to DNA sequencing. The sequencing result of the catalytic domain shows that this domain, *Chi-CatD*, contained the open reading frame (ORF) of 1278 bp encoding a polypeptide of 426 amino acid residues with a predicted molecular mass of 48 kDa (Figure 3.19).

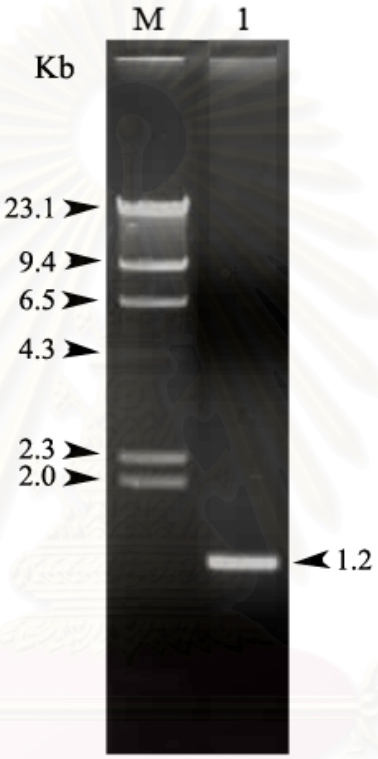


Figure 3.18 Agarose gel electrophoresis of amplified catalytic domain of chitinase cDNA. The PCR product was separated on 0.8% agarose gel and then visualized by ethidium bromide staining. Lane M is DNA marker, Lane 1 is PCR product.

```
      10        20        30        40        50        60
AACGATCTCTANATATTTTGTTTACTTTAGAAGAGATATACATATGGTGAGCGGCCGAGT
                                            M  V  S  G  R  V

        70        80        90       100       110       120
TTTGGCAGTCCTAGTGACGCTTGCAGCATTGGCAACACTAGTGTTGGCTGACCCGAGATT
 L  A  V  L  V  T  L  A  A  L  A  T  L  V  L  A  D  P  R  F

       130       140       150       160       170       180
CGAGCAAGAAGGAGCCCAGCGGCGGTGGGTGCGGCCCGAGGGCCAGGCGCGCCGCGTGTG
 E  Q  E  G  A  Q  R  R  W  V  R  P  E  G  Q  A  R  R  V  C

       190       200       210       220       230       240
CTACTACGAGGCGTGGGCCATCTACCGGCCCGGCGACGGCTTCTACGACATCGAGGACAT
 Y  Y  E  A  W  A  I  Y  R  P  G  D  G  F  Y  D  I  E  D  I

       250       260       270       280       290       300
CCCCGCCAACCTGTGCACGGACCTCATCTACTCGTTCATTGGCCTCTCCAACGTCACGTG
 P  A  N  L  C  T  D  L  I  Y  S  F  I  G  L  S  N  V  T  W

       310       320       330       340       350       360
GGAAGTGCTCATTCTCGACCCTGAGTACGACATCAACCTGAACGGTTTCCGGCGGTTCGT
 E  V  L  I  L  D  P  E  Y  D  I  N  L  N  G  F  R  R  F  V

       370       380       390       400       410       420
GGCGCTGAAGGACAAGTACCCTGACATGAAGACAAACATCGCCGTGGGCGGCTGGGCCGA
 A  L  K  D  K  Y  P  D  M  K  T  N  I  A  V  G  G  W  A  E

       430       440       450       460       470       480
GGGCGGAAGGAAGTACTCGCAGATGGTGATGGTGCCCGAAAGGAGGGCGTCTTTCATCAG
 G  G  R  K  Y  S  Q  M  V  M  V  P  E  R  R  A  S  F  I  R

       490       500       510       520       530       540
GAGCGTCGTCCAGCTGCTCACCGACTACGGCTTCGACGGGTTGGACTTGGACTGGGAATA
 S  V  V  Q  L  L  T  D  Y  G  F  D  G  L  D  L  D  W  E  Y

       550       560       570       580       590       600
CCCTGGCGCCACAGACCGAGGAGGCCAATATGCCGATAAGGACAACTTCCTTAAACTGGT
 P  G  A  T  D  R  G  G  Q  Y  A  D  K  D  N  F  L  K  L  V

       610       620       630       640       650       660
GCAGGAGCTGCGCGAAGCCTTCGACACCGTGGGGCTGGGCTGGGAAATCACGTGCGCCGT
 Q  E  L  R  E  A  F  D  T  V  G  L  G  W  E  I  T  C  A  V
```

```
       670       680       690       700       710       720
GCCCGTGGCCAAGTTCCGCCTGCAGGAGGGCTACCATGTGCCTCAGCTCTGCAGCCTGCT
 P  V  A  K  F  R  L  Q  E  G  Y  H  V  P  Q  L  C  S  L  L

       730       740       750       760       770       780
GGACGCCATCCACCTGATGACGTACGACCTGCGGGGCAACTGGGTCGGCTTCGCGGACGT
 D  A  I  H  L  M  T  Y  D  L  R  G  N  W  V  G  F  A  D  V

       790       800       810       820       830       840
GCACTCCATGCTGTACACTCGGCCCGGACTGGACGAGTGGGCCTACGAGAAGCTGAACGT
 H  S  M  L  Y  T  R  P  G  L  D  E  W  A  Y  E  K  L  N  V

       850       860       870       880       890       900
GAACGACGGCGCTCTCCTGTGGGTGGAATTCGGGTGTCCGCGTGATAAGTTGGTGGTCGG
 N  D  G  A  L  L  W  V  E  F  G  C  P  R  D  K  L  V  V  G

       910       920       930       940       950       960
GACGCCATTCTACGGGCGCACCTACACGCTGGGTGACCCCACCAACAACGGCCTGCACGC
 T  P  F  Y  G  R  T  Y  T  L  G  D  P  T  N  N  G  L  H  A

       970       980       990      1000      1010      1020
GCCCATCAAGAAGTGGGAGGGAGGCGGCAAGCCCGGCCCTTATACCAACGCCACCGGCAC
 P  I  K  K  W  E  G  G  G  K  P  G  P  Y  T  N  A  T  G  T

      1030      1040      1050      1060      1070      1080
TATGGCTTACTTCGAGATCTGCCTCATGATGAAGGAGGACTCCGAGTGGGTCGATCGCTA

 M  A  Y  F  E  I  C  L  M  M  K  E  D  S  E  W  V  D  R  Y

      1090      1100      1110      1120      1130      1140
CGATGACGTCGGCCTCGTCCCATTCACGCACAAAGGCGACCAGTGGGTGGGCTACGAGGA
 D  D  V  G  L  V  P  F  T  H  K  G  D  Q  W  V  G  Y  E  D

      1150      1160      1170      1180      1190      1200
CCCTGACAGTCTCAAGATCAAGATGGACTTCATCCGCGAGCAGGGCTACCTCGGCGCTAT
 P  D  S  L  K  I  K  M  D  F  I  R  E  Q  G  Y  L  G  A  M

      1210      1220      1230      1240      1250      1260
GACCTGGGCCATCGACCAGGACGACTTCCGGAACTGGTGTGGAAGGGGACAGAACCCGAT
 T  W  A  I  D  Q  D  D  F  R  N  W  C  G  R  G  Q  N  P  M

      1270      1280      1290      1300      1310      1320
GATGAACACCATTTACGATGGCATGAAGGACTACGTAGTGCCTGTTGCTCCCACTCTTTA
 M  N  T  I  Y  D  G  M  K  D  Y  V  V  P  V  A  P  T  L  *
```

```
      1330      1340      1350      1360      1370      1380
GGGATCCCTTTTTTCCTTAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATG

      1390      1400      1410
GAGAGCTCCCAANCGCGTGATCAATGGGG
```

Figure 3.19 The nucleotide sequence and deduced amino acid sequence of catalytic domain of chitinase (*Chi-CatD*) isolated from hepatopancreas of *P. monodon*. The open reading frame (ORF) of 1278 bp encodes a polypeptide of 426 amino acid residues. The underline letters are shown oligonucleotide primers. The asterisk represented a stop codon.

After the exact fragment of catalytic domain was confirmed by sequencing, the recombinant plasmid, pET17b-Chi-CatD, was transformed into *E. coli* BL21 (DE3) cell in order to express the protein as the same methods of *Chi-HP* expression. In the SDS-PAGE result, we found that the expected chitinase band was not detected where the expected size is based on deduce amino acid sequence (Figure 3.20). Surprisingly, the chitinase activity was detectable at 300 mU/ml crude enzyme (the unit of enzyme activity was defined as the amount of the enzyme capable to liberate 1 μmole of either *N*-acetylglucosamine or D-glucosamine per hour).

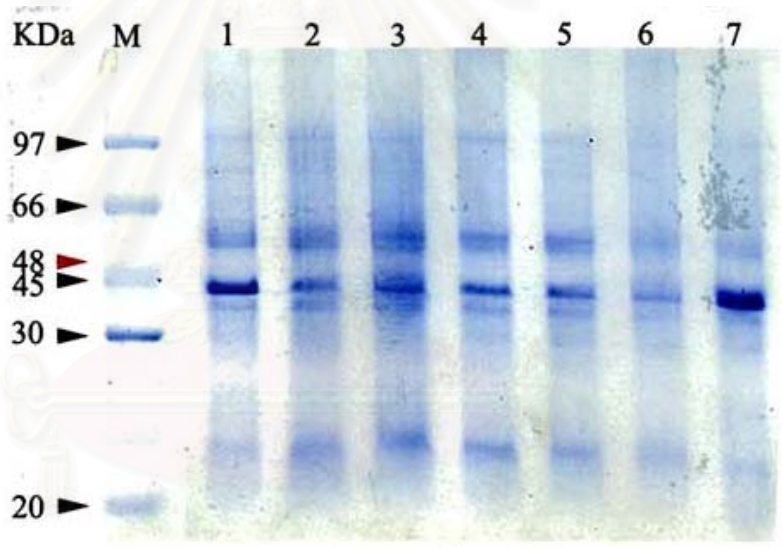


Figure 3.20 SDS-PAGE of the crude enzyme preparation of the pET-Chi-CatD-transformed *E. coli* BL21(DE3). Lane M is protein marker, Lane 1 is cell pellet of *E. coli* carried pET-17b, Lane 2 is cell pellet of *E. coli* carried pET-17b-ChiCatD for 0 h, Lane 3 is cell pellet of *E. coli* carried pET17b-ChiCatD for 1 h, Lane 4 is cell pellet of *E. coli* carried pET17b-ChiCatD for 2 h, Lane 5 is cell pellet of *E. coli* carried pET17b-ChiCatD for 3 h, Lane 6 is cell pellet of *E. coli* carried pET17b-ChiCatD for 4 h, and Lane 7 is cell pellet of *E. coli* carried pET17b-ChiCatD for overnight.

3.5 Characterization of chitinase

After the chitinase activity was monitored from an expression of pET-17b-Chi-CatD in BL21 host cells, the cells were cultured for a large scale production. Total cells were harvested by centrifugation and then broken by sonication. The crude enzyme was assayed by colorimetric method.

3.5.1 Optimum temperature of chitinase

To determine the optimum temperature of *Chi-CatD*, the chitinase activity was examined at several temperatures ranged from 20 ^{o}C to 90 ^{o}C. The 200 μl of crude enzyme was incubated with assay reaction composed of 75 μl of 0.1M phosphate buffer pH 7.0, 75 μl of 5% of beta-chitin, and 400 μl of distilled water, for 10 min. After incubation, chitinase activity was detected by measuring the amount of apparent reducing sugar using colorimetric method. The result shows the maximum chitinase activity was found at 60 ^{o}C, suggesting that this temperature is an optimal condition for crude chitinase as shown in Figure 3.21.

