วิศวกรรมโปรตีนของไคทิเนสจากแบคทีเรียเพื่อผลิตเอ็น-แอซีทิลไคโทโอลิโกแซ็กคาไรด์

นายสัญญา กุดั่น

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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PROTEIN ENGINEERING OF BACTERIAL CHITINASE FOR *N*-ACETYLCHITOOLIGOSACCHARIDE PRODUCTION

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biotechnology
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Thesis Title	PROTEIN ENGINEERING OF BACTERIAL CHITINASE FOR *N*-ACETYLCHITOOLIGOSACCHARIDE PRODUCTION
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สัญญา กุคั่น: วิศวกรรมโปรตีนของไคทิเนสจากแบคทีเรียเพื่อผลิตเอ็น-แอซีทิลไคโทโอลิโกแซ็กคาไรด์ (PROTEIN ENGINEERING OF BACTERIAL CHITINASE FOR *N*-ACETYLCHITOOLIGOSACCHARIDE PRODUCTION)
อ.ที่ปรึกษา: อ.ดร.รัฐ พิชญางกูร, 173 หน้า.

ในการศึกษานี้ได้ใช้เทคนิคทางพันธุวิศวกรรม และวิศวกรรมโปรตีนในการดัดแปลงไคทิเนสจากแบคทีเรียเพื่อศึกษากลไกในการทำงานและใช้ในการผลิตเอ็น-แอซีทิลกลูโคซามีน และ / หรือ ไคโทโอลิโกแซ็กคาไรด์ โดยทำการโคลนยีน และศึกษาสมบัติบางประการของไคทิเนสจากแบคทีเรียสายพันธุ์ *Bacillus licheniformis* SK-1 และ *Serratia* sp. ตลอดจนดัดแปลงโมเลกุลของ chitinase A1 (ChiA1), chitinase 60 (Chi60) และ chitinase B (ChiB) เพื่อนำไปใช้ในการผลิตน้ำตาลจากเบตาและคอลลอยด์ไคตินต่อไป

จากการศึกษา ChiA1, Chi60 และ ChiB เป็นเอนไซม์ที่สามารถเร่งปฏิกิริยาได้สองรูปแบบ คือ การย่อยสายไคตินจากปลายสายทางด้านรีดิวซ์ (ChiA1 และ Chi60) หรือ นอนรีดิวซ์ (ChiB) เข้ามา (exo-type) และการย่อยอย่างสุ่มภายในสายไคติน (endo-type) ทั้งนี้ขึ้นอยู่กับชนิดของสับสเตรทที่ใช้ ซึ่งรูปแบบในการทำงานที่แตกต่างกันสามารถนำไปใช้ในการเลือกผลิตน้ำตาลที่แตกต่างกันได้ โดยเอนไซม์ที่ทำงานแบบ exo-type สามารถนำไปใช้ในการผลิตเอ็น-แอซีทิลไคโทไบโอส ในขณะเอนไซม์ที่ทำงานแบบ endo-type สามารถใช้ในการผลิตเอ็น-แอซีทิลไคโทโอลิโกแซ็กคาไรด์

ได้ทำการสร้างอนุพันธ์ไคทิเนสโดยการตัดออกหรือเพิ่มโดเมนเข้าในโมเลกุล ChiA1 และ Chi60 เพื่อศึกษารูปแบบการทำงานของ chitinase A1 และ chitinase 60 พบว่าอนุพันธ์ของไคทิเนสที่มีรูปแบบการทำงานแบบ endo-type สามารถนำไปใช้ในการผลิตไคโทโอลิโกแซ็กคาไรด์ นอกจากนี้ไคเมอริกไคทิเนสที่สร้างขึ้นด้วยวิธีโฮโมโลกัสรีคอมบิเนชันระหว่าง chitinase 60 และ chitinase B (RecSK-1) สามารถย่อยสลายไคเมอร์ และ pNP-NAG_2 ให้ผลิตภัณฑ์เป็นมอนอเมอร์ ซึ่งสมบัติในการเร่งปฏิกริยานี้สามารถนำไปผลิตเอ็น-แอซีทิลกลูโคซามีนจากไคตินได้

ในการผลิตเอ็น-เอ็น-ไดแอซีทิลไคโทไบโอสจากเบตาไคตินด้วยไคทิเนส 60 กลายพันธุ์ที่ตำแหน่ง W33F/W245F และแยกผลิตภัณฑ์ออกจากปฏิกิริยาโดยวิธีไดอะไลซิส สามารถผลิตไคโทไบโอสได้ถึง 57 เปอร์เซ็นต์ของสับสเตรทเริ่มต้น โดยพบว่าหลังจาก 6 วัน สามารถผลิต NAG ได้ 85 มิลลิกรัม และ NAG_2 565 มิลลิกรัมเมื่อวิเคราะห์ด้วย HPLC นอกจากนี้ยังสามารถเตรียมไคโทโอลิโกแซ็กคาไรด์จากคอลลอยด์ไคตินด้วยไคทิเนส A1 ที่ถูกตัด chitin binding domain (ChiA1ΔChBD) ออก ซึ่งในภาวะที่ใช้อย่างเหมาะสมสามารถเตรียมไคโทเตตระโอสได้

สาขาวิชา..เทคโนโลยีชีวภาพ..... ลายมือชื่อนิสิต .....
ปีการศึกษา..2549..... ลายมือชื่ออาจารย์ที่ปรึกษา .....

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SANYA KUDAN: PROTEIN ENGINEERING OF BACTERIAL CHITINASE FOR *N*-ACETYLCHITOOLIGOSACCHARIDE PRODUCTION
THESIS ADVISOR: RATH PICHYANGKURA, Ph.D., pp.173.

In this study, genetic engineering and protein engineering techniques were used to modify bacterial chitinase for the production of *N*-acetylglucosamine and/or *N*-acetylchitooligosaccharide. β-chitin and colloidal chitin were used as substrate for sugar production by the chitinase derivatives. *Bacillus licheniformis* and *Serrtia* sp. chitinases were selected for studying of the oligosaccharide production. Chitinase A1 (ChiA1), chitinase 60 (Chi60) and chitinase B (ChiB) were cloned, expressed, partially purified and studied.

ChiA1, Chi60 and ChiB have both of exo- and endo-type modes for hydrolysis depending on the type of the substrate. The different hydrolysis mode of enzymatic hydrolysis can be used to prepare NAG or *N*-acetylchitooligosaccharide. Exo-chitinase can be use for the production of *N*-acetylchitobiose while endo-chitinase can be used for the production *N*-acetylchitooligosaccharide.

The engineered chitinase with deletion and insertion of the accessory domains in ChiA1 and Chi60 was constructed and characterized. The chitinase derivatives with high endo-type activity should be a good candidate for oligosaccharide production. The homologous recombination derivatives between chitinase 60 and chitinase B was constructed and characterized. Interestingly, the chimeric chitinase (RecSK-1) can hydrolyze pNP-NAG_2 and NAG_2 to produce monomer of NAG. In addition, the chimeric enzyme can be useful for the preparation of *N*-acetyl-D-glucosamine from chitin.

Chi60 mutant, Chi60W33F/W245F, was used to prepare *N*- *N'*-diacetylchitobiose from β-chitin and the product was separated by dialysis. The amounts of chitobiose from dialysis method yielded 57% (w/w) purity. After 6 days of incubation, the HPLC yields of NAG and NAG_2 from the hydrolysis of g β-chitin were 85 mg and 565 mg, respectively, representing 9% and 57% (w/w). In addition, the ChiA1ΔChBD was used to prepare oligosaccharide from colloidal chitin. In the condition used, chitotetraose can be prepared.

Field of study...Biotechnology..... Student's signature .....
Academic year...2006..... Advisor's signature .....

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CONTENTS

Page

Page

Page

Page

LIST OF TABLES

LIST OF FIGURES

Page

Page

ABBREVIATIONS

A	Absorbance
bp	Base pair
BSA	Bovine serum albumin
°C	Degree Celsius
CCMM	Colloidal chitin minimum medium
Da	Dalton (s)
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetrichloroacetic acid
et al.	Et. Alii (latin), and others
etc.	Et cetera (latin), other things
g	Gram
GlcNAc, NAG	*N*-acetyl-D-glucosamine
hr	Hour
HPLC	High performance liquid chromatography
i.e.	Id est (latin), that is
kb	Kilobase (s)
kDa	Kilodalton (s)
kb	Kilobase
L	Litre
M	Molar
mL	Mililitre
mg/mL	Miligram per mililitre
mg	Miligram
MW	Molecular weight
ng	Nanogram
µg	Microgram
µL	Microlitre
min	Minute
PAGE	Polyacrylamide gel electrophoresis

PCR	Polymerase Chain Reaction
PNAC	Partially *N*-acetylated chitin
rpm	Revolution per minute
w/w	Weight by weight
sp.	Species
NaCl	Sodium chloride
SDS	Sodium dodecyl sulfate
TLC	Thin layer chromatography

CHAPTER I
INTRODUCTION

Chitin, a β-(1,4)-linked polymer of *N*-acetyl D-glucosamine (NAG,GlcNAc), is widely distributed in nature, particularly as a structural polysaccharide in fungal cell walls, the exoskeleton of arthropods, the outer shell of crustaceans, nematodes, etc. Approximately 75% of the total weight of shellfish, such as shrimp, crab, and krill, is considered waste, and chitin comprises 20 to 58% of the dry weight of the said waste (Wang and Chang, 1997). Chitin has a broad range of applications in biochemical, food, and various chemical industries. It has antimicrobial, anticholesterol and antitumor activities (Patil *et al*., 2000; Gooday, 1999). Chitin and its related materials are also used in wastewater treatment (Flach et al., 1992), drug delivery (Kadowaki *et al*., 1997), wound healing, and dietary fiber (Dixon, 1995; Muzzarelli, 1977; Muzzarelli *et al*., 1999).

Chitinases, which hydrolyze chitin, occur in a wide range of organisms including viruses, bacteria, fungi, insects, higher plants, and animals (Park *et al*., 1997). The roles of chitinases in these organisms are diverse. In vertebrates, chitinases are usually part of the digestive tract. In insects and crustaceans, chitinases are associated with the need for partial degradation of old cuticle. Chitinases have been implicated in plant resistance against fungal pathogens because of their inducible nature and antifungal activities *in vitro* (Taira *et al*., 2002). Chitinase in fungi is thought to have autolytic, nutritional, and morphogenetic roles. In viruses, chitinases are involved in pathogenesis (Patil *et al*., 2000). In bacteria, chitinases play a role in nutrition and parasitism. In addition to the above roles in nature, chitinases can be applied for the production of *N*-acetyl-chitooligosaccharides and chitooligosaccharides, which have been found to function as antibacterial agents, elicitors of lysozyme inducers, and immunoenhancers (Wen *et al*., 2002). Chitinases can also be used in agriculture to control plant pathogens (Dahiya *et al*., 2005a; Karasuda *et al*., 2003). The findings in the catalytic and substrate-binding mechanisms of chitinolytic enzymes as well as their sequence homology and applications to plant protection against fungal pathogens and insect pests were reviewed in detail by Fukamizo, (2000). Various molecular and biotechnological

aspects such as regulation strategies, gene cloning of chitinase from microorganism and plants, and various industrial and agricultural applications of chitinases have been described by Patil *et al*., (2000).

Enzyme technology is an interdisciplinary field, and enzymes are routinely used in many environmental-friendly industrial sectors. With the advancement in biotechnology especially in the area of genetics, protein engineering, developments in bioinformatics, and the availability of sequence data have opened a new era of enzyme applications in many industrial processes.

Chitin occurrence and structure

Chitin is widely distributed in nature, particularly as a structural polysaccharide in fungus and exoskeleton of arthropods. It is one of the most abundant biopolymer found in nature, second to cellulose. Chitin is a β-(1,4) - linked *N*-acetyl-D-glucosamine, GlcNAc, polymer. Several reports are available regarding the presence of chitin in fungal cell walls (Table 1.1). In filamentous fungi and basidiomycetes, it comprises 16% of the dry weight of the organism. Cell walls of Mucoraceae are known to have chitosan in addition to chitin. In yeast, the amount of chitin in the cell wall is much lower, but bud scars have been shown to be largely composed of chitin (Kuranda and Robbins, 1991).

The X-ray diffraction studies revealed that chitin occurs in three polymorphic forms, that is, α-, β-, and γ-chitin, which differ in the arrangement of molecular chains within the crystal cell. In α-chitin, chains are arranged in an antiparallel fashion, whereas, in β-chitin, chains are parallel (Figure 1.1). In γ-chitin, chains are in mixed form (Peberdy, 1985). Among these, α-chitin is the most abundant form of chitin.

Chitin-degrading enzymes

Chitinases can be classified into two major categories. Endochitinases (EC 3.2.1.14) cleave chitin randomly at internal sites, generating low molecular mass multimers of GlcNAc, such as chitotetraose, chitotriose, and diacetylchitobiose. Exochitinases can be divided into two subcategories: chitobiosidases (EC 3.2.1.29), which catalyze the progressive release of diacetylchitobiose starting at the no reducing end of chitin microfibril, and β-(1,4) *N*-acetylglucosaminidases (EC 3.2.1.30), which cleave the oligomeric products of endochitinases and chitobiosidases,

Table 1.1 Chitin content of selected crustaceans, insects, molluscan organs and fungi (Tharanathan and Kittur, 2003)**.**

Organism	Chitin content (%)	Organism	Chitin content (%)
Crustacean		**Insects**	
Cancer (crab)	72.1[c]	*Periplaneta* (cockroach)	2.0[d]
Carcinus (crab)	64.2[b]	*Blatella* (cockroach)	18.4[c]
Paralithodes (king crab)	35.0[b]	*Colcoptera* (beetle)	27-35[c]
Callinectes (blue crab)	14.0[a]	*Diptera* (true fly)	54.8[c]
Crangon (shrimp)	69.1[c]	*Pieris* (sulphur butterfly)	64.0[c]
Alasakan shrimp	28.0[d]	*Bombyx* (silk worm)	44.2[c]
Nephrops (lobster)	69.8[c]	*Calleria* (wax worm)	33.7[c]
Homarus (lobster)	60-75[c]	**Fungi**	
Lepas (barnacles)	58.3[c]	*Aspergillus niger*	
Molluscan organs		*Penicillium notatum*	42.0[e]
Clamshell	6.1	*Penicillium chrysogenum*	18.5[e]
Oyster shell	3.6	*Saccharomyces cereviseae*	20.1[e]
Squid, skeleton pen	41.0	*Mucor rouxii*	2.9[e]
Krill, deproteinized shell	40.2	*Lactarius vaiiereus*	44.5
		(mushroom)	19.0

[a]Wet body weight

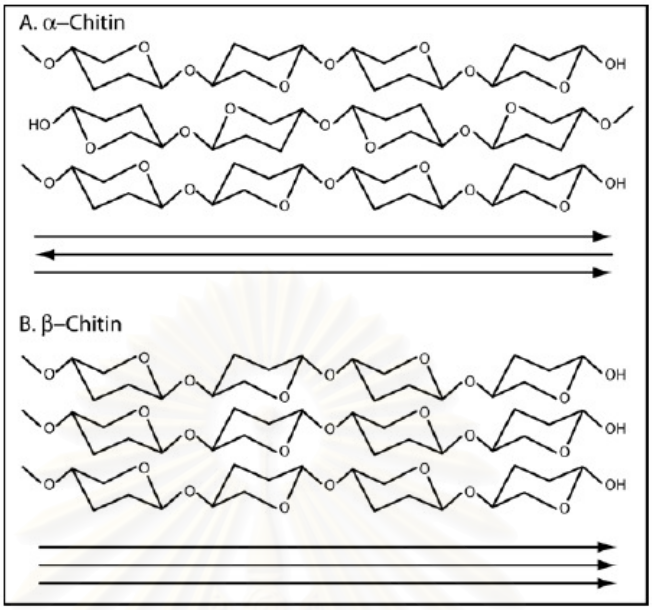
[b]Dry body weight

[c]Organic weight of cuticle

[d]Total weight of cuticle

[e]Dry weight of the cell wall

A



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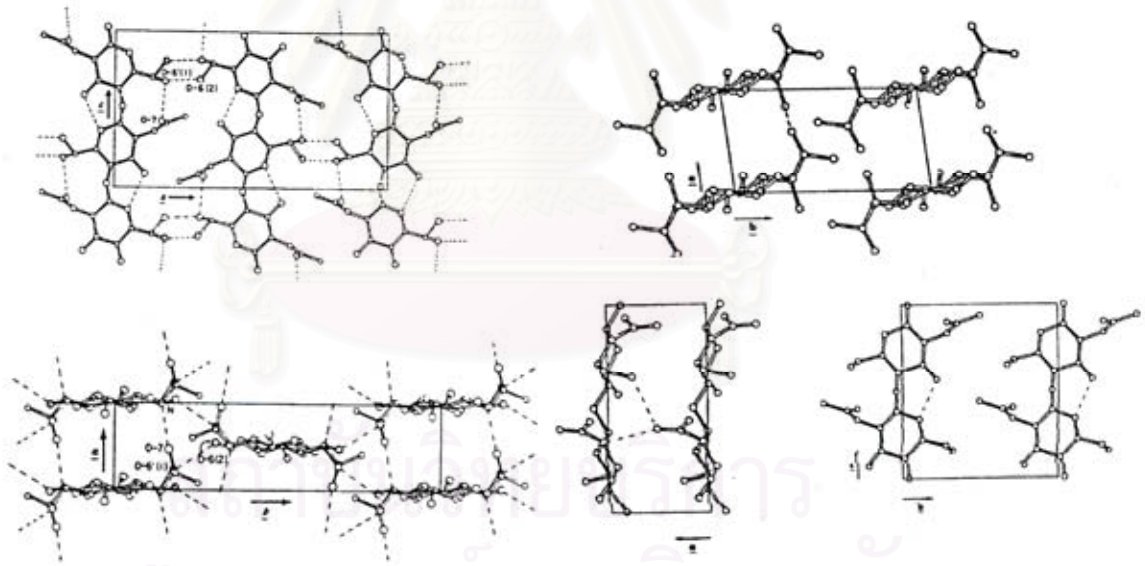


α-chitin **β-chitin**

Figure 1.1 Molecular structure of α- and β-chitin. Diagrammatic illustration of three types of chitin with arrangements as antiparallel (α-chitin), parallel (β-chitin) (A) (Tharanathan and Kittur, 2003). Structure of α-and β-chitin and hydrogen bond linkage between to C=O…NH groups (B) (Minke and Blackwell, 1978).

generating monomers of GlcNAc (Sahai and Manocha, 1993). An alternative pathway involves the deacetylation of chitin to chitosan, which is finally converted to glucosamine residues by the action of chitosanase (EC 3.2.1.132). Characteristics of some chitinases and their sources are summarized in Table 1.2.

Chitinase families

Based on amino acid sequence similarity, chitinolytic enzymes are grouped into 3 families, 18, 19, and 20 of glycosyl hydrolases (Henrissat and Bairoch, 1993). Family 18 is diverse in evolutionary terms and contains chitinases from bacteria, fungi, viruses, animals, and some plant chitinases. Family 19 consists of plant chitinases (classes I, II, and IV) and some *Streptomyces* chitinases (Hart *et al*., 1995). The chitinases of the two families, that is, 18 and 19, do not share amino acid sequence similarity. The amino acid residues found in the active site and includes glutamic acid (E) residue which played a crucial role in catalytic mechanism. Two glutamic residues are important in the mechanism of the action of Family 19 (Henrissat *et al*., 1991). Family 18 and 19 chitnase have completely different 3-D structures and molecular mechanisms and are therefore likely to have evolved from different ancestors in Figure 1.2 (Suzuki *et al*., 1999). Family 20 includes the β-*N*-acetylhexosaminidases from bacteria, *Streptomycetes*, and humans. Bacterial chitinases are clearly separated into three major subfamilies, A, B, and C (Figure 1.3), based on the amino acid sequence of individual catalytic domains (Watanabe *et al*., 1993). Subfamily A chitinases have the presence of a third domain corresponding to the insertion of an α+β fold region between the seventh and eighth $(\alpha/\beta)_8$ barrel. On the other hand, none of the chitinases in subfamilies A and B have this insertion. Several chitinolytic bacteria that possess chitinases belonging to different subfamilies like *Serratia marcescens* (Suzuki *et al*., 1999), *Bacillus circulans* WL-12 (Alam *et al*., 1995), and *Streptomyces coelicolor* A3 (2) (Saito *et al*., 1999) are reported.

Proposed catalytic mechanism of chitinases

Family 19 chitinase

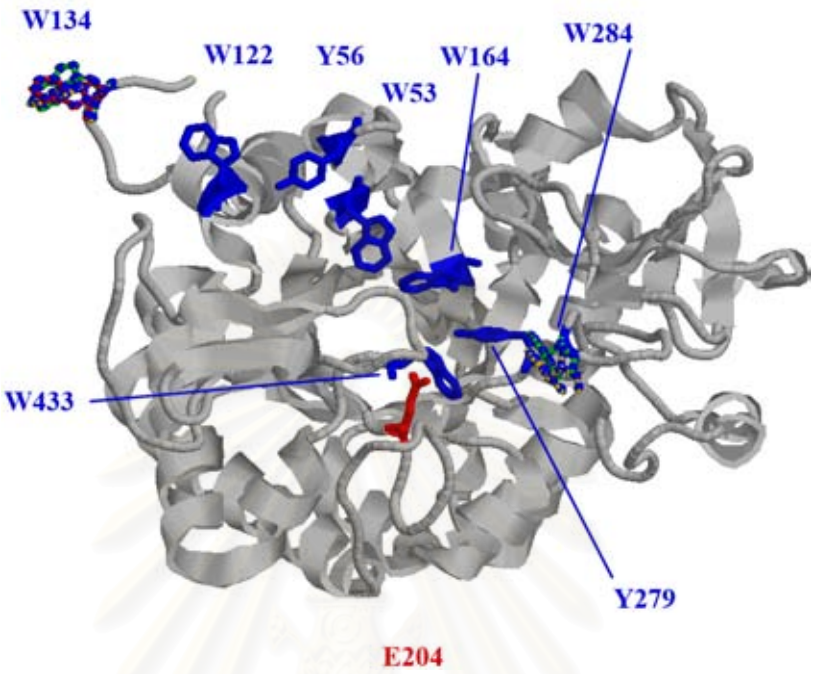
Family 19 chitinase from barley seeds has a three-dimensional structure similar to that of hen egg white lysozyme, especially in the substrate binding and

Table 1.2 Physiological properties of purified chitinase from microbial sources.

Microorganism	Optimum		Application	Inhibitors	Reference
	pH	Temperature (°C)			
Enterobacter sp. NRG4	5.5	45	Release of fungal protoplasts, production of GlcNAc, antifungal potential	*N*-bromo-succinamide	Dahiya et.al. 2005a,b,c
Enterobacter sp. G-1	7.0	40	n.s.	EDTA, PCMB	Park *et al*., 1997
Enterobacter aerogenes	6.0	55	n.s.	Hg^{2+}, Co^{2+}, Mg^{2+}	Tang *et al*., 2001
Alcaligens xylosoxydans	5.0	50	Antifungal potential	Cu^{2+}, Na^{2+}	Vaidya *et al*., 2003
Vibrio alginolyticus TK-22	4.0, 9.0	45	Preparation of chitopentoase and chitotriose	n.s.	Murao *et al*., 1992
Bacillus sp. BG-11	7.5-9.0	45-55	Antifungal potential	Allosamidin, iodoacetic acid, EDTA	Bhushan and Hoondal, 1998
Bacillus sp. NCTU2	7.0	60	n.s.	n.s.	Wen *et al*., 2002
Bacillus sp. 13.26	7.0-8.0	60	n.s.	Mn^{2+}, Ca^{2+}	Yuli *et al*., 2004
Serratia marcescens QMB1466	4.0-7.0	30	n.s.	n.s.	Roberts and Cabib, 1982
Serratia plymuthica HRO-C48	5.4	55	Antifungal potential	Co^{2+}, Cu^{2+}	Frankowski *et al*., 2001

n.s. Not specified

A



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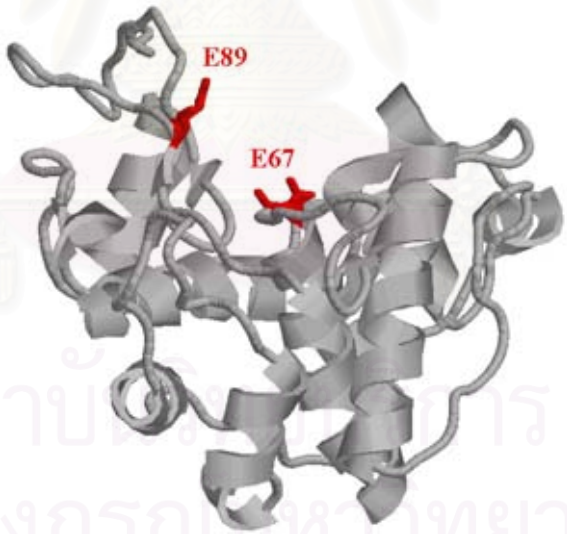


Figure 1.2 Three dimensional structure of family 18 and family 19 chitinase.