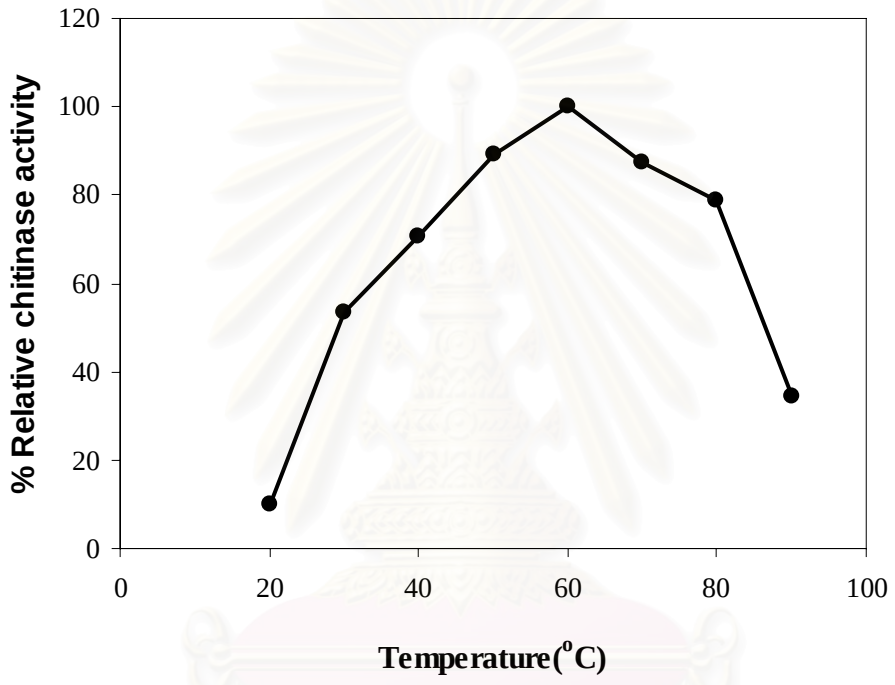


Figure 3.21 Effect of temperature on chitinase activity expressed from pET-17b-Chi-CatD. Crude enzyme was incubated at 20 – 90 ^{o}C in 0.1 M phosphate buffer pH 7.0 for 1 hour, when beta-chitin was used as a substrate.

3.5.2 Optimum pH of chitinase

To determine the optimum pH of *Chi-CatD*, the chitinase activity was examined at several pH ranged from 2.6 – 10 at 37 ^{o}C by using the universal buffer. The 200 μl of crude enzyme was incubated with assay reaction composed of 75 μl of 0.1M phosphate buffer pH 7.0, 75 μl of 5% of beta-chitin, and 400 μl of distilled water for 10 min. After incubation, chitinase activity was detected by measuring the amount of apparent reducing sugar using colorimetric method. The result shows that the crude chitinase has two ranges of optimum pH, acidic and basic ranges. The optimum pH of acidic range was pH 4.0. While, the optimum pH of basic range was pH 7-8 (Figure 3.22)

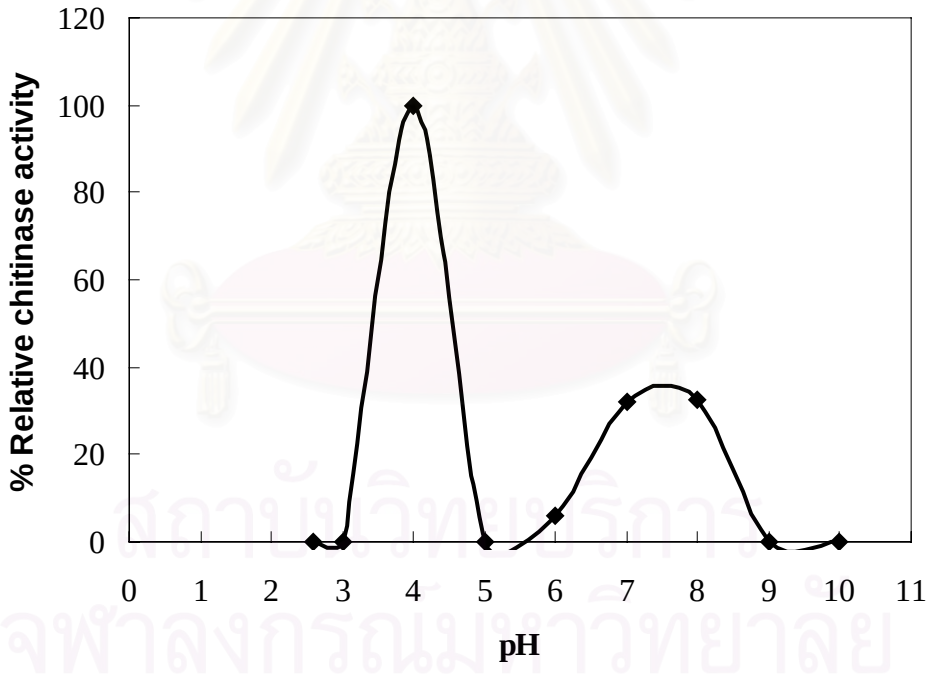


Figure 3.22 Effect of pH on chitinase activity expressed from pET-17b-Chi-CatD. Crude enzyme was incubated at several values of pH for 1 hour, using beta-chitin as a substrate.

3.5.3 Substrate specificity

To determine the substrate specificity of *Chi-CatD*, the chitinase activity was tested at pH4 and 60 ^{o}C. The 200 μl of crude enzyme was incubated with the assay reaction composed of 75 μl of 0.1M phosphate buffer, 250 μl of several types of substrate, 25 μl 1.0 mg/ml colloidal chitin, and 75 μl 1.0 mg/ml β-chitin, adjust to a final volume of 750 μl with distilled water, for 10 min. Several types of substrate are 0.1 mg/ml partially *N*- acetyl chitin (PNAC), 1.0 mg/ml regenerated chitin, 0.1 mg/ml 75% DD of chitosan, and 0.1 mg/ml 100% DD. After incubation, chitinase activity was detected by measuring the amount of apparent reducing sugar colorimetric method. The result shows several types of substrates were degraded by chitinase from *Chi-CatD*; however, beta- chitin is the best substrate for *Chi-CatD* (Figure 3.23).

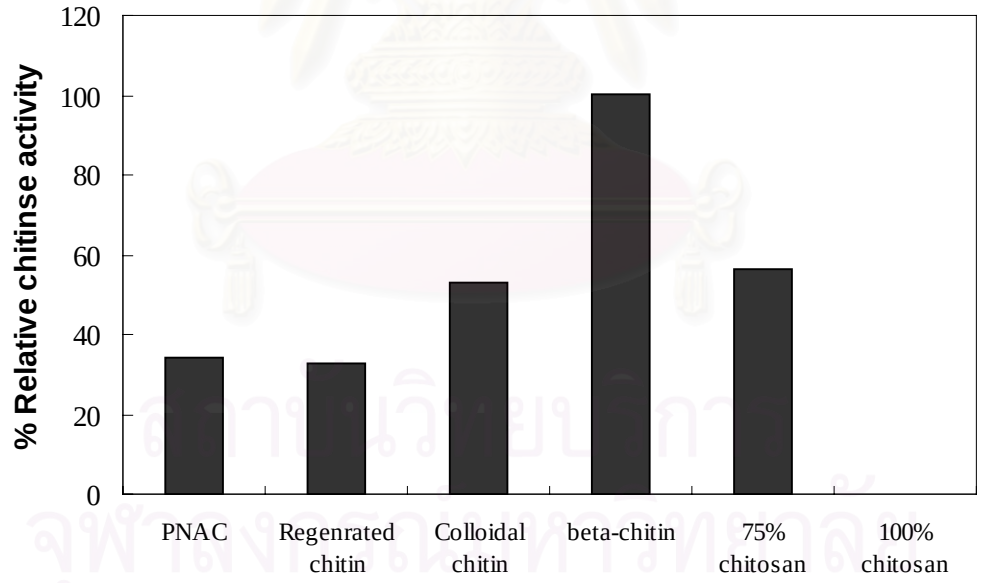


Figure 3.23 Effect of substrate on chitinase activity expressed from pET-17b-Chi-CatD. Crude enzyme was incubated at 60 ^{o}C in 0.1 M phosphate buffer pH 4.0 for 1 hour.

3.5.4 Determination of hydrolytic products

To determine the products hydrolyzed by *Chi-CatD*, HPLC and TLC methods were performed. The chitinase activity was examined at pH 4.0 and pH 7.0. At each value of pH, the reaction was incubated at 37 ^{o}C and 60 ^{o}C for overnight. For TLC method, The 500 μl of crude enzyme was incubated with the assay reaction composed of 100 μl of either 0.1M phosphate buffer pH 7.0 or universal buffer pH 4.0, 150 μl of 5% of beta-chitin, and 350 μl of distilled water. After the reaction was incubated for overnight, the pellet remaining in the completely hydrolyzed reaction was removed by centrifugation. The supernatant was collected and dried by vacuum centrifugation, subsequently, resuspended with 10 μl of de-ionized water and then spotted on siliga gel for thin layer chromatography (TLC). The result shows the product produced by the crude chitinase is disaccharide as shown in Figure 3.24. For HPLC method, the hydrolytic product of crude chitinase was incubated under the same conditions of TLC method. The hydrolysate was harvested by centrifugation. The 0.3 ml of a supernatant was mixed with 0.7 ml of acetonitrile, and filtrated through a 0.45 micron filter. The results showed the products similar to those determined by TLC method (Figure 3.25 A-F).

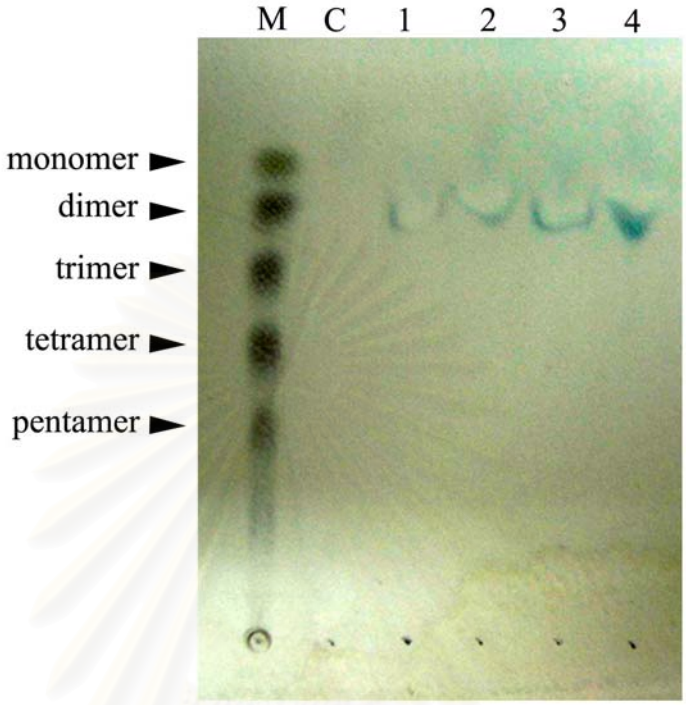
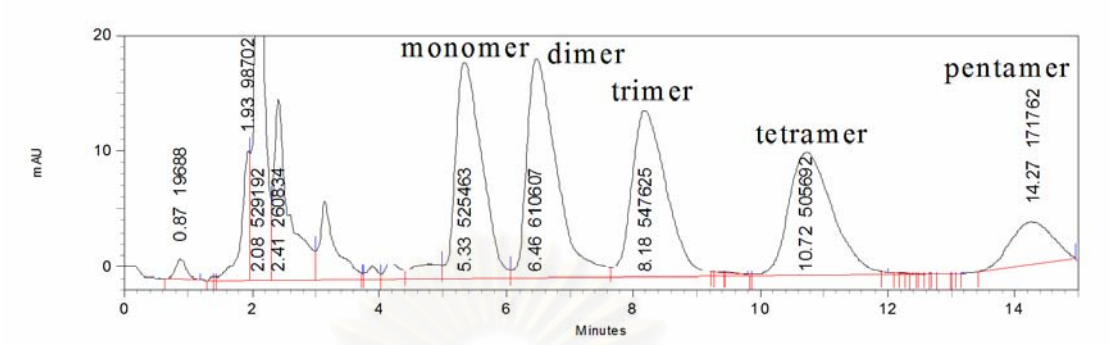
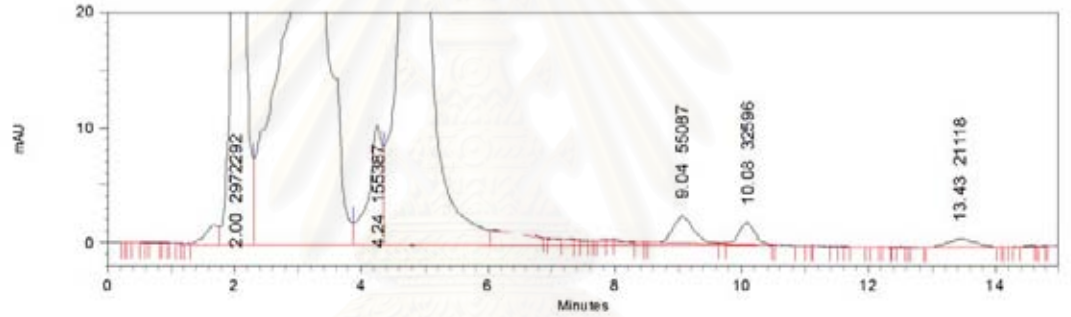


Figure 3.24 The hydrolytic product of *Chi-CatD* determined by TLC method. Crude enzyme was incubated at 37 and 60 ^{o}C in pH 4 and pH 7 for overnight, when beta-chitin was used as a substrate.

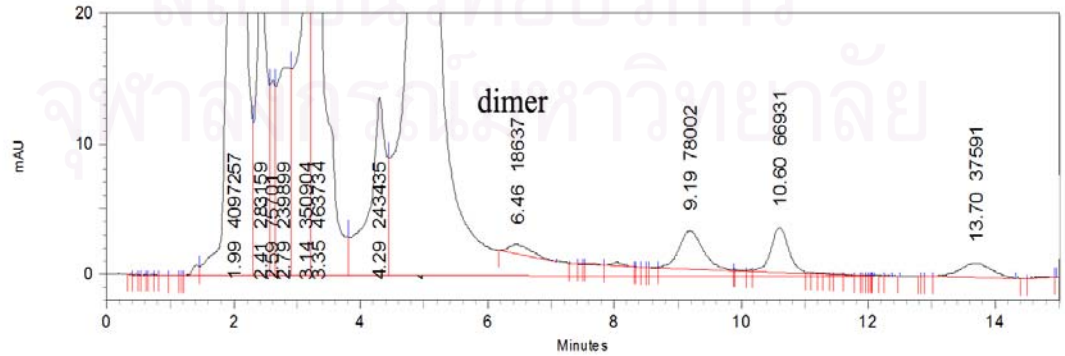
A) Standard



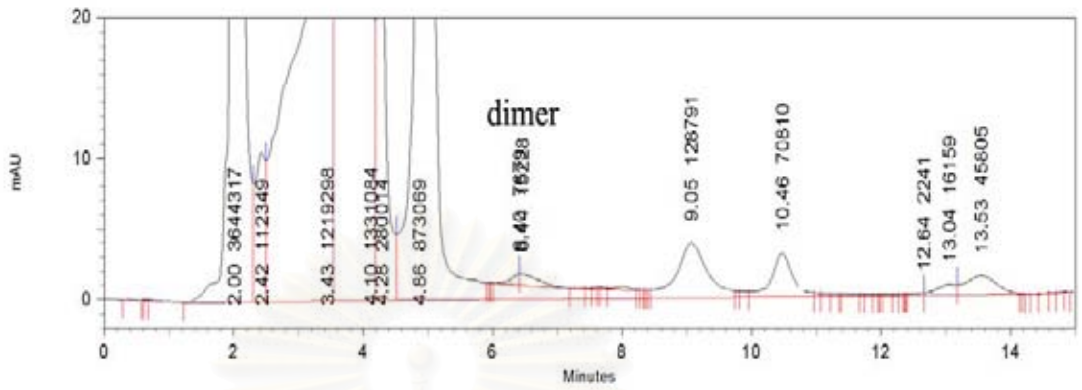
B) Control



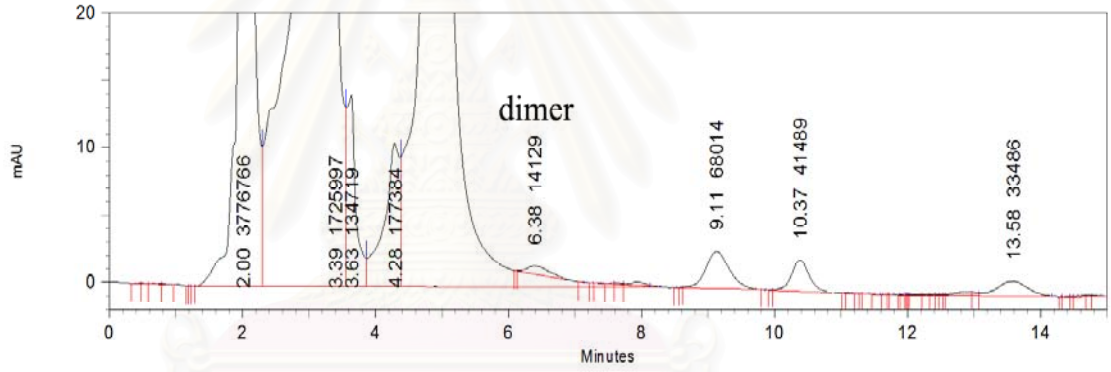
C) The product of crud enzyme was incubated at 37 °c, pH 4



D) The product of crud enzyme was incubated at 37 °c, pH 7



E) The product of crud enzyme was incubated at 60 °c, pH 4



F) The product of crud enzyme was incubated at 60 °c, pH 7

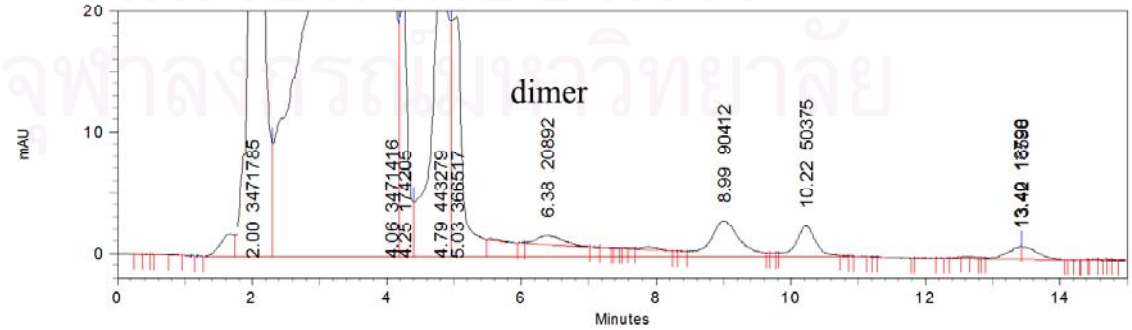


Figure 3.25 The hydrolytic product of *Chi-CatD* was determined by HPLC method. Crude enzyme was incubated at 37 and 60 ^{o}C in pH 4 and pH7 for overnight, when beta-chitin was used as a substrate as shown in A-F

3.6 Recombinant expression of chitinase gene in the *Pichia pastoris* expression system

3.6.1 Amplification of cDNA encoding chitinase

A chitinase gene was amplified from cDNA by using specific primers. For construction an expression cassette, the designs of forward and reverse primers were performed. In addition to the convenience of cloning, a *Mfe*I site was added to 5'-end of forward PCR primers. Besides, a *Not*I site was added to 3'-end of reverse primer. Primer sequences were:

NHPyfor : 5'GCGCAATTGATGGTGAGCGGCCGAGTT3'

NHPyRewII: 5'AAGGAAAAAAGCGGCCGCTCAATGGTACACTTT AGTTC3'

These primers were used to amplify the chitinase gene in order to in-frame into the *Pichia* expression vector pPIC9K and transformed to *E. coli* XL-10 GOLD cells.

The PCR amplification was performed as following: denaturation at 94 ^{o}C for 5 minute, 25 cycles of denaturation at 94 ^{o}C for 30 second, annealing at 55 ^{o}C for 30 second, extension at 72 ^{o}C for 4 minute. The final elongation step was performed at 72 ^{o}C for 10 minute. The result shows the prominent PCR product of chitinase gene which approximate size was 2 kb as shown in Figure 3.26.

Figure 3.26 Agarose gel electrophoresis of amplified chitinase cDNA. The PCR product was separated on 0.8% agarose gel and then visualized by ethidium bromide staining. Lane M is DNA marker and Lane 1 is PCR product.

3.6.1 Construction of recombinant plasmid pGEM-T easy vector with chitinase gene

The PCR product from a previous step was purified and ligated into pGEM-T easy vector. The ligated reaction was transformed into *E. coli* XL-10 GOLD cells. The recombinant clone was selected on LB agar plate containing ampicillin, IPTG, and X-Gal. The white colonies were randomly selected and cultured in LB broth containing ampicillin for overnight. After incubation, the cultures were subjected to plasmid extraction and digested with *Mfe*I and *Not*I restriction enzymes. Subsequently, theses reactions were analyzed on 0.8% agarose gel electrophoresis. This result showed the recombinant plasmid contained chitinase gene which approximate size was 1.95 kb. In the next step, inserted DNA was purified from the agarose gel for further ligation into pPIC9K.

3.6.3 Construction of recombinant plasmid pPIC9K vector with chitinase gene

This chitinase gene was eluted from 0.8% agarose gel by using Qiaquick Gel Extraction Kit (Qiagen, Germany). Afterwards, the samples were ligated into pPIC9K expression vector at the *ECoR*I and *Not*I sites. The recombinant plasmids were transformed into *E. coli* XL-10 GOLD cells. Then, the recombinant clone was selected on LB agar plate containing ampicillin. The selected plasmids were extracted and digested with *Pvu*II restriction enzymes to confirm the exact inserted gene. The reaction was analyzed on 0.8% agarose gel electrophoresis (Figure 3.27). These results indicated that the constructing gene contained chitinase gene in pPIC9K, revealed three bands: 6.5, 2.9, and 1.7 kb in lane 3 which were observed after digested with *Pvu*II restriction enzymes. The recombinant plasmid which carried chitinase gene was called *pPIC9K-ChiHP*74. In the next step, pPIC9K-ChiHP74 was linearized by digested with restriction enzyme *Sac*I (Figure 3.28) for transforming into *Pichia pastoris*. The 250 μl of cell suspension was spreaded on the YPD agar plates and incubated at 30 ^{o}C until colonies appear. After incubation, colonies were

subsequently screened for G418 resistance at a final concentration of 0.25, 0.5, 0.75, 1, and 1.5 mg/ml G418. The result shows *P. pastoris* can grow on YPD plate containing 1.5 mg/ml G418. The colony PCR was used to confirm the target DNA that had recombined into the chromosomes of recombinant yeast before use in protein expression. The resulting PCR product was run on 1% agarose gel to determine whether the DNA fragment was successfully amplified. In the PCR reaction, yeast genomic DNA was used as a template and 5'AOX and α-factor were used as primers. The first step to extract yeast genomic DNA, a single colony was picked and resuspended in 10 μl of water. Then add 5 μl of a 5 U/ μl solution of lyticase and incubate at 30 ^{o}C for 10 minute and freeze the sample at -80 ^{o}C for 10 minute. The amplification reaction was described in section 2.8.2.1.5. The result shows the PCR product of *P. pastoris* carrying pPIC9K-ChiHP74 was not observed as shown in Figure 3.29.