Bacillus circulans chitinase A1 (PDB number 1ITX); Panel A and barley chitinase (PDB number 2BBA); Panel B, binding sites are show in blue, and catalytic sites are in red. This figure was created using RasMol 2.7.3.

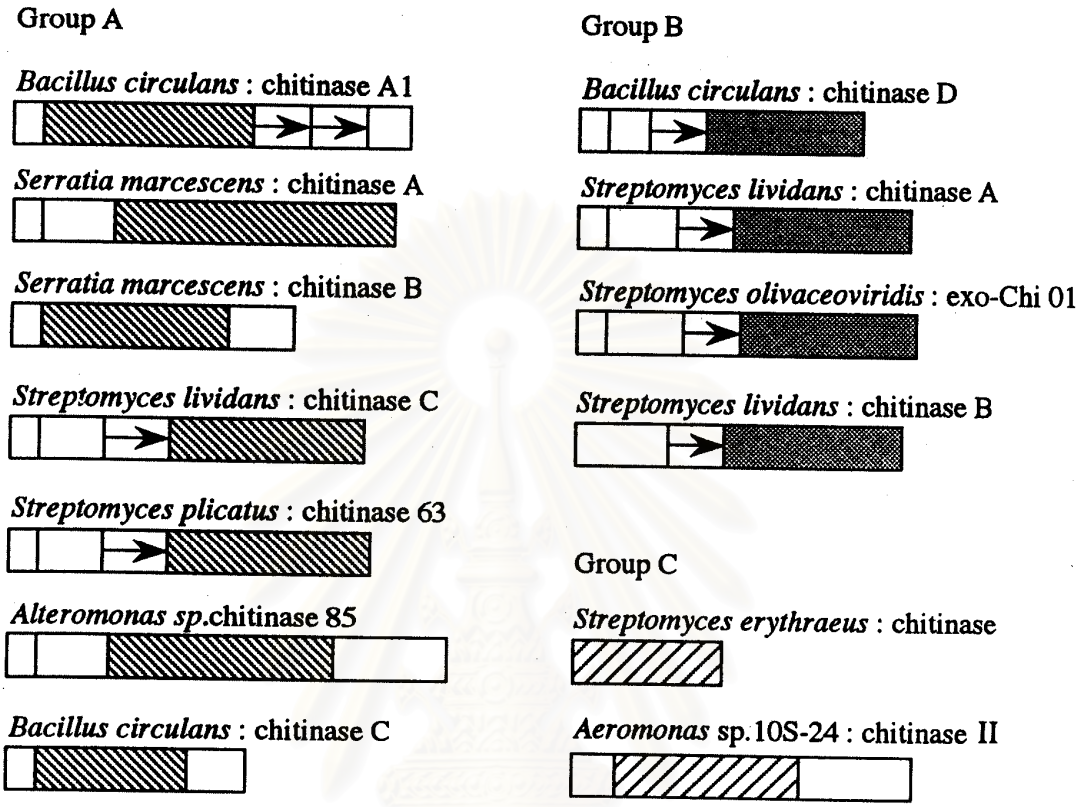


Figure 1.3 Classification of the bacterial chitinases based on the homology of similarity amino acid sequence of individual catalytic domains. Shadowed boxes indicate the homologous regions of individual chitinases to the catalytic domain of *Bacillus circulans* chitinase A1; Group A, *B. circulans* chitinase D; Group B, or *Streptomyces erythraeus* chitinase; Group C. Arrows indicate fibronectin type III like domain (From Hart *et al.*, 1995). Amino acid similarity within the catalytic domain of *B. circulans* chitinase was used to classify other bacterial chitinases into 3 groups. Group A is similar with chitinase A1, Group B is similar with chitinase D, and Group C with no similarity with chitinases from *B. circulans*.

catalytic core composed of a three stranded β-sheet and two α- helices (Monzingo *et al*., 1996). From this finding, it can be speculated that barley chitinase has a catalytic mechanism similar to that of hen egg white lysozyme. Contrary to speculation, hydrolytic products from barley chitinase reaction were found to be in α-form as determined by ^{1}H-NMR spectroscopy, indicating that the chitinase inverts the anomeric form through its catalytic reaction (Hollis *et al*., 1997). Regardless of the structural similarity, the catalytic mechanism of family 19 chitinase is different from that of hen egg white lysozyme. Some structural difference in the catalytic center between the barley chitinase and hen egg white lysozyme would result in the different catalytic mechanisms. As reported by Withers and his co-workers, the distance between the two catalytic residues is closely related to the catalytic mechanism (Wang *et al*., 1994). In the case of retaining enzymes, the average distance between the two catalytic residues is about 4-5 Å, while the distance is about 10-11 Å in inverting enzymes. In fact, the distance between Glu35 and Asp52 in hen egg white lysozyme is 4.6 Å. In the site-directed mutagenesis study of barley chitinase, the mutation of Glu67 to Gln completely eliminated its activity, and that of Glu89 impaired the activity to 0.25 % of that of the wild type. Glu67 and Glu89 are most likely to be a proton donor and a second catalytic residue like Asp52 in the lysozyme, respectively (Andersen *et al*., 1997). In the crystal structure, the distance between the two catalytic residues is 9.3 Å. Obviously, the difference in catalytic mechanism between hen egg white lysozyme and barley chitinase is ascribed to the distance between the two catalytic residues. The longer separation between the catalytic residues seems to be a structural feature characteristic of family 19 chitinase. The reaction of inverting glycosyl hydrolases which have two largely separated catalytic residues is often explained by a single displacement mechanism (Kuroki *et al*., 1995). The mechanism is shown in Figure 1.4, scheme I. At first, the general acid, Glu67, protonates the β-1,4- glycosidic oxygen atom, forming an oxocarbonium ion intermediate, and then the water molecule activated by the general base, Glu89, attacks the C1 atom of the intermediate state from the a-side to complete the reaction. The separated location of the two catalytic residues might permit the water molecule to be located in-between the anomeric C1 atom and the carboxyl oxygen of the general base (Glu89). This location of the water molecule would result in the anomeric inversion of the reaction

products. From the molecular dynamics simulations (Brameld and Goddard, 1998), however, Glu89 was found not only to activate the nucleophilicity of the water molecule but to act as a stabilizer of the carbonium ion intermediate. In addition, the simulation study indicated that the $(GlcNAc)_6$ substrate binds to barley chitinase with all sugar residues in a chair conformation; that is, no sugar residue distortion was found in family 19 chitinase complexed with the substrate. Chitinase from yam (*Dioscorea opposita*) was reported to produce a-form of the product, indicating that the chitinase is an inverter (Fukamizo *et al*., 1995). Chitinase from another plant was reported to be an inverter as well (Dahlquist *et al*., 1969 and Iseli *et al*., 1996). All of these inverting chitinases from plant should have a similar catalytic mechanism.

Family 18 Chitinases

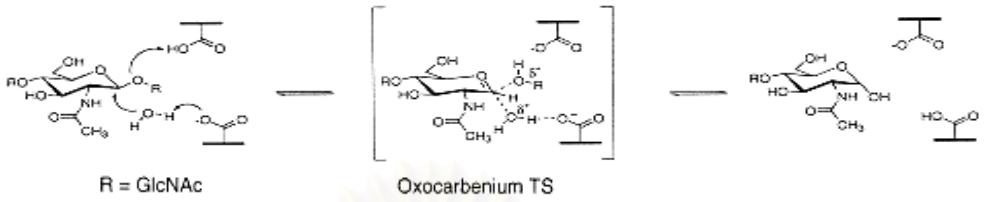
Family 18 chitinases have not been studies as extensively as those from family 19. They were reported to yield hydrolysis products which retain the anomeric configuration at C1′ (Brameld *et al*., 1998; Brameld and Goddard, 1998 and Yannis *et al*., 2001) and two proposed catalytic mechanism.

The earlier proposed catalytic mechanism (shown in figure 1.4, Scheme II) invoked a substrate assistance mechanism (Brameld *et al*., 1998). That is, the *N*-acetyl group at position 2 for the scissile sugar may itself facilitate the reaction via formation of a transient oxazolinium intermediate (Terwisscha *et al*., 1995). Unlike the enzymes described thus far, family 18 chitinases have a catalytic $(\alpha/\beta)_8$-barrel domain. The catalytic residues of this enzyme family were first reported by Watanabe and his co-worker for chitinase A1 from *Bacillus circulans* WL-12 (Watanabe *et al*., 1993). Site-directed mutagenesis of Glu204 completely eliminated its activity, and the residue was considered to be a proton donor in its catalysis. From the sequence comparison, the glutamic acid residue was found to be conserved in all chitinases in family 18. In *Serratia marcescence* chitinase A, the catalytic carboxylate corresponding to Glu204 of *B. circulans* chitinase A1 is Glu315. Like hen egg white lysozyme, *B. circulans* chitinase A1 produce β-anomer (Armand *et al*., 1994), hence is a retaining enzyme. As described above, in retaining enzymes, the location of the second carboxylate is close to that of the proton donor carboxylate (< 5 Å). In the consensus region of the catalytic domain of family 18 chitinases, there are several

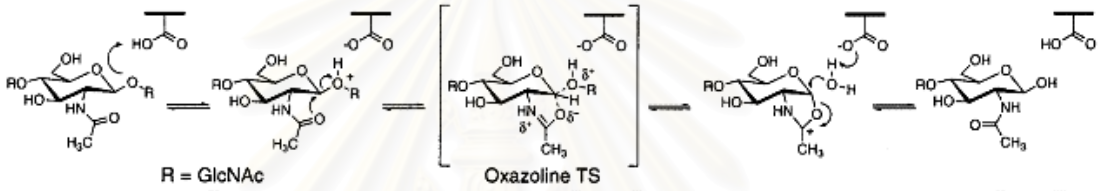
conserved carboxylic amino acid residues, for example, Asp200 and Asp202 in chitinase A1 from *B. circulans*, Asp311 and Asp313 in chitinase A from *S. marcescens*. Site-directed mutagenesis of Asp200 and Asp202 in *B. circulans* chitinase A1 impaired the enzymatic activity, but did not completely eliminate the activity (Watanabe et al., 1993 and Watanabe et al., 1994). The location of these residues does not correspond to that of the second carboxylate in lysozyme (Asp52) or in family 19 barley chitinase (Glu89). Thus, the second carboxylate cannot be identified in any family 18 chitinase. The family 18 chitinases should have a different mechanism of catalysis. Recent studies on the family 18 chitinases indicate that the catalytic reaction of the enzymes takes place through a substrate-assisted mechanism. A putative oxocarbonium ion intermediate is stabilized by an anchimeric assistance of the sugar *N*-acetyl group after donation of a proton from the catalytic carboxylate to the leaving group. Such stabilization might occur either through a charge interaction between the C1 carbon and the carbonyl oxygen of the *N*-acetyl group or via an oxazoline intermediate with a covalent bond between C1 carbon and the carbonyl oxygen. The mechanism does not require the second carboxylate and can rationalize the anomer retaining reaction of the enzymes without the second carboxylate. This mechanism was first proposed for the spontaneous acid-catalyzed hydrolysis of 2-acetamido-substituted polysaccharides in solution, and applied to the lysozyme mechanism (Lowe *et al*., 1967). Experimental evidence of the substrate assistance in family 18 chitinase has been first provided by the crystal structure of the inhibitor allosamidin bound to chitinase from *Hevea brasiliensis* (Terwisscha *et al*., 1995). Recent studies by quantum mechanical calculation supported the substrate-assisted mechanism in family 18 chitinase (Brameld *et al*., 1998).

The last proposed catalytic mechanism in chitinase A from *Serratia marcescens* suggest that residues Asp313 and Try390 along with Glu315 play a central role in the catalysis (Yannis *et al*., 2001). Yannis *et al*. proposed that after the protonation of the substrate glycosidic bond, Asp313 that interacts with Asp311 flips to its alternative position where it interacts with Glu315 thus forcing the substrate acetamido group of -1 sugar to rotate around the C2-N2 bond. As a result of these structural changes, the water molecule that the hydrogen-bonded to Try390 and the NH of the acetamido group is displaced to a position that allows the completion of hydrolysis (Scheme III). In this mechanism, we will not observe an oxazoline ring

Scheme I



Scheme II



Scheme III

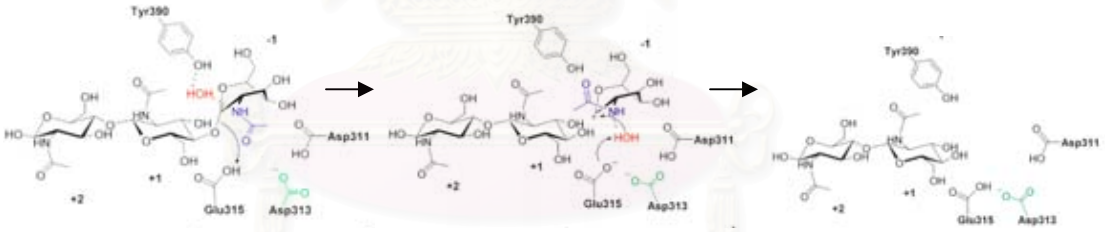


Figure 1.4 Chitinase mechanisms. The single-displacement hydrolysis mechanism proposed for family 19 chitinases. Two acidic residues are required in the active site, and the hydrolysis product shows inversion of the anomeric configuration (scheme II). The double-displacement hydrolysis mechanism proposed for family 18 chitinases. Protonation of a GlcNAc residue in a boat conformation leads to an oxazoline intermediate, which may be hydrolyzed to form a product with retention of the anomeric configuration (scheme II, III).

intermediate, the acetamido group of -1 sugar comes close to O5 atom in a way that could allow a modified "substrate assisted" reaction, shown in Figure 1.4, scheme III.

Substrate binding mechanism

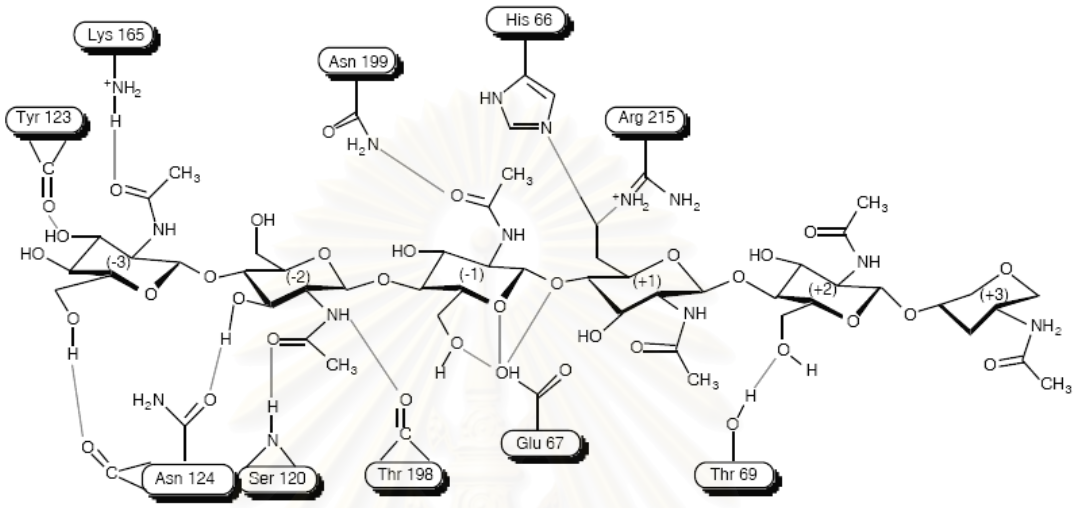
Family 19 chitinases

The binding mode of $(GlcNAc)_6$ to family 19 chitinase from barley seeds was estimated by energy minimization based on the X-ray crystal structure of the enzyme (Hart *et al*., 1995). They reported that the enzyme has six subsites, (-4)(-3)(-2)(-1)(+1)(+2), like hen egg white lysozyme. In barley chitinase, the sugar residue interaction at subsite (-4) is unlikely to be significant, but the binding cleft seems to be longer on the reducing end side than that of the lysozyme. Therefore, the binding cleft of the barley chitinase is most likely (-3)(-2)(-1)(+1)(+2)(+3). The theoretical model of the hexasaccharide degradation by the chitinase was constructed by a slight modification of the lysozyme model. In the energy minimized structure of the chitinase complexed with $(GlcNAc)_6$, the sugar residues at sites (-2) and (-3) makes hydrogen bonds with Tyr123 and Asn124 (Figure 1.5A). These amino acid residues are conserved for all chitinases in class I and class II chitinases from plant origin, and are considered to be the most important for substrate binding.

Family 18 chitinases

For family 18 chitinases, the entire substrate binding cleft was first revealed by superposition of the structure of *H. brasiliensis* chitinase complexed with $(GlcNAc)_4$ and that of *S. marcescens* chitinase A complexed with $(GlcNAc)_2$ (Tews *et al*., 1997). Brameld and Goddard have done the molecular dynamics simulations of $(GlcNAc)_6$ binding to *S. marcescens* chitinase A (Brameld and Goddard, 1998). Both works indicated that the binding cleft are represented by (-4)(-3)(-2)(-1)(+1)(+2) in family 18 chitinases. The Figure 1.5 shows the hydrogen bonding interaction between *S. marcescens* chitinase A and $(GlcNAc)_6$. The sugar residue at site (-1) is distorted, and positioned near the central axis of the barrel structure. The distorted sugar residue is likely to somewhat slip into the central core of the barrel, but the other sugar residues are to be accommodated on the upper surface of the barrel. Asp230, Thr276, and Asn474 are responsible for sugar residue binding at sites (- 4) and (-3).

A



B

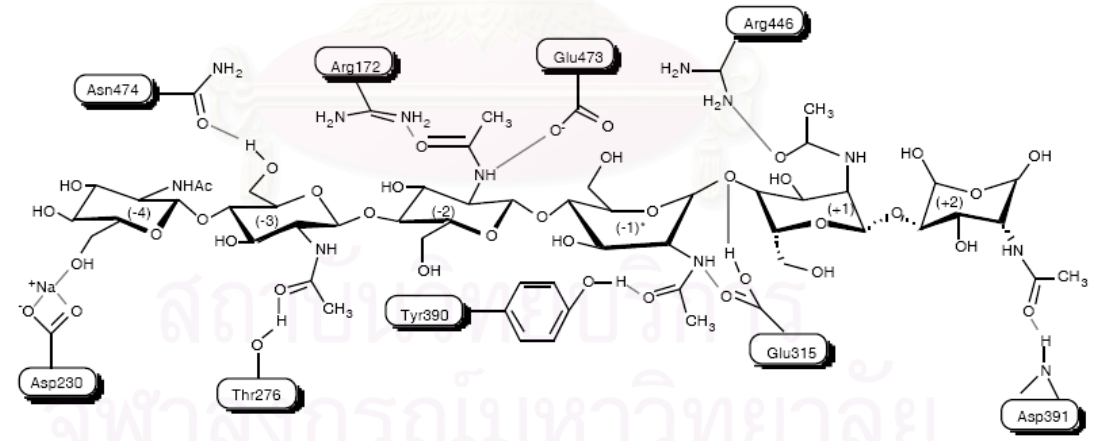


Figure 1.5 Interaction between barley chitinase and $(GlcNAc)_6$ (A) and *S. marcescens* chitinase A and $(GlcNAc)_6$ (B). Hydrogen bonding interaction was from molecular dynamics simulation (Brameld and Goddard, 1998).

In addition, the aromatic residues, Trp167, Tyr163, and Trp 539, are considered to participate in the sugar residue binding by hydrophobic contacts at sites (-3), (-1), and (+1), respectively (Figure 1.5B). At site (-2), Arg172 and Glu473 are likely to be important. The sugar residues are considered to be less tightly bound to the sites (+1) and (+2). In the early stage of the reaction, the enzyme produced β-anomer of $(GlcNAc)_2$ from the substrate $(GlcNAc)_6$ together with the equilibrium amounts of α- and β-anomers of $(GlcNAc)_4$. This result leads us to consider a different binding cleft, (-2)(-1)(+1)(+2)(+3)(+4). But the structural information rules out the sugar residue binding at sites (+3) and (+4). The subsite structure of family 18 chitinases seems to remain unclear. Further evidences from oligosaccharide digestion experiments are needed to define the substrate binding subsites of the family 18 chitinase.

Reaction mechanism of chitinase.

Makino *et al*., 2006 proposed the postulated reaction mechanisms of chitinase in (A) hydrolysis of chitin and (B) polymerization of GlcN β-(1,4) GlcNAc monomer 1. In the hydrolysis, the glycosidic oxygen of the saccharide chain placed between the donor site and the acceptor site of chitinase is protonated by the carboxylic acid in the active center of the enzyme immediately after recognition as illustrated in stage 1. Then, the acetamido oxygen at the C2 position of the saccharide unit at the donor site attacks the neighboring C1 carbon to form the corresponding oxazolinium ion stabilized by another carboxylate in the active center, leading to scission of the glycosidic linkage (stage 2) (Herissat, 1991; Henrissat *et al*., 1996; Sakamoto *et al*., 2001 and Tews *et al*., 1997; htttp://afmb.cnrs-mrs.fr/CAZY/index.html.). Nucleophilic attack by a water molecule from the β-side opens the ring of the oxazolinium to accomplish the hydrolysis reaction, giving rise to the hydrolyzate having a β-configuration (stage 3). In the polymerization, the oxazoline monomer is effectively recognized at the donor site of chitinase as a TSAS (Kobayashi *et al*., 2006). The nitrogen atom in the oxazoline ring is immediately protonated by the carboxylic acidafter the recognition to form the corresponding oxazolinium ion (stage1′), which is stabilized there. Then, the hydroxy group at the C4 of another monomer or the growing chain end attacks the C1 of the oxazolinium from the β-side (stage 2′),

resulting in the formation of β−(1,4) glycosidic linkage (stage 3′). Repetition of these reactions is a ring-opening polyaddition, leading to the formation of a chitin-chitosan hybrid polysaccharide under total control of regioselectivity and stereochemistry. The key-point is the structure of the transition state (or the intermediate), which is commonly involved in both stages 2 and 2′ as a protonated oxazolinium moiety. Monomer 1 is very close to the moiety, showing the importance of the concept of a transition-state analogue substrate (TSAS) monomer in Figure 1.6. (Kobayashi *et al*., 1995; Kobayashi, 1997; Kobayashi 1999; Kobayashi *et al*., 2001a,b,c; Kobayashi, 2005; and Kobayashi *et al*., 2006)

Synergistic action of multiple forms of chitinases

Most of the chitinolytic organisms produce multiple isomeric forms of chitinases, which may result from posttranslational processing of a single-gene product or, more often, the products of multiple genes. The heterogeneity of chitinases was attributed to posttranslational modifications such as differential glycosylation and/or proteolysis.

Multiple chitinolytic enzymes have been reported in several microorganisms such as *S. marcescens* (Suzuki *et al*., 2002), *Aeromonas* sp. No. 10S-24 (Ueda, *et al*.1995), *Pseudomonas aeruginosa* K-187 (Wang and Chang, 1997), *B. circulans* WL-12 (Mitsutomi *et al*., 1998), *Bacillus licheniformis* X-7u (Takayanagi *et al*., 1991), *Streptomyces* sp. J. 13-3 (Okazaki *et al*., 1995), and *Streptomyces griseus* HUT 6037 (Itoh *et al*., 2002).

Suzuki *et al*., (2002) reported the synergistic action of chitinases Chi A, Chi B, and Chi C1 of *S. marcescens* 2170 on chitin degradation. They proposed that despite having similar catalytic domains, Chi A and Chi B were considered to digest chitin chains in the opposite direction. Chi A was proposed to degrade the chitin chain from the reducing end, whereas Chi B, from the nonreducing end. Addition of Chi A after treatment of powdered chitin with Chi B and vice versa was generally improved chitin degradation efficiency.