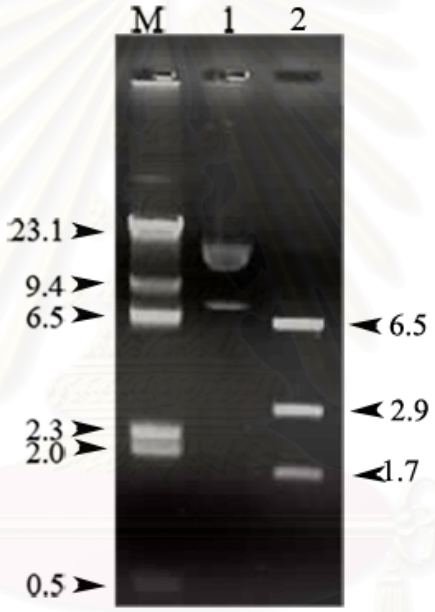


Figure 3.27 Agarose gel electrophoresis of the constructing chitinase gene in pPIC9K (pPIC9K-Chi-HP74) separated on 0.8% agarose gel and then visualized by ethidium bromide staining. Lane M is the DNA marker, Lane 1 is pPIC9K-Chi-HP74, and Lane 2 is pPIC9K-Chi-HP74 digested with *Pvu*II restriction enzymes shown three band: 6.5, 2.9, and 1.7 kb

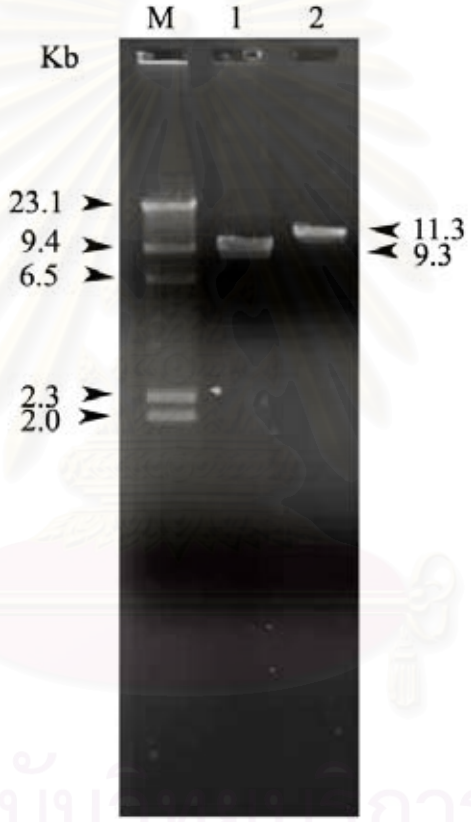


Figure 3.28 Agarose gel electrophoresis of pPIC9K and pPIC9K-Chi-HP74 digested with *Sac*I. The digestion reaction was separated on 0.8% agarose gel and then visualized by ethidium bromide staining.
Lane M is DNA marker, Lane 1 is pPIC9K/*Sac*I, and Lane 2 is pPIC9K-Chi-HP74/*Pvu*II

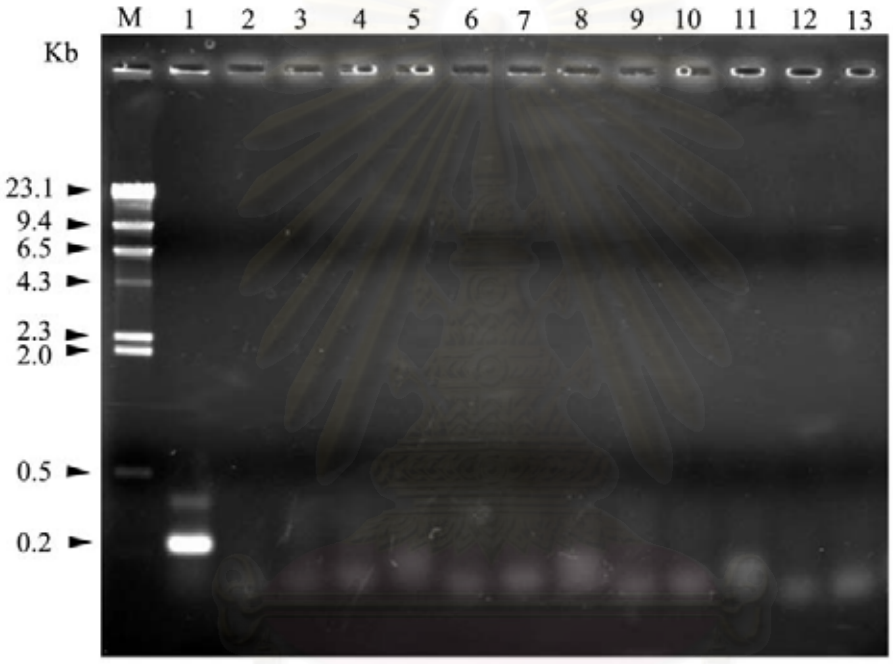


Figure 3.29 Agarose gel electrophoresis of colony PCR of *P. pastoris* carried pPIC9K-ChiHP74. The PCR product was separated on 0.8% agarose gel and then visualized by ethidium bromide staining. Lane M is the DNA marker, Lane 1 is PCR product of *P. pastoris* carried pPIC9K, and Lane 2-13 is PCR product of *P. pastoris* carried pPIC9K-ChiHP74.

3.6.4 Expression of recombinant yeasts

The *P. pastoris* carrying pPIC9K-ChiHP74 was cultured in BMMY medium and induced with absolute methanol at the final concentration of 0.5% (v/v) in every 24 hour. One milliliter of the expression culture was harvested everyday for 7 days interval. After that the express protein was analyzed as the same methods of *Chi-HP* expression but staining with silver stain. In the SDS-PAGE result, we found that the expected chitinase band was not detected where the expected size is based on deduce amino acid sequence as shown in Figure 3.30.

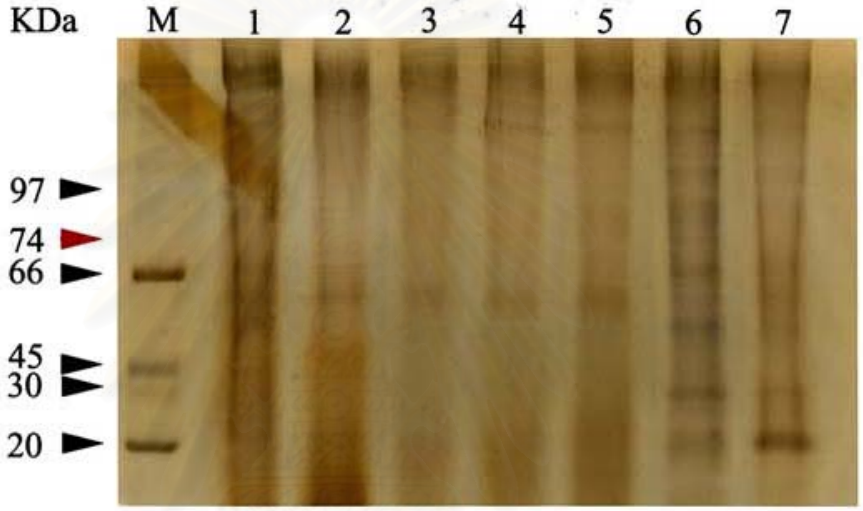


Figure 3.30 SDS-PAGE of the crude enzyme preparation of the pPIC9K-ChiHP74 transformed *P. pastoris* and induce with 0.5 % methanol. Lane M is the protein marker, Lane 1 is *P. pastoris* carried pPIC9K, Lane 2 is *P. pastoris* carried pPIC9K-ChiHP74 at o h, Lane 3 is *P. pastoris* carried pPIC9K-ChiHP74 at 1 day, Lane 4 is *P. pastoris* carried pPIC9K-ChiHP74 at 2 day, Lane 5 is *P. pastoris* carried pPIC9K-

ChiHP74 at 3 day, Lane 6 is *P. pastoris* carried pPIC9K-ChiHP74 at 4 day, Lane 3 is *P. pastoris* carried pPIC9K-ChiHP74 at 5 day.

3.7 The comparison of ChiHP76 with other chitinase genes

Based on previous finding, Chitinase are classified into three different arrangements of domains and hydrolytic products. ChitinaseA from *Serratia marcescens*, *Chi*A, comprises the catalytic domain (CatD) and N-terminal domain at C-terminal and N-terminal, respectively. The hydrolytic products of *Chi*A are di-saccharide [$(GlcNAc)_2$]. The chitinaseB from *Serratia marcescens*, *Chi*B, comprises the catalytic domain (CatD) and chitin binding domain (ChBD). The hydrolytic products of ChiB are tri-saccharide [$(GlcNAc)_3$]. Besides, ChitinaseA1 from *Bacillus circulan* WL-21 comprises the catalytic domain (CatD), two fibronectin type III-like domains, and C-terminal chitin-binding domain (ChBD). Furthermore, ChiA1 was already known the structure and the necessary amino acids. To align the sequence, amino acids sequence of *Chi*A from *Bacillus circulan* WL-21 and interested chitinase from hepatopancreas of *Penaeus monodon* (*ChiHP*74) were compared together. The results reveal the same located amino acids which ChiA consists of Y56, W53, W433, W164, W134, W284, Y279, and E204. Additionally, ChiHP74 consists of Y56, W53, W433, W164, Y134, W284, Y279, and E204 (Figure 3.25). These amino acids are important for the role of hydrolyzing activity of chitinase.