A thermophilic bacterium, *B. licheniformis* X-7u, possesses four chitinases, I, II, III, and IV. Chitinases II, III, and IV produced $(GlcNAc)_2$ and GlcNAc, whereas chitinase I predominantly produced $(GlcNAc)_2$. Chitinases II, III, and IV also

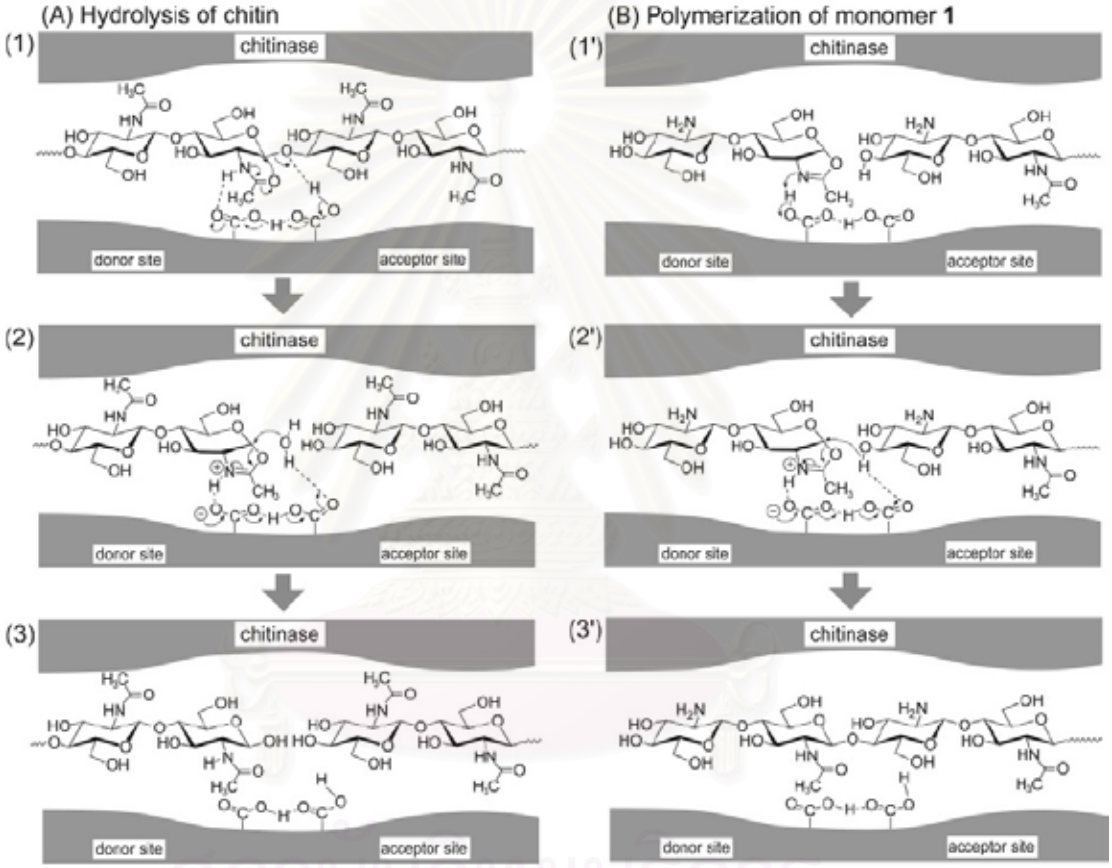


Figure 1.6 Postulated reaction mechanisms of chitinase catalysis (Makino *et al*., 2006)**.**

catalyzed a transglycosylation reaction that converted $(GlcNAc)_4$ into $(GlcNAc)_6$ (Takayanagi *et al*., 1991).

Chitinase production

Microbial chitinase has been produced by liquid batch fermentation, continuous fermentation, and fed-batch fermentation. In addition to these, solid-state fermentation and biphasic cell systems have also been used for the production of chitinase. Generally, chitinase produced from microorganisms is inducible in nature.

Extracellular chitinase production is reported to be influenced by media components such as carbon sources, nitrogen sources, and agricultural residues such as rice bran, wheat bran, etc. (Bhushan, 1998; Dahiya *et al*., 2005b). An enhancing effect of glucose on chitinase production was reported by Bhushan (1998) when glucose was used with chitin in production medium. However, a suppressing effect of glucose on chitinase production was reported by Miyashita *et al*., (1991).

Several other physical factors such as aeration, pH, and incubation temperature also affect chitinase production. The addition of amino acids and their analogs such as tryptophan, tyrosine, glutamine, and arginine (0.1 mM) in the growth medium stimulated chitinase production from Bacillus sp. BG-11 (Bhushan, 1998).

Some other methods, such as cell immobilization (Bhushan, 1998), biphasic cell systems (Chen and Lee, 1995), solid-state fermentations, etc., have been used for improving chitinase production from different microorganisms (Bhushan, 1998). In an immobilized system, whole cell immobilization of an organism to a solid support such as polyurethane foam was applied. Chitinase production was enhanced up to 4.8-fold over a period of 72 hr in submerged fermentation.

Enhanced production of extracellular chitinase by *S. marcescens* in an aqueous two-phase system (ATP) of PEG and dextran was reported by Chen and Lee, (1995). They reported a maximum chitinase activity of 41.5 units in ATPs [2% (w/w) PEG 20,000 and 5.0% (w/w) dextran T500] compared with 13.6 units in a polymer-free system.

In solid substrate fermentation using flake chitin as the solid substrate, the maximum chitinase yield obtained from *Enterobacter* sp. NRG4 was 616 U/g solid substrate after 168 h of growth at 30°C and 75% moisture level. When wheat bran was used in combination with chitinous substrates, the chitinase yield increased.

Maximum enzyme yield was 1,475 U/g at wheat bran to flake chitin ratio, 1; moisture, 80%; and inoculum, 2.6 mL after 168 hr (Dahiya *et al*., 2005b).

Bacterial chitinases

Bacterial chitinases generally consist of multiple functional domains, such as chitin binding domains (ChBDs) and fibronectin type III-like domain (FnIIID) linked to the catalytic domain (CatD). Many chitinases are composed of a catalytic domain joined to one of more chitin-binding domains, as in the case of various insoluble polysaccharide hydrolases including cellulases. This kind of substrate-binding domain is functional not only for accumulating catalytic sites on the surface of substrates but also for disrupting hydrogen bonds in the crystalline region of substrates and thereby facilitating subsequent hydrolysis by the catalytic domains (Tomme *et al*., 1995).

Catalytic domains (CatD)

The three dimensional (3D) structures of some members of family 18 chitinase have been determined such as *S. marcescesns* chiA and B. circulans WL-12 chiA. The 3D structure reveal that they share a similar $(\beta/\alpha)_8$ barrel (as show in Figure 1A). That is eight strands of parallel β sheet are laid sown with the helices the return stroke. The eight strands of the sheet bend into barrel with the helices forming a ring toward the outside. However, the structure of the only structure of the member of family 19 chitinase has been solved from *Hordwum valgare* (barley) as show in Figure 1.2B). The 3D structure of the protein revealing a mixture of secondary structures, including 10 α-helical segments, and on three-stranded β sheet.

Chitin binding domain (ChBD)

There are three families of chitin binding domains. ChBD family 1 is found of chitinases from *Bombyx mori* and *Penaeus japonicas*. ChBD family 2 is found in chitinases from *Arabidopsis thaliana*, *H. vulgare* and *Oryza sativa*. ChBD family 3 is found in chitinases from *A. caviae*, *B. ciruclans* and *V. harveyi*. However, only 3D structure of ChBD from B. circulans WL-12 is completely solved using NMR technique. This domain contained two antiparallel β-sheets. One sheet is composed of

three strands (β_2, β_3 and β_5) and the other is composed of two strands (β_1 and β_4). No region characteristic of α-helix exists. These five b-strands form the hydrogen bond networks. The two antiparallel β-sheets formed by these hydrogen bond networks fold into the topology of a twisted b-sandwich with an angle of about 45 between the sheets. The β sheet formed by β_2, β_3 and β_5 makes a flat surface on the molecule, and the loop connecting β_4, and β_5 run on the opposite side to the surface. The core region formed by the hydrophobic and aromatic residues makes the overall structure rigid and compact as shown in Figure 1.7A. ChBD of this chitinase is required for the enzyme to bind specifically of insoluble chitin and to hydrolyze it efficiently (Takashisa, 2000). In contrast, chitinase A from *S. marcescens* contains a putative ChBD in its *N*-terminal region (ChiN) but on sequence similarity exists between ChiN and ChBD from *B. circulans* WL-12. Moreover, important differences are found in the surface residues of these two domains. ChiN has adjacently arranged tryptophans exposed on a continuous surface with the conserved aromatic residues of catalytic domain. These play important roles in guiding a chitin chain into the catalytic site (Uchiyama *et al*., 2001).

Fibronectin type III like domain (FnIIID)

FnIIID has been indentified in a set of depolymerase from bacterial and an animal fibronectins. It is likely that the bacterial modules were initially acquired form an animal source and were then spreaded further between distantly related bacteria by horizontal transfers (Bork and Doolittle, 1992). Three-dimensional structure of FnIIID of chitinase A1 from *B. circulans* WL-12 was solved by NMR techniques. Structure of $FnIIID_{chiA1}$ 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-sheets. There are three loops in the each direction of *N*-terminus and *C*-terminus connects to the seven β-strands, as shown in Figure 1.7B. FnIIID comprise domain-intrinsic and domain-specific regions. The former, made up of relatively conserved residues, are responsible for forming the FnIIID scaffold, which comprises a hydrogen-bond network and hydrophobic core. The scaffold is common to all FnIIID structures and endows the domain with it's the mechanical extensibility against tension and its high refolding speed. In contrast, exposed residues that are not well conserved across the

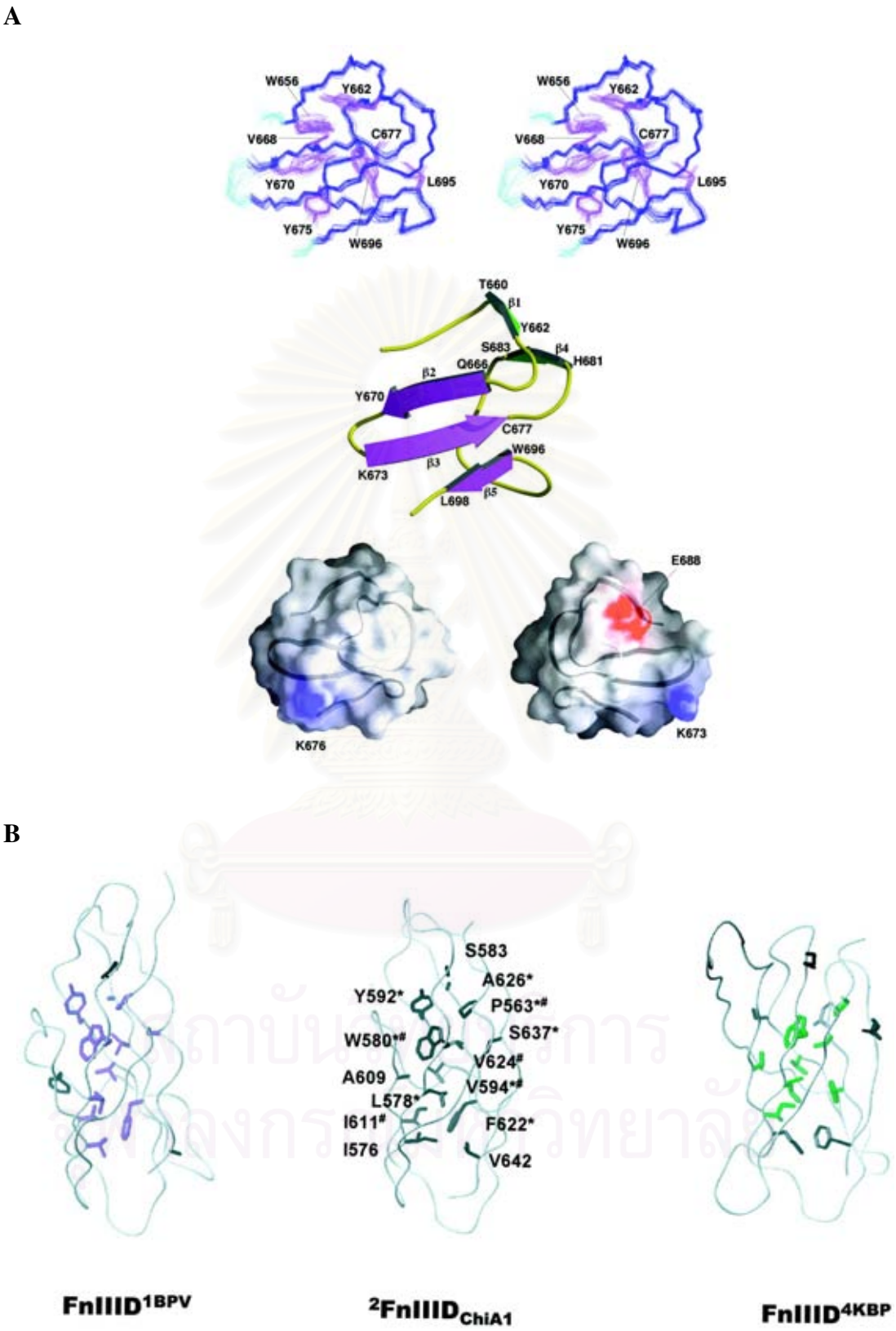


Figure 1.7 Tertiary structures of $ChBD_{ChiA1}$ (A) and $FnIIID_{ChiA1}$ (B) from *B. circulans* (Uchiyama *et al*., 2001; Goo *et al*., 2002)**.**

FnIIID family form the domain-specific regions. These residues often form the recognition site for the FnIIID of an interacting partner protein. It has been reported that the deletion of $FnIIID_{chiA1}$ has no impact on the chitin binding activity of ChiA1, but causes a significant decrease in the colloidal chitin hydrolyzing activity. The natural function of the FnIIID remains unclear (Goo *et al*., 2002).

Chitinase in *Bacillus* sp.

When *Bacillus circulans* was grown in chitin, six distinct chitinase molecules were detected in the culture supernatant. These chitinases (A1, A2, B1, B2, C, and D) showed the following distinct sizes and isoelectric points: Mr 74,000, p*I* 4.7 (A1); Mr 69,000, p*I* 4.5 (A2); Mr 38,000, p*I* 6.6 (B1); Mr 38,000, p*I* 5.9 (B2); Mr 39,000, p*I* 8.5 (C); and Mr 52,000, pI 5.2 (D). Among these chitinases, A1 and A2 had the highest colloidal-chitin-hydrolyzing activities. Chitinase A1 showed a strong affinity to insoluble substrate chitin. Purified chitinase A1 released predominantly chitobiose [$(GlcNAc)_2$] and a trace amount of *N*-acetylglucosamine (GlcNAc) from colloidal chitin. *N*-terminal amino acid sequence analysis of chitinases A1 and A2 indicated that chitinase A2 was generated from chitinase A1, presumably by proteolytic removal of a *C*-terminal portion of chitinase A1. Since chitinase A2 did not have the ability to bind to chitin, the importance of the *C*-terminal region of chitinase A1 to the strong affinity of chitinase A1 to substrate chitin was suggested. Strong affinity of the chitinase seemed to be required for complete degradation of insoluble substrate chitin. Chitinase A1 is the key enzyme in the chitinase system of this bacterium.

The functional domains of the *B. circulans* ChiA1 were determined separately: CatD by X-ray crystallography and ChBD and FnIIID by NMR (Watanabe *et al*., 1990; Watanabe *et al*., 1993). $CatD_{chiA1}$ consists of an $(\beta/\alpha)_8$-(triosephosphate isomerase) TIM barrel, and two insertion domains, β-domain 1 and β-domain 2, that are attached on top of the TIM-barrel provide a deep cleft for substrate binding. The crystal structure of inactivated $CatD_{chiA1}$ complexed with NAG_7 suggests that cleavage of the chitin chain occurs at the second linkage from the reducing end and the presence of seven subsites, numbered -5 to +2 , in the binding cleft was deduced form the complexed structure. In addition, outside of the subsite binding cleft, two exposed tryptophan residues (W122 and W134) were found to be aligned on the

extension of the oligomer chain bound to the cleft. These two aromatic residues have been shown to be essential for hydrolysis of crystalline chitin and have been proposed to play an important role on guiding a chitin chain into the substrate-binding cleft during crystalline-chitin hydrolysis. When compared *B. circulans* ChiA1 3D structures to that of *S. marcescens* ChiA and ChiB. The 3D structure of CatD of *Serratia* ChiA is basically very similar to $CatD_{chiA1}$. Two exposed aromatic residues outside of the substrate-binding cleft and four aromatic residues in the cleft of $CatD_{chiA1}$ are all conserved in *S. marcescens* ChiA. In addition, two additional aromatic residues were found on the surface of the *N*-terminal domain of *S. marcescens* ChiA. All four exposed aromatic residues outside of the cleft have been shown to be essential determinants for crystalline-chitin hydrolysis. Three of them, two in the *N*-terminal domain and one in the catalytic domain, play vital roles in chitin binding and the remaining residue appears to be important for guiding the chitin chain into the substrate-binding cleft. Based on these observations, a model for the possessive hydrolysis of crystalline chitin by ChiA has been proposed (Watanabe *et al.*, 2001; Papannikolau *et al*., 2001).

Chitinases in *Serratia marcescens*

When grown with chitin as the source of carbon, *S. marcescens* produces 4 chitinases called ChiA, ChiB, ChiC1 and ChiC2 (Perrakis *et al*., 1994; Suzuki *et al*., 1999; van Aalten *et al*., 2000). ChiA and ChiB consist of a catalytic domain and a smaller domain putatively involved in chitin binding (Figure 1.8). ChiC1 consists of a catalytic domain and two *C*-terminal putative chitin-binding domains. ChiC2 is produced *in vivo* by proteolytic processing of ChiC1 and consists of the catalytic domain only (Suzuki *et al*., 1999). The crystal structures of ChiA (Perrakis *et al*., 1994; Papanikolau *et al*., 2001) and ChiB (van Aalten *et al*., 2000; van Aalten *et al*., 2001; Kolstad *et al*., 2002; Vaaje-Kolstad *et al*., 2004), with and without substrates and with different mutations have been solved. There are no crystal-structures for ChiC, and hence less is known about this enzyme. Figure 1.8 shows the structures of inactive point mutants of ChiA (Papanikolau *et al*., 2001) and ChiB (van Aalten *et al*., 2001) in complex with short fragments of substrate. The catalytic domains of the enzymes have a $(\beta\alpha)_8$- (TIM-barrel) fold. Both ChiA and ChiB contain a large insert (78 and 73 residues, respectively) between strand β_7 and helix α_7 of the barrel,

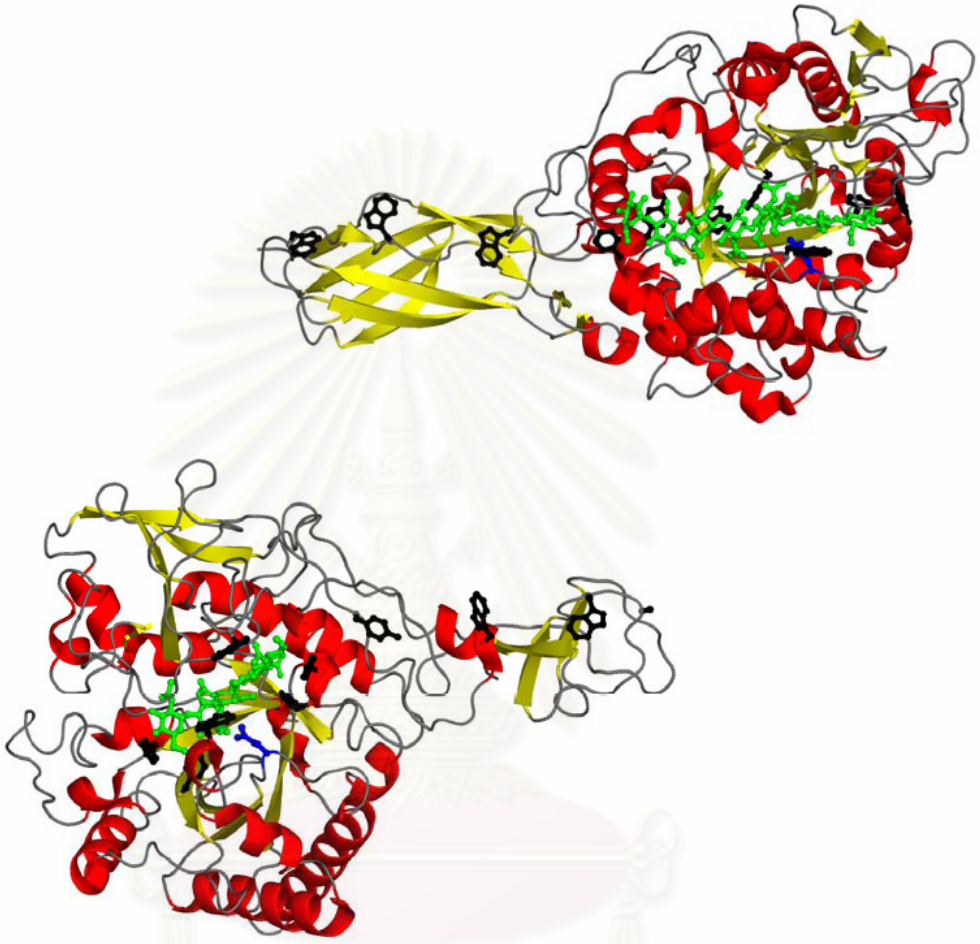


Figure 1.8 Structure of ChiA (pdb id: 1EHN, upper panel) and ChiB (pdb id: 1E6N, lower panel) from *Serratia marcescens* in complex with substrate. The catalytic acids (Glu315 in ChiA, Glu144 in ChiB) are mutated to Gln and shown as blue sticks. ChiA binds $(GlcNAc)_8$ (colored green) in subsites -6 to +2; ChiB binds $(GlcNAc)_5$ (colored green) in subsites -2 to +3. Side chains of aromatic residues involved in substrate binding are shown as black sticks. The picture was made with PyMol (DeLano, 2002).

the so-called α+β domain (Perrakis *et al*., 1994). Both enzymes have an additional domain, *N*-terminal in ChiA and *C*-terminal in ChiB, which have different structures, but which are both assumed to be involved in substrate binding (Perrakis *et al*., 1994; van Aalten *et al*., 2000).

Both ChiA and ChiB have deep substrate-binding clefts with almost a "tunnel-like" character. This characteristic, which is partly due to the presence of the α+β domain, is usually considered to be characteristic for exochitinases, which may act processively (Davies and Henrissat, 1995). Sequence alignments (based on homology to hevamine, a family 18 plant chitinase from rubber tree; Terwisscha van Scheltinga *et al*., 1994) show that the catalytic domain of ChiC lacks the α+β domain and that the substrate-binding groove of ChiC may have a more shallow and open character. Thus, ChiC is likely to be an endochitinase, like hevamine (Bokma *et al*., 2000; Suzuki *et al*., 2002; Eijsink *et al*., 2003; Horn *et al*., 2004). The substrate binding grooves, and the surface of the chitin-binding domains of the enzymes are lined with aromatic residues, and it has been shown by site-directed mutagenesis that these aromatic residues contribute to substrate binding (Uchiyama *et al*., 2001; Watanabe *et al*., 2001; Houston *et al*., 2002). Generally, substrate-binding clefts of glycosyl hydrolases have defined subsites related to binding of individual sugar units. These subsites are labeled from –n to +n, where –n represents the non-reducing end and +n the reducing end. Cleavage takes place between the -1 and +1 subsites (Davies *et al*., 1997). In ChiA the groove is extended in the direction where the non reducing end of the sugar binds ("-"subsites), whereas in ChiB the groove is extended in the direction where the reducing end of the sugar binds ("+" subsites). Both enzymes act primarily as exoenzymes, but apparently ChiA degrades from the reducing end, whereas ChiB degrades from the non-reducing end (Brurberg *et al*., 1996; van Aalten *et al*., 2000; Uchiyama *et al*., 2001; Suzuki *et al*., 2002; Horn *et al*., 2004). This could be one explanation for the observed synergistic effect between the two enzymes (Brurberg *et al*., 1996; Suzuki *et al*., 2002).

Molecular cloning and protein engineering

A number of attempts have been made to clone and express genes from several organisms such as *B. circulans* WL-12 (Mitsutomi et al. 1998), *Enterobacter*

agglomerans (Chernin *et al*., 1997), and *S. marcescens* 2170 (Suzuki *et al*., 1998) into *E. coli*.

These proteins were expressed in *E. coli* to study the degradation of chitin by chitinases ChiA, ChiB, and ChiC from *S. marcescens* (Suzuki *et al*., 2002). The chitinase gene from *S. marcescens* was also expressed in *E. coli* and *Pseudomonas fluorescens* 701E1, and it was reported that the gene was expressed more efficiently in *E. coli*, as compared with *Pseudomonas* (Fuchs *et al*., 1986). The chitinase gene from *Streptomyces lividans* was cloned in *E. coli* to study induction pattern (Miyashita *et al*., 1991). They suggested the role of a 12-bp direct repeat in the induction of chitinase by chitin and its repression by glucose.

Recently, chitinase A from *Enterobacter* sp. G-1 and chitosanase A from *Matsuebacter chitosanotabidus* were cloned in the yeast *Schizosaccharomyces pombe* to study the functional expression of these enzymes and their effect on morphogenesis in *S. pombe*. In this host, chitinase was expressed inside the cells, whereas chitosanase was expressed as a secretion product (Shimono *et al*., 2002).

A number of reports are available on the cloning of chitinases either to increase biocontrol efficiency of *Bacillus thuringiensis* to prepare highly active chitinase preparation or to produce transgenic plants for increased resistance against insects. Sampson and Gooday, (1998) reported chitinolytic activities of two strains of *B. thuringiensis*. They reported enhanced virulence of *B. thuringiensis* with increased chitinase production. Two chitinases, Chi35 and Chi25, from *Streptomyces thermophilus* OPC 520 were cloned in *E. coli*, and it was reported that the polysaccharide binding domain of Chi 35 is involved in the hydrolysis of insoluble chitin and antifungal activity (Tsujibo *et al*., 2001). A family 18 chitinase gene, *chiA*, from thermophile *Rhodothermus marinus* was cloned and expressed in *E. coli*. It was reported to be the most thermostable chitinase isolated from bacteria (Hobel *et al*. 2005). In another report, two chitinase genes encoding ChiCH and ChiCW of *Bacillus cereus* 28-9 were cloned in pGEX-6P-1 and expressed in *E. coli* cells as soluble glutathione S-transferase-chitinase fusion proteins (Huang and Chen, 2005).