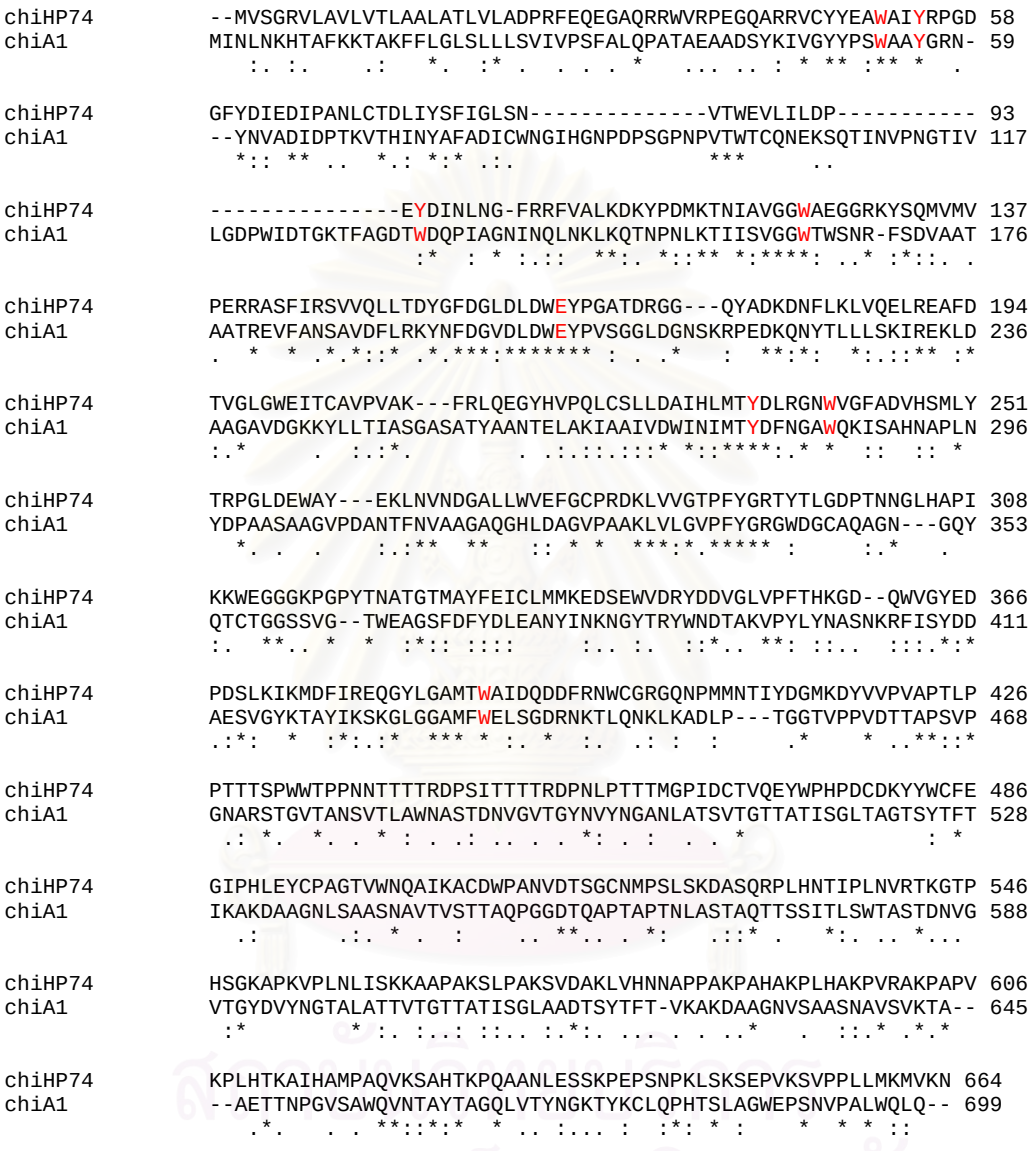


Figure 3.31 An alignment of *ChiHp*74 amino acid sequence compared with *ChiA*1 amino acid sequence. The amino acids that are important to the role of hydrolyzing activity of chitinase were shown in red letter.

CHAPTER IV
DISCUSSION

Three putative chitinase genes were obtained from a total of 10,100 EST sequences in *Penaeus monodon* EST database: two from a hemocyte library and another from a hepatopancreas library. After these sequences were reconfirmed by alignment with BLASTN program, the results show all sequences are similar to chitinase gene. Two clones found in the hemocyte library were similar to chitinase precursor of beetroot *Beta vulgaris*. The reason that two clone were not further characterized is because of low similarity when they were blast against the NCBI database. Also, chitinase clone found in the hepatopancreas library, named HPa0029, shows 87% homology to the *Penaeus monodon* chitinase 1, named *PmChi*-1. Thus, this clone, Hpa0029, was selected for study in all experiments.

Several studies reported about known gene in its database, Pan, D. et al. (2005) found that 3 chitinase clones from a total of 400 clones were found in the hepatopancreas cDNA library of WSSV-resistant shrimp *Penaeus japonicus* by suppression subtractive hybridization accounting for 0.75%. Compared to this study, we found only 3 clones from a total of 10,100 EST clones representing for 0.03%. This result indicated that chitinase gene from *P. japonicus* was expressed more twenty-five times more than that of *P. monodon*. However, the total clone from *P. japonicus* EST library resulted from a probe hybridization which is not like in *P. monodon* that the total clones were randomly sequenced. This result was concordant that both *P. japonicus* and *P. monodon* EST database show the ratio of chitinase clone at very low rate which is less than 1%.

In a previous study, chitinaseA1 gene, *PmChi*-1, isolated from the hepatopancreas library encodes a 621- amino acid protein possessing the functional domains of the chitinase protein family. The similarity of a product encoded from this gene is 81.8% identical to a chitinase1 protein expressed in the hepatopancreas of *Penaeus japonicus* (*Pj-chi1*). The result indicated that chitinase gene of the study, Hpa0029, was also a homologue of *Pj-chi1* also. An analysis by reverse transcription-

polymerase chain reaction (RT-PCR) indicates that *PmChi*-1 messenger RNA is detectable in the hepatopancreas and the gut. (Siok H. T. *et al*., 2000). Furthermore, *PmChi*-1 activity increases during molting cycle, suggesting that hepatopancreas expressing chitinase was involved in the degradation of endogenous chitin in the gut peritrophic membrane prior to molting (Siok H. T. *et al*., 2000), however, the characterization of *PmChi*-1 has been unknown.

In this study, chitinase isolated from the hepatopancreas cDNA library of *P. monodon* was clone into expression vector, pET-17b, for further expression and characterization. This gene was amplified with PCR using designed primers incorporated with restriction enzyme sites at their 5' ends for convenience in cloning this gene to the expression vector. With two different restriction sites incorporated, the gene could be cloned directionally into the vector. For some reason, the restriction digested PCR product could not be cloned directly into the pET-17b expression vector. Thus, the PCR product was first cloned into pGEM-T easy vector and then subcloned into the pET-17b. This recombinant plasmid was named *Chi-HP*. Subsequently, *Chi-HP* was sequenced and expressed.

The sequence alignment of *ChiHP* and *PmChi*1 indicated that *Chi-HP* has one added adenine base causing frame shift at downstream of gene resulting in loss stop codon which differed from *PmChi*-1 sequence. For this reason, *Chi-HP* could not been expressed in *E. coli* expression host or expressed incorrectly. The new location of reverse primer that can bind to a DNA template was redesigned for finding the exact stop codon of *Chi-HP* gene at 3' terminus. After that the PCR product was cloned using the same method of *Chi-HP* and sequenced. The sequence of this recombinant plasmid has the stop codon and was called *ChiHP*74.

ChiHP74 transformant was cultured and induced with IPTG in order to produce the expecting protein, chitinase. The supernatant and cell pellet were assayed for chitinase activity and analyzed by electrophoresis on a 10% SDS-PAGE. The chitinase activity and the approximate 74 kDa protein were not detected after induction for 20 hours in the both supernatant and cell pellet. This may be due to the fact that the sequence of *ChiHP74* showed a tachycitin like domain. Tachycitin is an invertebrate Cys-rich chitin binding protein with antimicrobial activity against both fungi and bacteria. Suetake et al. (2002) found that tachycitin from horseshoe crab

Tachypleus tridentatus has 10 cystein residues which form 5 disulfide bridges. This effect made inclusion bodies in *E. coli*; however, they found chitinase activity after denatured and refolded this protein. In contrast to this study, *ChiHP*74 has 6 cystein residues which form 3 disulfide bridges. The harvested cells and supernatant were subjected to determine the chitinase activity. The result shows chitinase activity is not expressed in both fractions expecting that the inclusion bodies may occur within the cells. Thus, the cells and the supernatant of culture were run on 10% SDS-PAGE, the band of chitinase was not found, compared with negative control. The result indicated the inclusion bodies was not observed within the host, thus this enzyme may express in a soluble form. Tachycitin from horseshoe crab expressed in inclusion body form can be denatured and refolded successfully. This may be resulted from a small molecular size of 8.5 KDa, while ChiHP74 has a size of 74 KDa which hard to refold in an actual structure containing chitinase activity. From a negative result of SDS-PAGE, this occurrence may be caused that the picked colony grown on ampicillin plate has not contained the chitinase activity, but, in contrast, the colony which contained chitinase activity can not grow on ampicillin plate since the chitinase may harm to the host cell. Several studies reported that protein containing cystein-rich domain expressed antimicrobial activity. Chiou, T.T. et al. (2005) found that a synthetic penaeidin-like antimicrobial peptide (AMP) from *P. monodon* consisting of a proline rich domain and 6 cystein-rich residues contains microbial activity. Also in penaeidin-3 from *Litopenaeus vannamei*, Yang, Y. et al. (2003) reported that this protein expressed in *Saccharomyces cerevisiae* showed similar antimicrobial activity. Penaeidin-3 from *L. vannamei* displays a proline-rich domain stabilized by three conserved disulfide bonds whose arrangement has not been characterized. Penaeidin isolated from hemolymph of *P. vannamei* shows antimicrobial activity against fungi and bacteria. After the mature molecule characterized, the molecule composes of proline-rich domain and disulfide bridge (Destoumieux, D. et al, 1997). Interestingly, Ahmad, T. et al. (2003) attemped to express the chitinase from *Helicoverpa armigera* comprised peritrophin chitin binding region at C-terminus which contains six conserved cysteins in *E. coli* expression system. They found that the specific activity of chitinase was very low as compared to those of the insect cell expressed and the

native molting fluid chitinase. Hence, whether ChiHP74 was expressed in insect cell expression system may show the chitinase activity.

From no expression of ChiHP74 in expression host, only was the catalytic domain, CatD, cloned and observed for chitinase expression. The recombinant plasmid pET17b-Chi-CatD was introduced into Rosetta(DE3)pLysS, BL 21 (DE3), and BL21(DE3) pLys expression host. After 1 ml of culture was plated on a solid medium for overnight, Rosetta(DE3)pLysS and BL21(DE3) pLys expression host were not found any bacterial colony, but found only a few numbers of colonies in BL21(DE3). One colony of BL21(DE3) was picked and grown in a large scale for monitoring the chitinase activity. In induction for expression of protein encoded from CatD, IPTG was used as a synthetic inducer to open the lac operon. The IPTG was added to the final concentration of 0.25 and 1 mM, using autoclaved water as a negative inducer. After overnight incubation, the cells were harvested by centrifugation. Obviously, the amount of cell in negative control was obtained more than that of 0.25 and 1 mM, respectively. Contrasted with that previous study, Nakapong, S. (2004) indicated that chitinase from Bacillus sp. PP8 has the highest activity by induction with the IPTG final concentration of 1 mM. Unfortunately, although the chitinase activity of this study was observed in BL21(DE3) cells, the activity was found at a very low level and this activity was less stable because after stored within a week, the activity was completely abolished. This result of low production of chitinase was confirmed by SDS-PAGE. After cells were harvest at several time intervals, both supernatant and cell pellet were load on 10% SDS-PAGE. There is no different intensity of expected band which is a result from low protein production. Suggest that a gel should be stained with silver staining. It may show the different intensity of the expected band in several time intervals. By the way, surprisingly, when pET17b-Chi-CatD was transformed into other strains which are not expression host such as, JM109, Top10, and DH5α, a large number of colonies was observed after 10 μl of culture was plated and incubated for over night. Moreover, when attempted to transform pGEM-Chi-CatD into both those three of expression host and other non-expression hosts, the results show that many colonies were grown on medium after overnight incubation. From all experiments on the expression of CatD gene, this may be based on the fact that protein from CatD may

have an antimicrobial activity which harms to the expression host cells; however, nowadays, there is no report concerning chitinase found in *P. monodon* that has an antimicrobial activity, but some studies reported that chitinase found in arthropod involved in nutrient digestion, the partial breakdown of the exoskeleton prior to molting, and the regulation of physical properties of the peritrophic membrane. Furthermore, as much as we searched for any experiment about the expression of chitinase gene found in *P. monodon*, there are no reports that chitinase gene can be successfully expressed in *E. coli* expression system. Several studies were tried to monitor the chitinase gene (*PmChi-1*) at transcription level instead. Tan, S.H. (2000) studied the transcription level of chitinase by reverse transcription. The result shows that mRNA of *PmChi*-1 was transcripted in both the hepatopancreas and the digestive tract during the molting cycle fluctuates markedly. Unlike in Watanabe, T. (1998), they found no different level of chitinase *PjChi*-3 mRNA during molting cycle; they suggested that *PjChi*-3 found in hepatopancreas may involve in digestion instead. However, this study was the first one which can be successfully expressed the partial chitinase gene in BL21(DE3). Nevertheless, the protein expressed from CatD has not shown only low activity but instability also.