Such studies are essential for designing a more efficient chitinase producer and production of transgenic plants that can be used for the control of fungal and insect pathogens. Furthermore, biochemical and molecular studies could lead to a

better understanding of the chitinase secretory process and the development of cloning strategies suitable for secretion of desired products.

Protein engineering

There are three major and principally different routes to obtain enzyme variants with improved properties such as increased stability: (1) isolating enzymes from organisms living in appropriate extreme environments (e.g. Vieille and Zeikus, 2001; Schiraldi and De Rosa, 2002; van den Burg, 2003), (2) rationale-based mutagenesis (=classical protein engineering) (e.g. de Kreij *et al*., 2002; O'Fagain, 2003; Eijsink *et al*., 2004), and (3) directed evolution (e.g. Arnold *et al*., 2001; Zhao *et al*., 2002; Cherry and Fidantsef, 2003; Turner, 2003; Lutz and Patrick 2004; Robertson and Steer, 2004). Classical protein engineering utilizes information on enzyme structure and on the molecular basis of an enzyme property to rationally predict beneficial mutations, while the two other methods depend on evolution, either natural evolution or *in vitro* evolution. Several studies (e.g. Cherry *et al*., 1999; Martin *et al*., 2001; Richardson *et al*., 2002) have shown that the most efficient strategies are often based on the simultaneous use of all these three methods. Naturally occurring enzymes isolated from organisms growing in extreme environments may be a good starting point for protein-engineering efforts (van den Burg, 2003), but this approach has not been used in the present study and will hence be treated only superficially. The protein engineering efforts described in this thesis were directed towards increasing knowledge of catalysis and improving mode of catalysis. To accomplish this both rational engineering methods and directed evolution have been used.

Application of chitinases

Chitinases have shown an immense potential for increasing the production of several useful products in the most economic way. The major applications of chitinases are discussed in the following subsections.

Cytochemical localization of chitin/chitosan using chitinase chitosanase gold complexes

Chitin and chitosan are the most ubiquitous polymers of fungal cell walls. Although biochemical analysis can provide precise information about their structures, cytochemical localization studies can reveal the functional specialization of these polymers. Wheat germ agglutinin-gold complex and chitinase gold complex have been used as probes for the detection of GlcNAc residues in the secondary cell walls of plants and in pathogenic fungi (Benhamou and Asselin, 1989). Grenier *et al*., (1991) report the tagging of a barley chitosanase with colloidal gold particles for the localization of chitosan in spore and hyphal cell walls of fungi. This technique was used for the detection of chitosan in the cell walls of *Ophiostoma ulmi* and *Aspergillus niger*. Chitinase gold-labeled complexes have also been used for the immuno-cytochemical and cytochemical localization of chitin and *N*-acetyl-D-glucosamine residues in a biotrophic mycoparasite, *Piptocephalis virginiana* (Manocha and Zhonghua, 1997).

Production of single cell protein

The solid waste from shellfish processing is mainly composed of chitin, $CaCO_3$, and protein. Revah-Moiseev and Carrod, (1981) suggested the use of shellfish waste for the bioconversion of chitin to yeast single-cell protein (SCP) using chitinolytic enzymes. They used the *S. marcescens* chitinase system to hydrolyze the chitin and *Pichia kudriavazevii* to yield SCP (with 45% protein and 8–11% nucleic acids). The commonly used fungi as the source of SCP are *Hansenula polymorpha*, *Candida tropicalis*, *Saccharomyces cerevisiae*, and *Myrothecium verrucaria*. Vyas and Deshpande, (1991) utilized the chitinolytic enzymes of *M. verrucaria and S. cerevisiae* for the production of SCP from chitinous waste. The total protein content was reported to be 61%, with very low contents of nucleic acids (3.1%). Cody *et al*. (1990) suggested the enzymatic conversion of chitin to ethanol. The criteria used to evaluate SCP production are growth yield, total protein, and nucleic acid contents. The protein content in organisms used was between 39 and 73%, whereas the nucleic acid contents were 1–11%. The best reported was that of *S. cerevisiae*, which exhibited >60% proteins and 1–3% nucleic acid contents.

Isolation of protoplasts

Fungal protoplasts have been used as an effective experimental tool in studying cell wall synthesis, enzyme synthesis, and secretion, as well as in strain improvement for biotechnological applications. Since fungi have chitin in their cell walls, the chitinolytic enzyme seems to be essential along with other wall-degrading enzymes for protoplast formation from fungi. Dahiya *et al*., (2005a) reported the effectiveness of *Enterobacter sp*. NRG4 chitinase in the generation of protoplasts from *Trichoderma reesei, Pleurotus florida, Agaricus bisporus,* and *A*. *niger*. Mizuno *et al*., (1997) isolated protoplast from Schizophyl-lum commune using the culture filtrate of *B. circulans* KA-304. An enzyme complex from *B*. *circulans* WL-12 with high chitinase activity was effective in generating protoplasts from *Phaffia rhodozyme* (Johnson *et al*., 1979).

Chitinase as a target for biopesticides

Chitin is present in the exoskeleton and gut lining of insects. The molting enzyme chitinase has been described from *Bombyx mori* (silkworm), *Manduca sexta* (tobacco hawkmoth), and several other species. Similarly, chitinases have been implicated in different morphological events in fungi (Villagomez-Castro and Lopez-Romero, 1996). Allosamidin, a potent inhibitor of chitinase, was found to be inhibitory to the growth of mite (*Tetranychus urticae*) and a housefly larva (*Musca domestica*) after ingestion (Sakuda *et a*l., 1987). Chitinase inhibitors can be explored as potential biopesticides.

Estimation of fungal biomass

A variety of methods have been described to quantify fungi in soil. The techniques include direct microscopic observation and extraction of fungus-specific indicator molecules such as glucosamine ergosterol. A strong correlation has been reported between chitinase activity and fungal population in soils. Such correlation was not found for bacteria and actinomycetes. Thus, chitinase activity appears to be a suitable indicator of actively growing fungi in soil. Miller *et al*., (1998) reported the correlation of chitinase activity with the content of fungus-specific indicator molecules 18:2ωb phospholipid fatty acid and ergosterol using specific

methylumbelliferyl substrates. Similarly, chitinase and chitin-binding proteins can be used for the detection of fungal infections in humans (Laine and Lo, 1996).

Mosquito control

The worldwide socioeconomic aspects of diseases spread by mosquitoes made them potential targets for various pest control agents. In case of mosquitoes, entomopathogenic fungus such as *Beauveria bassiana* could not infect the eggs of *Aedes aegypti*, a vector of yellow fever and dengue, and other related species due to the aquatic environment. The scarabaeid eggs laid in the soil were found to be susceptible to *B. bassiana* (Ferron, 1985). *M. verrucaria*, a saprophytic fungus, produces a total complex of an insect cuticle-degrading enzyme (Shaikh and Desphande, 1993). It has been seen that both first and fourth instar larvae of mosquito *A. aegypti* can be killed within 48 h with the help of the crude preparation from *M. verrucaria* (Mendonsa *et al*., 1996). Though 100% mortality was observed within 48 hr, purified endochitinase lethal times (LT50) were 48 and 120 h for first and fourth instar larvae, respectively. However, the time period was found to be decreased, corresponding to 24 hr and 48 hr, when the purified chitinase was supplemented with lipolytic activity.

Morphogenesis

Chitinases play an important role in yeast and insect morphogenesis. Kuranda and Robbins, (1991) reported the role of chitinases in cell separation during growth in *S. cerevisiae*, and Shimono *et al*., (2002) studied the functional expression of chitinase and chitosanase and their effects on morphogenesis in the yeast *S. pombe*. When the *chiA* gene was expressed in *S. pombe*, yeast cells grow slowly and cells become elongated, but when the *choA* gene was expressed, cells become swollen. Expression of both *chiA* and *choA* genes resulted in elongated and fat cells.

Medical application

Chitinases can be employed in human health care, such as making ophthalmic preparations with chitinases and microbiocides. A direct medical use has been suggested for chitinases in the therapy for fungal diseases in potentiating the activity of antifungal drugs (Pope and Davis, 1979; Orunsi and Trinci, 1985). They can also

be used as potential additives in antifungal creams and lotions due to their topical applications.

Control of plant pathogenic fungi

Biological control or the use of microorganisms or their secretions to prevent plant pathogens and insect pests offers an attractive alternative or supplement for the control of plant diseases. Therefore, biological control tactics have become an important approach to facilitate sustainable agriculture (Wang *et al*., 2002).

Chitin application increased the population of chitinolytic actinomycetes, fungi, and bacteria. The increase is shown to be correlated with the reduction in pathogenic fungi and nematodes and, more importantly, with the reduction of infectivity and, hence, crop damage (Wang *et al*., 2002). A biological control agent of fungal root pathogen should exert a sufficient amount of antagonistic activity.

The chitinase produced by *Enterobacter* sp. NRG4 was highly active toward *Fusarium moniliforme*, *A. niger*, *Mucor rouxi*, and *Rhizopus nigricans* (Dahiya *et al*., 2005a). The chitinase from *Alcaligenes xylosoxydans* inhibited the growth of *Fusarium udum* and *Rhizoctonia bataticola* (Vaidya *et al*., 2001).

Mahadevan and Crawford, (1997) reported the antagonistic action of *Streptomyces lydicus* WXEC108 against *Pythium ultimum* and *Rhizoctonia solani*, which cause disease in cotton and pea. Horsch *et al*., (1997) suggested the use of *N*-acetylhexosaminidase as a target for the design of low molecular weight antifungals. Chitinases can be added as a supplement to the commonly used fungicides and insecticides not only to make them more potent but also to minimize the concentration of chemically synthesized active ingredients of the fungicides and insecticides that are otherwise harmful to the environment and health. Bhushan and Hoondal, (1998) studied the compatibility of a thermostable chitinase from *Bacillus* sp. BG-11 with the commonly used fungicides and insecticides.

A *Fusarium chlamydosporum* strain, a mycoparasite of groundnut rust (*Puccinia arachidis*), produces endochitinase that inhibits germination of uredospores of rust fungus (Mathivanan *et al*., 1998). Chitinolytic enzymes of *T. harzianum* were found to be inhibitory to a wide range of fungi than similar enzymes from other sources (Lorito *et al*., 1993). Govindsamy *et al*., (1998) reported the use of purified preparation of *M. verrucaria* chitinase to control groundnut rust, *P. arachidis*.

Penicillium janthinellum P9 caused mycelial damage in *Mucor plumbus* and *Cladosporium clados*poriodes (Giambattista *et al*., 2001). Partially purified chitinase from *T. harzianum* destroys the cell wall of *Crinipellis perniciosa*, the casual agent of witches' broom disease of cocoa (DeMarco *et al*., 2000). Chitinase from *B. cereus* YQ 308 inhibited the growth of plant pathogenic fungi such as *Fusarium oxysporium*.

Production of chitooligosaccharides, glucosamine, and NAG

Chitooligosaccharides, glucosamines, and NAG have an immense pharmaceutical potential. Chitooligosaccharides are potentially useful in human medicines. For example, chitohexaose and chitoheptaose showed antitumor activity. A chitinase from *Vibrio alginolyticus* was used to prepare chitopentaose and chitotriose from colloidal chitin (Murao *et al*., 1992). A chitinase preparation from *S. griseus* was used for the enzymatic hydrolysis of colloidal chitin. The chitobiose produced was subjected to chemical modifications to give novel disaccharide derivatives of 2-acetamido 2-deoxy D-allopyranose moieties that are potential intermediates for the synthesis of an enzyme inhibitor, that is, *N*, *N*′-diacetyl-β-chitobiosyl allosamizoline (Terayama *et al*., 1993).

Specific combinations of chitinolytic enzymes would be necessary to obtain the desired chain length of the oligomer. For example, the production of chitooligosaccharides requires high levels of endochitinase and low levels of *N*-acetylglucosaminidase and exochitinase, whereas the production of GlcNAc requires higher proportion of exochitinase and *N*-acetylglucosaminidase (Aloise *et al*., 1996). Alternatively, transglycosylation activity of a variety of endochitinases and *N*-acetylglucosaminidases will also be useful to generate desired chitooligomers, oligomers with changed glycosidic linkages and glycopeptides.

Nanjo *et al*., (1989) observed the accumulation of hexamer when tetramer or pentamer was incubated with *Nocardia orientalis* chitinase. A chitinase from *T. reesei* also exhibited a similar type of efficient transglycosylation reaction. They reported the accumulation of hexamer and dimer as the major product when the enzyme was reacted with tetramer (Usui *et al*., 1990). They also observed a chain elongation from dimer to hexamer and heptamer using lysozyme catalysis in the presence of 30% ammonium sulfate in a buffered medium. Chi-26 from *Streptomyces kurssanovii*

showed the accumulation of hexamer in the reaction mixture containing tetramer and pentamer (Stoyachenko *et al*., 1994).