Protein, Chi-CatD, encoded from CatD gene was partially characterized by the determination of optimum temperature, optimum pH, substrate specificity, and the product generated by hydrolysis reaction. Firstly, to determine the optimum temperature, *Chi-CatD* has an optimum temperature in the range of 60 ^{o}C while chitinase from *B. cepacia* has the optimum temperature at 37 ^{o}C and 55 ^{o}C (Kattiyawong et al, 2001) which is a result from multiple enzymes with different optimum temperatures. Despite the fact that the tiger shrimp lived in the sea which has temperature equaled to its surrounding, *Chi-CatD* should also have the optimum temperature equaled to its habitat temperature which ranged around 12-36 ^{o}C. This may be due to the fact that *Chi-CatD* was expressed from the catalytic domain of chitinase gene, thus, the conformation at an active site may be differed from the active site of chitinase expressed from full-length gene resulted in changing the optimum temperature. Secondly, when determined the optimum pH of *Chi-CatD*, two optimum pH values of 4 and 7 were observed suggesting that this protein can work in a wide range of pH. Like the previous report, Lesya et al (1996) found that chitinase from

Bacillus licheniformis B-6839 has shown two optimum pH values of 5 and 8 which use glycol chitin as a substrate. Furthermore, silk worm (Koga et al, 1997) and plant yam (Tsukamoto et al, 1984) also showed two optimum pH values of 4 and 8-10 toward glycol chitin. Thirdly, in order to assess the specificity of substrate for chitinase, several types of substrates were evaluated. The result shows beta chitin has the highest specificity with 100% of relative hydrolytic activity, followed by 75%-Chitosan, colloidal chitin, partially N-acetyl chitin (PNAC), regenerated chitin, and 100%-Chitosan with 56%, 52%, 34%, 32% and 0% of relative hydrolytic activity, respectively. These results suggest that the structure of substrate effects on the binding affinity to enzyme. The structure of beta chitin is crystalline form but not for other types which indicated that chitinase from *P. monodon* has more preferred to hydrolyze the crystalline form of substrate. Moreover, concordant with the previous report, Watanabe et al (2003) found that *chitinase*A1 from *Bacillus circulans* has more preferred on crystalline chitin than do other types. The amino acids which important to the role of hydrolyzing activity of chitinase consists of Y56, W53, W433, W164, W134, W284, Y279, and E204. Interestingly, Y56, W53 are very essential for crystalline chitin hydrolysis. This report was supported the present results due to there are the similar amino acid. Furthermore, the hydrolyzing activity against crystalline beta-chitin and colloidal chitin has been highly reduced when *Chi*A1 was mutated by site directed mutagenesis at position of Y56, W53. Therefore we also indicated that *Chi-CatD* may also possibly hydrolyze the crystalline chitin. However, the activity against soluble substrate has not yet been enhanced. Fourthly, to determine the products catalyzed by *Chi-CatD,* HPLC and TLC methods were performed. The chitinase activity was examined at pH 4.0 and pH 7.0. The result shows the products catalyzed by the crude chitinase at pH 4.0 are disaccharide. Corresponding to other study, for HPLC method, the hydrolytic product of crude chitinase was incubated under the same conditions of TLC method. The completely hydrolysis was harvested by centrifuged. The 0.3 ml of supernatant was mixed with 0.7 ml of acetonitrile, and filtrated through a 0.45 micron filter. The result showed the products as same as determine by TLC method (Figure 3.22).

According to the expression experiment, chitinase gene from hepatopancreas of *P. monodon* could not be expressed in *E. coli* system. Therefore,

we have attempted to further study in other expression systems. Thus, *P. pastoris* expression system was chosen for expression the full-length gene. For instance of *P. pastoris* expression system, characterization of the recombinant *Pisum sativum* defensin 1, a pea defensin which presents four disulfide bridges and high antifungal activity, was expressed in *Pichia pastoris* (Cabral, K.M.S. et al. 2003). Chitinase B1 from *Aspergillus fumigatus* has also been characterized in *P. pastoris* expression system (Jaques, A.K. et al. 2003). Furthermore, in order to produce large quantities of recombinant human chitinase, *P. pastoris* expression system was used to develop this process (Goodrick, J.C. et al. 2001). In our study, chitinase gene from hepatopancreas of *P. monodon* was cloned into expression vector, pPIC9K into *P. pastoris*. The inserted fragment was digested with two types of restriction enzyme, *MfeI* and *NotI*; pPIC9K vector was digested with *EcoRI* and *NotI*. At the cloning site of vector, *SnaBI*, *AvuII*, *EcoRI*, and *NotI* restriction sites were located. Also, when considered the restriction site within the inserted fragment, *SnaBI* and *EcoRI* restriction sites were found, thus *SnaBI* and *EcoRI* restriction enzymes were no longer used to digest this fragment. One way to solve this problem that is, use specific primers added with *MfeI* and *NotI* at each 5'-end of each primer to amplify the inserted fragment. After amplification, the inserted fragment contained the *MfeI* and *NotI* sites at each end. The expression vector which not contained *MfeI* site was digested with *EcoRI* and *NotI* restriction enzymes. This result can be explained that *MfeI* and *EcoRI* sites have the complementary sequences at its protruding end, two restriction site can be ligated together; recombinant plasmid was successfully constructed which confirmed by sequencing.

Normally, selection of yeast containing pPICK9K was performed by selection on MD plate and followed by YPD plate containing various concentrations of geneticin. Yeast containing either pPICK9K or pPICK9K-ChiHP74 was grown on these plates. These can be inferred that pPICK9K and pPICK9K-ChiHP74 had been transformed into the host successfully. To confirm this result, colony PCR was performed by using specific primers. The specific primers were designed from two specific regions: one on pPICK9K vector and another on ChiHP74. Yeast carried pPICK9K was successfully amplified with specific primer to the vector when the expected band was observed. Yeast carrying pPICK9K-ChiHP74 was completely

failed in amplification with both types of specific primers. Although, the amplification of pPICK9K-ChiHP74 was failed, yeast carrying this recombinant plasmid was still grown on YPD medium containing geneticin. The results may be due to the fact that the inserted fragment, ChiHP74, maybe harm to host cell, and that yeast carrying pPICK9K-ChiHP74 can grow on selective plate causes host cell may eliminate the inserted fragment including the region around the cloning sites. The specific primer that specify to the vector binds closely to region of cloning site, so, specific primer to the vector can not produce the target band also. These results were reconfirmed by spreading the wild type yeast on YPD plate containing geneticin. Surprisingly, there was no any colony of yeast grown on this medium. By the way, if yeast contains pPICK9K-ChiHP74 within the cell and expression of this gene was occurred, chitinase may kill the host. There are several lines evidence of indicating that chitinase may harm the host cell. Mali, et al. (2004) suggested that chitinase from a hydroid *Hydractinia echinata* also fulfills a role in host defense, probably against fungal and nematode pathogens. Moreover, Aguilar-Uscanga, and Francois (2003) reported that chitin was the composition of yeast cell wall. Chitinase from this study may affect growth of this organism. Thus, expression of this chitinase gene both full-length was unsuccessful in prokaryotic and eukaryotic expression system. For further study on the expression of this gene, other expression systems such as baculovirus and mammalian cell were suggested for the expression of this gene.

CHAPTER V
CONCUSSION

cDNA Chitinase gene from Hepatopancreas of the black tiger shrimp, *Penaeus monodon* was successfully amplified by using specific primers. This gene was cloned into pET-17B and transformed to *E. coli* Top10. The nucleotide sequence has revealed a 1995 open reading frame encoding 665 amino acids which comprise signal peptide corresponding to 74 kDa, called *ChiHP*74. The *ChiHP*74 sequence showed high identity compared with *PmChi*-1 sequence from *Penaeus monodon* by 98%.

*ChiHP*74 was expressed in two expression systems: *E. coli* and yeast expression systems. In *E. coli* expression system, *ChiHP*74 was cloned into pET-17b in both *E. coli* Rosetta(DE3)pLysS and BL21(DE3). The result shows that both strains have shown no activity of chitinase. In yeast expression system, ChiHP74 was cloned into pPIC9K and transformed to *Pichai pastoris*. The result above shows no expression activity.

Based on no expression results of *ChiHP74* full-lengths, several domains were considered to be separately cloned for monitoring the activity of chitinase. A catalytic domain was the one chosen and cloned into pET-17b. The nucleotide sequence has revealed a 1278 open reading frame encoding 426 amino acids which comprises a signal peptide corresponding to 48 kDa, called *Chi-CatD.*

Chi-CatD was expressed in *E. coli* Rosetta(DE3)pLysS and BL21(DE3). The activity of chitinase was found in BL21(DE3) harboring pET-17b-Chi-CatD, when incubated with shaking at 37 ^{o}C, 250 rpm for 20 hour after induction with 0.25 mM IPTG. The optimum pH has been shown at pH values of 4 and 7. Besides, the optimum temperature has been shown at 60 ^{o}*C*. Moreover, β-chitin has been shown to have highest specificity for *Chi-CatD*. The major product of this reaction is (GlcNAc)

REFERENCES

Abdel-Banat, B.M.A., Koga, D., 2001. A genomic clone for a chitinase gene from the silkworm, *Bombyx mori*: Structural organization identifies functional motifs. *Insect Biochem. Mol. Biol*. 31, 497–508.

Aguilar-Uscanga B. and Francois J.M. (2003). A study of the yeast cell wall composition and structure in response to growth conditions and mode of cultivation. *Appl. Micorobio*. 37:268-274.

Ahmad, T., Rajagopal, R., and Bhatnagar, R.K. (2003). Molecular characterization of chitinase from polyphagous pest *Helicoverpa armigera*. *Biochem. Biophys. Res. Commun*. 310: 188-195.

Austin, P.R., Brine,C.J., Castle, J.E. and Zikakis, J.P. (1989). *Science* 49: 2127-2135

Baily-Brook, J. H., and Moss, S. M. (1992). Penaeid taxonomy, biology and zoogeography (Eds), Marine shrimp culture: Principles and practices. Amsterdam: *Elsevier Science Publishers*. 60: 896-915.

Blake, C.C.F., Johnson, L.N., Mair, G.A., North A.C.T., Phillips, D.C., and Sarma, V.R. (1967). Crystallographic studies of the activity of hen egg-white lysozyme. *Proc.Roy. Soc. Ser B.* 167: 378-388.

Bodek, K. (2002). Effect of microcrystalline chitosan on the solubility of *ibuprofen*. *Acta. Pol. Pharm*. 59: 105-108.

Boller, T. (1985). In “Plant-Microbe interactions: Molecular and Genetic Perspectives” (Eds.). *MacMillan, New York*. 2: 384-414.

Boot, R. G., Renkema, G. R., Strijland, A., Zonneveld, A. J., and Aerts, M. F. G. (1995). Cloning of a cDNA encoding chitotriosidase, a human chitinase produced by macrophages. *J. Biol. Chem.* 270: 26252-26256.

Bork, P. and Doolittle, R.F. (1992). Proposed acquisition of an animal protein domain by bacteria. *Proc. Natl. Acad. Sci.* USA. 89: 8990-8994.

Buchholz, F. (1989). Moult cycle and seasonal activities of chitinolytic enzymes in the integument and digestive tract of the antarctic krill, *Euphausia superba*. *Polar Biol*. 9: 311-317.

Bulet, P., Stocklin, R., and Menin, L. (2004). Anti-microbial peptides: from invertebrates to vertebrates. *Immunol. Rev*. 198: 169-184.

Cabral, M.S., Almeida, S., Valente, A., Almeida, C.L., and Kurtenbach E. (2003). Production of the active antifungal *Pisum sativum* defensin 1 (Psd1) in *Pichia pastoris*: overcoming the inefficiency of the STE13 protease. *Protein Expr. Purif*. 31: 115-122.

Casteels-Josson1, K., Capaci, T., Casteels, P., and Tempst, P. (1993). Apidaecin multipeptide precursor structure: a putative mechanism for amplification of the insect antibacterial response. *The EMBO J*.12, 4:1569 – 1578.

Chiou, T.T., Wu, J.L., Chen, T.T., and Lu, J.K. (2005). Molecular cloning and characterization of cDNA of penaeidin-like antimicrobial peptide from tiger shrimp (Penaeus monodon). *Mar. Biotech.* 7: 119-127.

Choi, H.K., Choi, K.H., Kramer, K.J., Muthukrishnan, S., 1997. Isolation and characterization of a genomic clone for the gene of an insect molting enzyme, chitinase. *Insect Biochem. Mol. Biol*. 27, 37–47.

Cue, R. (1999). Antimicrobial action of exogenous chitosan. EXS. 87:315-333.

de la Vega, H., Specht, C.A., Liu, Y., Robbins, P.W., 1998. Chitinases are a multi-gene family in *Aedes*, *Anopheles* and *Drosophila*. *Insect Mol. Biol*. 7, 233–239.

Destoumieux, D., Bulet, P., Loew, D., Dorsselaer, A.V., Rodriguez, J., and Bachere, E. (1997). Penaeidins, a new family of antimicrobial peptides isolated from the shrimp *Penaeus vannamei* (Decapoda). *J. Biol. Chem*. 272: 28398-28406.

Estrella, A. H., and Chet, I. (1999). Chitinase in biological control. Chitin and chitinases. 14: 171-180.

Felt, O., Buri, P., and Gurny, R., (1998). Chitosan: a unique polysaccharide for drug delivery. *Drug Dev. Ind. Pharm*. 24: 979-993.

Flach, J., Pliet, P. E., and Jolles, P. (1992). What's new in chitinase research? *Experientia.* 48: 701-716.

Fukamizo, T., Ohkawa, T., Ikeda, Y., and Goto, S. (1995). Specificity of chitosanase from *Bacillus pumilus*. *Biochim. Biophys. Acta*. 1205:183-188.

Fukamizo, T., Koga, D., and Goto, S. (1995). Comparative biochemistry of chitinases-anomeric form of the reaction product. *Biosci. Biotechnol. Biochem.* 59: 311-313.

Gooday, G. W. (1994). Physiology of microbial degradation of chitin and chitosan. *Biochem. Micro. Degrad*. 26: 279-312.

Goodrick, J.C., Xu, M., Finnegan, R., Schilling, B.M., Schiavi, S., Hoppe, H., and Wan, N.C. (2001). High-level expression and stabilization of recombinant

human chitinase produced in a continuous constitutive *Pichia pastoris* expression system. *Biotechnol. Bioeng*. 74:492-497.

Goosen, M. (1997). Application of chitin and chitinase. Lancaster. *Technomic*

Hart, P.J., Pfluger, H.D., Monzingo, A.F., Hollis, T., and Robertus, J.D. (1995). The refined crystal structure of an endochitinase from *Hordeum Vulgare* L. seeds at 1.8 Å resolution. *J. Mol Biol* 248: 402-413.

Helander, I., Nurmiahoo-Lassila, E., Ahvenainen, R., Rhoades, J., and Roller, S. (2001). Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. Int. *J. Food Microbiol*. 71: 235-244.

Henrissat, B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similarity. *Biochem J*. 280:309-316.

Hetru, C., Bulet, P., Cociancich, S., Dimaecq, J.L., Hofmann, D., and Hoffmann J.A. (1994). The insect host defense. *Phylogen. Immun.*89: 43-66.

Hirano, S., Gebelein, C.G., and CarraherJr, C.E. (1995). Industrial biotechnological polymers. *Technomic.* 189: 277-296

Hirano, S. (1999). Chitin and chitosan as novel biotechnological materials. *Polym. Int.* 48: 732.

Hollis, T., Honda, Fukamizo, T., Marcotte, E.M., and Robertus J.D. (1997). Kinetic analysis of barley chitinase. *Arch. Boichem. Biophys.* 344: 335-342

Hudson, S. M. (1997). Application of chitin and chitosan as fiber and textile chemicals.(Eds). *Advances Science*. 20: 590-599.

Ilango, R., Kavimani, S., Jaykar, B., and Umamaheshwari, G. (1999). Dissolution studies on tablets of ibuprofen using chitosan. *Indian J. Exp. Biol*. 37: 505-508.

Illum, L. (1998). Chitosan and its use as a pharmaceutical excipient. *Pharm. Res*. 15: 1326-1331.

Imoto, T. and Yagishita, K. (1971). A sample activity measurement of lysozyme. *Agr. Biol. Chem*. 35: 1154.

Jaques, A.K., Fukamizo, T., Hall D., Barton, R.C., Escott, G.M., Parkinson T., Hitchcock, C.A., and Adams D.J. (2003). Disruption of the gene encoding the ChiB1 chitinase of *Aspergillus fumigatus* and characterization of a recombinant gene product. *Microbiology*. 149: 2931-2939.

Jha, N., Leela, I., and Prabhakar Rao, A.V.S. (1988). Removal of cadmium using chitosan. *J. Environ. Eng*. 114: 962.

Kuttiyawong, K. (2001) Cloning and nucleotide sequencing of chitinase gene from Burkholderia cepacia TU09. M. Sc. Thesis, Department of Biochemistry, Graduate school, Chulalongorn University. 95 pp.

Kawabata, S., Nagayama, R., Hirata, M., Shigenaga, T., Agarwala, K. L., Saito, T., Cho, J., Nakajima, H., Takagi, T., and Iwanaga, S. (1996) *J. Biochem.* 120, 1253–1260

Kelly, J.A., Sielecki, A.R., Sykes, B.D., James M.N.G., and Phillips D.C. (1979). X-ray crystallography of the binding of the bacterial cell wall trisaccharide NAM-NAG-NAM to lysozyme. *Nature.* 282: 875-878.

Kim, M.G., Shin, S.W., Bae, K.S., Kim, S.C., Park, H.Y., 1998. Molecular cloning of chitinase cDNAs from the silkworm, *Bombyx mori*, and the fall webworm, *Hyphantria cunea*. Insect Biochem. *Mol. Biol*. 28, 163–171.

Knorr, D. (1991). Recovery and utilization of chitin and chitosan in food processing waste management. *Food Technol.* 114: 378-390.

Koga, D., Sasaki, Y., Uchiumi, Y., Hirai, N., Arakane, Y., and Nagamatsu, Y. (1997). Purification and Characterization of *Bombyx mori* chitinase. *Insect Boichem. Mol. Bio*. 27: 759-767.

Kono, M., Wilder, M.N., Matsui, T. Furukawa, K., Koga, D., and Aida, K. (1995). Chitinolytic enzyme activities in the hepatoopancreas, tail fan and hemolymp oof kuruma prawn *Penaeus japonicus* during molting cycle. *Fisheries Sci*.61: 727-728.

Kramer, K. J. and Koga, D. (1986). Insect chitin: physical state, synthesis, degradation and metabolic regulation. *Insect Biochem*. 16:851-877.

Kramer, K.J., Corpuz, L., Choi, H.K., Muthukrishnan, S., 1993. Sequence of a cDNA and expression of the gene encoding epidermal and gut chitinases of *Manduca sexta*. *Insect Biochem. Mol. Biol*. 23, 691–701.

Kramer, K.J. and Muthykishnan, S. (1997). Insect chitinase: molecular biology and potential use as biopesticides. *Insect Biochem Mol. Biol*. 27: 887-900.

Lam, S.K., and Ng, T.B. (2000). Isolation of a small chitinase-like antifungal protein from *Panax notoginseng* (sanchi ginseng) roots. *J. Biochem. Cell Biol*. 33: 287-292.

Lesya, A. T., Lyudmila, P. R., Tatyana, M. S., Galina, G. C., and Valentin, M. S. (1996). Chitinase of *Bacillus likeniformis* B-6839: Isolation and properties. *Can. J. Microbiol*. 42: 307-315.

Madhavan, P. (1992). Chitin, chitosan and their novel applications. *Science Lecture Series*. 1.

Mali, B., Mohrlen, F., Frohme M., Frank, U. (2004). A putative role of a chitinase in a cindarian: pattern formation and immunity. *Dev. Comp. Immunol*. 28: 973-981.

Maris J. P., De Wit, J. L.,and Quicke G. V. (1966). A critical examination of the Nelson-Somogyi method for the determination of reducing sugars. *Anal Biochem*.3: 373-381.

Matsuzaki, K. (2001). Mechanisms of innate immunity based on antimicrobial peptides. *Tanpakushitsu Kakusan Koso*. 46: 2060-2065.

McKay, G., Blair, H.S. and Gardner, J.R. (1989). Adsorption of dyes chitin. I. equilibrium studies. *J. Appl. Polym. Sci.* 27: 3043.

Mikitani, K., Sugasaki, T., Shimada, T., Kobayashi, M., Gustafsson, J.A., 2000. The gene of the silkworm, *Bombyx mori*, contains a novel Tc-like transposable element. *J. Biol. Chem*. 275, 37725–37732.

Murao, S., Kawada, T., Itoh, H., Oyama, H., and Shin, T. (1992). Purification and characterization of a novel type of chitinase from *Vibrio alginolyticus*. *Biosci. Biotech. Biochem*. 56: 368-369.

Muzzarelli, R.A.A. (1998). Management of hypercholesterolemia and overweight by oral administration of chitosans. *New Biomedical Materials - Applied and Basics*, POI Press. London.

Muzzarelli, R., Mattioli-Belmone, M., Pugnaloni, A. and Biagini, G. (1999). Biochemistry, histology and clinical uses of chitins and chitosans in wound heating. EXS. 87: 251-264.

Muzzarelli, R.A. (1999). Clinical and biochemical evolution of chitosan for hypercholesterolemia and overweight control (Eds.), *chitin and chitinases, Birkhauser, Basel*.

Nakapong, S. (2004). Necleotide sequencing and expression of chitinase gene from *Bacillus circulans* PP8. M. Sc. Thesis, Department of Biochemistry, Graduate school, Chulalongorn University. 143 pp.

Nanjo, F., Ishikawa, M., Katsumi, R., and Sakai, K. (1990). Purification, properties, and transglycosylation reaction of β-N-acetylhexosaminidase from *Nocardia orientalis*. *Agric. Biol. Chem*. 54: 899-906.

Ohno, T., Armand, S., Hata, T., Nikaidou, N., Henrissat, B., Mitsutomi, M., and Watanabe, T. (1996). A modular family 19 chitinase found in the prokaryotic organism *Steptomyces griseus* HUT6097. *J. Bacteriol* 178:5065-5070.

Oshima, H., Miyazaki, R., Ohe, Y., Hayashi, H., Kawamura, K., and Kikuyama, S. (2002). Isolation and sequence of a novel amphibian pancreatic chitinase. *Comp. Biochem. Physiol.* B. 132: 381-388.

Pan, D., He, N., Yang, Z., Lui, H., Xu X. (2005). Differential gene expression profile in hepatopancreas of WSSV-resistant shrimp (*Penaeus monodon*) by suppression subtractive hybridization. *Dev. Comp. Immunol*. 29: 103-112.

Pawlowska, E. (1997). The assensment of influence of chitosan on he dental plup rats (Eds). *Advances in Chitin Science*. 20: 705-710.

Primavera, J. H. (1990). *External and internal anatomy of adult penaeid prawns/shrimps*. SEAFDEC, Aquaculture Department.

Sambrook and Russel (2001). Molecular cloning A laboratory manual, Third edition. New York: Cold Spring Harbor Laboratory. 1.32-1.34.

Sambrook and Russel (2001). *Molecular cloning A laboratory manua*l, Third edition. New York: Cold Spring Harbor Laboratory. 1.35-1.37.

Shahidi, F., Arachichi, J., and Jeon, Y. (1999). Food applications of chitin and chitosan. *Trends Food Sci. Technol.* 10: 37-51.

Shaikh, S. A., and Deshpande, M. V. (1993). Chitinolytic enzymes: their contribution to basic and applied research. *J. Microbial. Biotech*. 9: 468-475.

Shinoda, T., Kobayashi, J., Matsui, M., Chinzei, Y., 2001. Cloning and functional expression of a chitinase cDNA from the common cutworm, *Spodoptera litura*, using recombinant baculovirus lacking the virus-encoded chitinase gene. Insect Biochem. Mol. Biol. 31, 521–532.

Singla, A. and Chawla, M. (2001). Chitosan: some pharmaceutical and biological aspects. An update. *J. Pharm. Pharmacal*. 53: 1047-1067.

Spindler-Barth, M., Van Wormhoudt, A. and Spindler, K.D. (1990). Chitinolytic enzymes in the integument and midgut-gland of the shrimp *Palaemon serratus* during the molting cycle. *Mar. Biol*. 106: 49-52.

Spreen, K.A., Zikakis, J.P., Austin, P.R. and in: Zikakis, J.P. (Ed.). (1984). Chitin, chitosan and related enzymes. *Academic Press, Orlando*. 57: 288-298.

Suetake, T., Aizawa1, T., Koganesawa1, N., Osaki, T., Kobashigawa, Y., Demura1, M., Kawabata, S.I., Kawano, K., Tsuda, S. and Nitta1, K. (2002). Production and characterization of recombinant tachycitin, the Cys-rich chitin-binding protein. *Prot. Engine*. 15: 763-769.

Tan, S.H., Degnan, B.M., and Lehnert, S.A. (2000). The *Penaeus monodon* chitinase 1 Gene is differentially expressed in the hepatopancreas during the molt cycle. *Mar. Biotechnol*. 2: 126-135.

Tokoro, A., Tatewaki, N., Suzuki, K., Mikami, T., Suzuki, S., and Suzuki, M. (1988). Growth-inhibitory effects of Hexa-*N*-acetylchitohexose and chitohexose againt Meth-A Solid Tumer. *Chem. Pharm. Bull*. 36: 784-790.

Tracey, M. V. (1957). Chitin. *Rev. Pure. Appl. Chem.* 7: 1-14.

Tsukamoto, T., Kago, D., Ide, A., Ishibashi, T., Horino-Matsushige, M., Yagashita, K., and Imoto, T. (1984). Purification and some properties of chitinase from yam, *Discorea opposite* THUNB. *Agric. Biol. Chem*. 48: 931-939.

Usui, T., Hayashi, Y., Nanjo, F., Sakai, K., and Ishido, Y. (1987). Transglycosylation reaction of a chitinase purified from *Norcardia orientails. Biochem. Biophys. Acta.* 923: 302-309.

Watanabe, T., Ito, Y., Yamada, T., Hashimoto, M., Sekine, S. and Tanaka, H. (1994). The roles of the C-terminal domain and type III domains of chitinase A1 from *Bacillus circulans* WL-12 in chitin degradation. *J Bacteriol*.176: 4462-4472.

Watanabe, T., Kono, M., Aida, K. and Nagasawa, H. (1996). Isolation of cDNA encoding a putative chitinase precursor in the Kuruma prawn *Penaeus japonicus. Mol. Mar. Biol. Biotechnol*. 5: 299-303.

Watanabe,T. and Kono,M. (1997). Isolation of a cDNA encoding a chitinase family protein from cuticular tissues of the Kuruma prawn Penaeus japonicus. *Zool. Sci*. 14 (1), 65-68.

Watanabe, T., Kono, M., Aida, K. and Nagasawa, H. (1998). Purification and molecular cloning of chitinase expressed in the hepatopancreas of the penaeid prawn *Penaeus japonicus*. *Biochim. Biophys. Acta.* 1382: 181-185.

Watanabe, T., Ariga, Y., Sato, U., Toratani, T., Hashimoto, M., Nikaidou, N., Kezuka, Y., Nonaka, T., and Sugiyama, J. (2003). Aromatic residues within the substrate-binding cleft of *Bacillus circulans* chitinase A1 are essential for hydrolysis of crystalline chitin. *Biochem J.* 376: 237-244.

Yang, Y., Poncet, J., Garnier, J., Zatylny, C., and Bachere, E. (2003). Solution structure of the recombinant Penaeidin-3, a shrimp antimicrobial peptide. J. *Biol. Chem*. 278: 36859-36867.

Ylitalo, R., Lehtien, S., Wuolijoki, E., Ylitalo, P. and Lehtimaki, T. (2002). Cholesterol-lowering properties and safety of chitosan. *Arzneimittelforschung.* 52:1-7.

Zheng, Y., Zheng, S., Cheng, X., Ladd, T., Lingohr, E.J., Krell, P.J., Arif, B.M., Retnakaran, A., Feng, Q., 2002. A molt-associated chitinase cDNA from the spruce budworm, *Choristoneura fumiferana*. *Insect Biochem. Mol. Biol*. 32, 1813–1823.

Zikakis, J.P., Saylor, W.W. and Austin, P.R. (Eds.) (1982). Chitin and Chitosan. *The Japanese Society of Chitin and Chitosan*, Tottori. 17: 233-245.

APPENDICES

APPENDIX A

Phenol-chloroform precipitation method

The sample was mix with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and gently mixed. The mixture was centrifuged at 5,000 rpm, 4 ^{o}C for 15 minutes. Consequently, two volumes of absolute ethanol were added, mixed and stored at -20 ^{o}C for 30 minutes. The plasmid was collected by centrifugation at 5,000 rpm, 4 ^{o}C for 15 minutes and air dried for 10 minutes. The pellet was dissolved in TE or sterile water.

APPENDIX B

The A-tail procedure for blunt-end PCR fragments

One to seven microliter of purification PCR fragment, generated by a *Pfu* DNA polymerase, was add 1 µl of 10x reaction buffer with $Mgcl_2$, 0.2 mM dNTP, 2U of Taq DNA polymerase, and deionized water was adjust to final volume 10 µl. Subsequently, the reaction was incubated at 70 °C for 30 minutes.

APPENDIX C

Preparation of competent cells

A single colony of *E. coli* was cultured, acts as a starter, into 2 ml of LB-broth and incubated at 37 ^{o}C with 250 rpm shaking for overnight. The starter was diluted in 500 ml of LB-broth and incubated 37 ^{o}C with 250 rpm shaking until the optical density at 600 nm of the cells reached 0.4-0.6 (~3-4 hours). Consequently, the culture was chilled on ice for 30 minutes. The pellet was harvested by centrifugation at 5,000 rpm for 15 minutes at 4 ^{o}C. Subsequently, the pellet was washed twice with 1 and 0.5 volume of cold sterile ultrapure water, respectively. The pellet were resuspended, centrifuged at 5,000 rpm for 15 minutes at 4 ^{o}C, and washed with 10 ml of cold sterile 10% (v/v) glycerol. The solution mixture was centrifuged. The pellet was resuspended with 1-2 ml of cold sterile 10% (v/v) glycerol. Finally, the cell suspension was divided into 40 μl aliquots and stored at -80^{o}C.

APPENDIX D

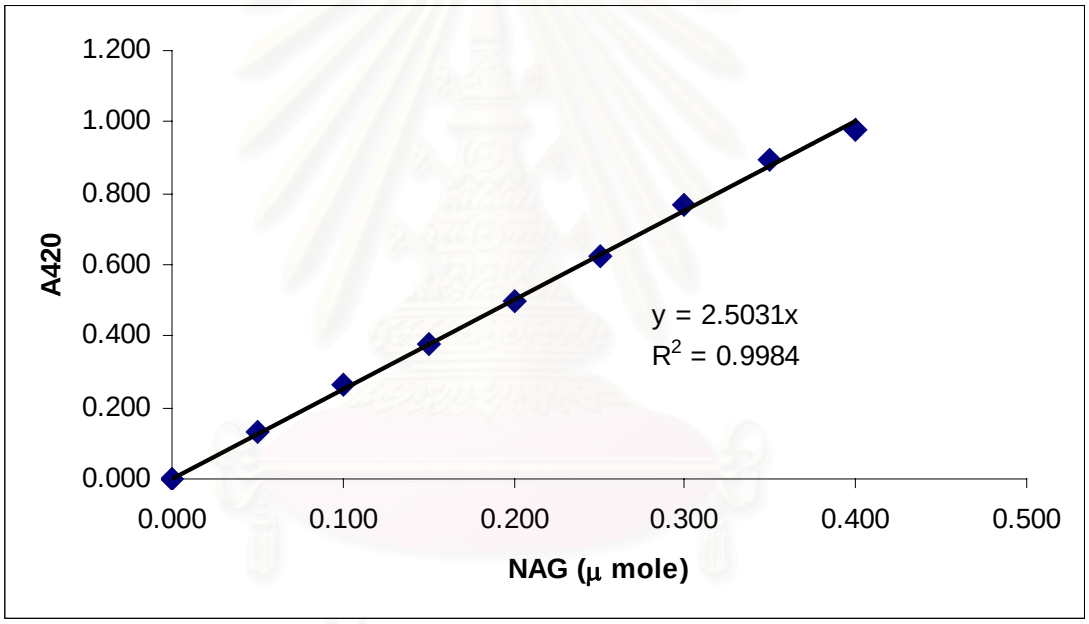
Completent cells yeast preparation

A single colony of *pichia pastoris* KM-71 was cultured and used as the starter 5 ml in YPD [2% (w/v) peptone, 1% (w/v) bacto yeast and 2% (w/v) glucose] in a 50 ml conical tube at 30 ^{o}C with shaking for overnight. Inoculate 0.1 ml of the overnight culture in 100 ml of fresh medium used a 1 liter flask. Grow overnight again to an optical density at 600 nm (OD_{600}) reached 1.3-1.5. Cells were then chilled on ice for 15-30 minutes and harvested by centrifugation at 3500 rpm for 5 minutes at 4 ^{o}C. The supernatant was removed as much as possible. The cell pellet was washed by resuspending in 100 ml of cold steriled water, gently mixing and centrifugation. The pellet was washed further with 50 ml of cold steriled water, followed with 4 ml of 1 M sobitol. In the final step, the pellet was resuspened in 200 μl of 1 M sobitol. This cell suspension was divided into 80 μl aliquots and stored at -80 ^{o}C until used.

APPENDIX E

Standard curve *N*-acetyl glucosamine for chitinolytic enzyme assay by colorimetric method.

Standard curve GlcNAc was made by monitoring the absorbance at 420 nm of standard concentration GlaNAc according to the Schale's method.



APPENDIX F

Silver staining

The gel was submerged for 30 min in 10% acetic acid. Then, the gel was briefly rinsed 3 times with ultrapure water for 2 min each and incubated in a staining solution (0.1% silver nitrate containing 1.5 ml/liter of 37% formaldehyde) for 30 min. The excess silver ions were eliminated by brief rinse with the ultrapure water. Gel was developed in a cold (8-18^{o}C) developing solution (3% sodium carbonate containing 1.5 ml/liter of 37% formaldehyde and 200 μl/liter of 10 mg/ml sodium thiosulfate) and agitated until the first band were visible. The developing solution was poured off and the fresh developing solution was added. The gel was continued development until optimal image intensity was obtained. The developing reaction was stopped by using 10% acetic acid with agitating for 5 min. The gel was rinsed 2 times with the ultrapure water for 2 min each.

BIOGRAPHY

Miss Panutda youdsang was born on May 31, 1981. She graduated with the Bachelor of Science from the Department of biochemistry at Chulalongorn University in 2002. She has studied for the degree of Master of Science at Biotechnology, Chulalongorn University.