The transglycosylation reaction of *Mucor hiemalis* endo-β-*N*-acetyl glucosaminidase was used for the preparation of sugar derivatives modified at C-1 or C-2 for the synthesis of glycopeptides (Yamanoi *et al*., 2004).

A chitinolytic enzyme preparation from *N. orientalis* IFO12806 was used for the preparation of GlcNAc from chitooligosaccharides (Sakai et al. 1991). Crude bacterial chitinases from *Burkholderia cepacia* TU09 and *B. licheniformis* SK-1 were used for the hydrolysis of α-chitin (from crab shells) and β-chitin (from squid pens) to produce NAG (Pichyangkura *et al*., 2002). Sashiwa *et al*., (2002) produced NAG from α-chitin using crude chitinolytic enzymes from *Aeromonas hydrophila* H-2330.

Preparation of Chitin Oligomers by Enzymatic Hydrolysis

In contrast to chemical hydrolysis, enzymatic hydrolysis of chitin and chitosan by chitinase, chitosanase, lysozyme, and cellulase has several benefits to produce more readily available oligomers with a high DP with milder reaction conditions. Uchida *et al*., 1989 explained that the enzymatic hydrolysis was a useful method for the preparation of the oligomers from chitin and chitosan because the yield of high DP oligomers was greater in enzymatic hydrolysis than in acid hydrolysis. Chitin and chitosan oligomers may therefore be prepared by enzymatic means, as shown in Figure 1.9.

Chitin may be degraded via enzymatic hydrolysis by lysozyme and chitinase. Lysozyme hydrolyzes partially *N*-acetylated chitosans (PNACs) under homogeneous conditions. The lysozyme digestibility of PACNs increases with the increase of the degree of *N*-acetylation of PNACs because lysozyme recognizes GlcNAc sequence with more than three residues (Aiba, 1994). Chitinases are found in bacteria, fungi, plants, and insects, but not in most mammals except for cows, goats, and sheep. Therefore, chitin is thought to be degraded mainly by lysozyme in most mammalian animals (Takamori *et al*., 1996). Chitinase and β-*N*-acetylglucosaminidase are the enzymes from bacteria which are involved in the degradation of chitin. Most chitinases from bacteria are of the endo-type and produce oligomers more than dimer, NAG_2, thus chitinase is often used conveniently to produce oligosaccharides. β-*N*-

Acetylglucosaminidase is involved in hydrolytic action of *N*-acetyl-chitooligosaccharides or NAG_2 as substrates and releases free *N*-acetylglucosamine.

Although many kinds of chitinase-producing microorganisms have been isolated, little is known about enzymes which are able to specifically produce the oligomers with high DP. Takiguchi and Shimahara, 1989) reported that the production of only dimer with enzyme from a thermophilic bacterium. Takayanagi *et al*., (1991) reported that four kinds of thermostable chitinases isolated from the cell-free culture broth of *Bacillus licheniformis* X-7u produced $(GlcNAc)_2$ or GlcNAc. Mitsutomi *et al*., (1995) revealed that chitinase A1 and D from *Bacillus circulans* WL-12 specifically hydrolyzed the *N*-acetyl-β-D-glucosaminidic bonds in a 50% *N*-acetylated chitosan to produce heterooligosaccharides with GlcNAc at the reducing end residue and heterooligosaccharides with DP2 or 3 were produced as major hydrolysis products. Ohtakara *et al*., (1991) and Mitsutomi *et al*., (1990) also reported that main oligomers produced during the course of hydrolysis of partially *N*-acetylated chitosan by chitinase from *Streptomyces griseus* and *Aeromonas hydrophila* were heterochitooligosaccharides with 2 to 4 residues. In acting modes of most chitinolytic enzymes from microorganisms, chitin is cut randomly by chitinase in the solid state and then higher oligomers produced are immediately hydrolyzed by the enzyme in the solution. Such behavior of the enzyme is not good for preparation of higher oligomers (Aiba and Muraki, 1996). Recent studies on enzymatic transglycosylation have revealed production of higher oligomers, such as hexamer and heptamer, from lower oligomers. Usui *et al*., (1987) found that transferase activity of a chitinase purified from Nocardia orientalis IFO 12806 could be used for the preparative scale synthesis of NAG_6 and NAG_7 from NAG_4 and NAG_5, respectively. Nanjo *et al*., (1990) found a chitinase from *N. orientalis*, which is essentially a hydrolase that could catalyze a transglycosylation reaction in an excess of NAG_4 as an initial substrate and described an effective synthesis of NAG_6 from NAG_4 by the transferase activity of the enzyme in the presence of ammonium sulfate. Similarly, Usui *et al*., 1987.

Application of chitin and chitosan oligomers

Chitin, chitosan, and their oligomers have much functionality, especially physiological activities. Their functions have led to progressively increased utilization

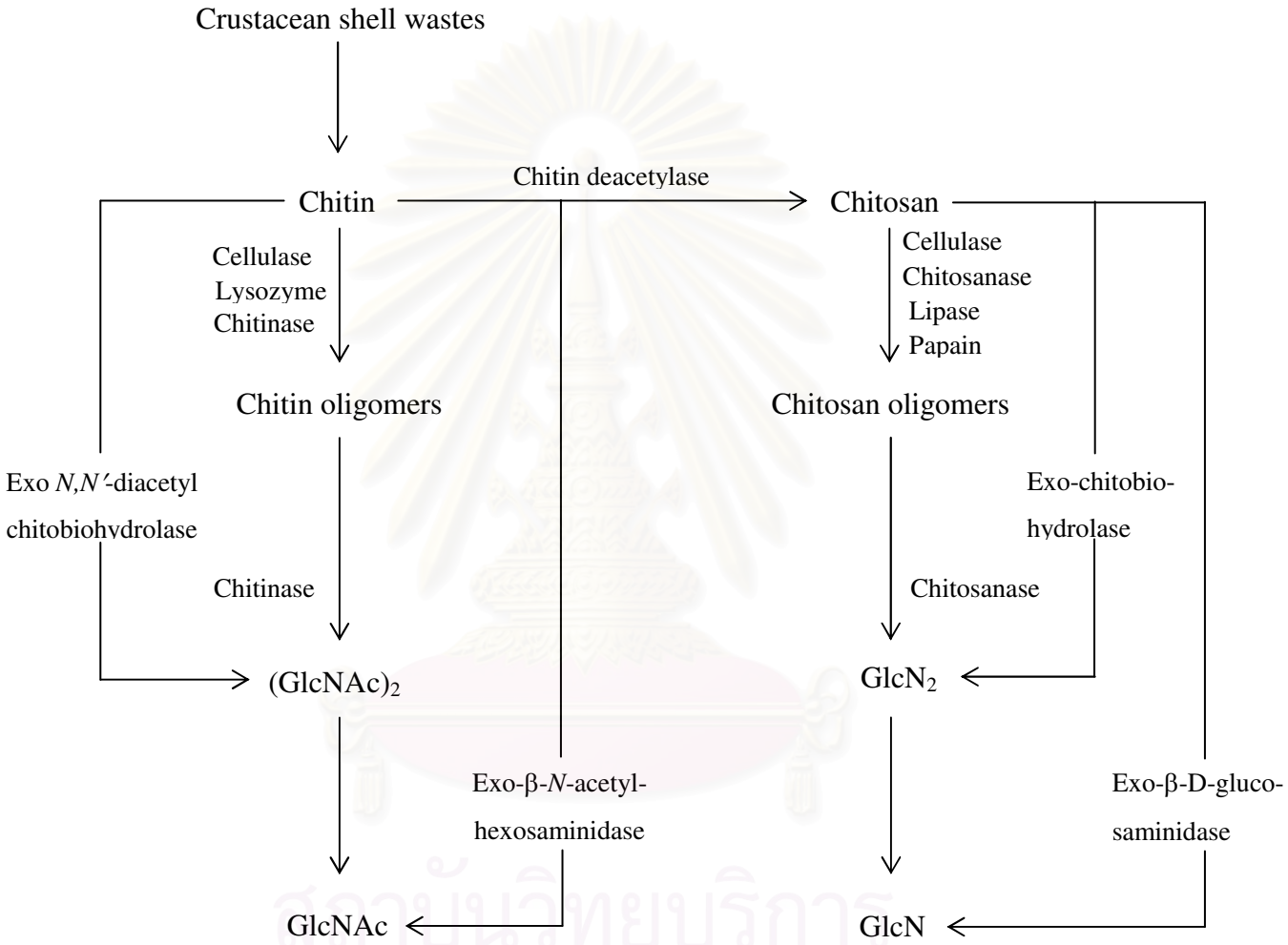


Figure 1.9 The pathway for the conversion of chitin and chitosan into their oligomers by enzymatic means.

in the food and pharmaceutical fields for human health. The functional properties of chitin and chitosan oligomers have clearly revealed their high dependency on DP (Hirano and Nagao, 1989; Suzuki, 1996). The high-DP oligomers from pentamer to heptamer have been reported to possess better functional characteristics compared with those of relatively low DP (Kendra and Hadwigr, 1984).

The functional properties of chitin and chitosan and their oligomers, antibacterial activity, antitumor activity, protective effect on bacterial infection in vivo, hypocholesterolemic effect, and calcium absorption acceleration effectin vivo are discussed.

Antibacterial effect

Studies on chitin and chitosans have already revealed inhibition of growth of several fungi and microbacteria, especially phytopathogens (Uchida *et al*., 1989). Chitosan also induced a plant-defense enzyme, chitinase, in plant tissues which degrades fungal cell walls (Hirano and Nagao, 1989), induces the accumulation of the antifungal phytoalexin pisatin in pea pods (Walker-Simmons *et al*., 1983), and elicites the antimicrobial phytoalexin and pisatin in pea pods (*Pisum sativum*) (Hadwiger and Beckman, 1980). Allan and Hadwiger, (1979) reported that chitosan has a higher antifungal activity than chitin because positively charged amino groups on chitosan inhibit the growth of fungi or microbacteria by polyelectrolyte complexes with negatively charged carboxyl anion groups present in their cell walls (Young and Kauss, 1983). Furthermore, chitosan oligomers have been shown to inhibit the growth of several phytopathogens (Uchida *et al*., 1989; Yamada *et al*, 1993). Hirano and Nagao, (1989) studied the relationship between the degree of polymerization of chitosan and the degree of pathogen inhibition. They showed that chitosan oligomers (DP 2-8) as well as partially hydrolyzed chitosan with a low molecular weight possessed stronger growth inhibition than high molecular weight chitosan against several phytopathogens including *Fusarium oxyporum*, *Phomopsis fukushi*, and *Alternaria alternata*, among others. Kendra *et al*., (1989) explained that some chitosan oligomers with biological activity present in pea/*Fusarium* interactions appear to inhibit fungal growth. Uchida *et al*., (1989) found that oligomers with a higher molecular weight, which were slightly hydrolyzed with chitosanase, were more active in both antifungal and antibacterial activities than native chitosan and lower

molecular weight oligomers. With respect to antimicrobial activity of partially hydrolyzed chitosan oligomers, Jeon and Kim, (1998) reported that the highest molecular weight oligomers (MW 5,000-10,000 Da), among the three fractions of oligomers produced and separated using the ultrafiltration membrane enzymatic reactor system, showed the strongest bactericidal and fungicidal activities against most pathogens tested. Ueno *et al*., (1997) also reported that chitosan oligomers with a MW of less than 2000 Da did not easily suppress microbial growth while an oligomer with MW 9300 Da almost completely suppressed microbial growth at a very low concentration. On studies of modified oligomers for antibacterial activity, Muraki and Aiba (1996) reported that partially *N*-lauroyl (PNL)-chitooligosaccharides (DP 7-8), PNL-$(GlcN)_7$ and PNL-$(GlcN)_8$, with a degree of *N*-lauroylation of about 50% had a fairly strong antibacterial activity against the growth of *E. coli*, compared to all $(GlcN)_n$ and PNL-$(GlcN)_n$ with a chain length of less than seven residues.

On the other hand, higher plants have various defense reactions which are the production of phytoalexins, enzymes such as chitinase and β-glucanase, proteinase inhibitors, hydroxyproline-rich glycoproteins, proteinase active oxygen species, and lignification, when they are attacked by pathogens such as fungi, bacteria, and viruses (Yamada *et al*., 1993). Elicitors have been shown to induce these defense systems in plants and contributed to an increase in the yield of secondary products in various plant cell cultures (Murak and Aiba, 1996). Chitin, chitosan, and their oligomers have been reported to exhibit elicitor activities toward several plants, and have been widely used as elicitors for the induction of secondary products in plant cell cultures (Akiyama *et al*., 1995; Brodelius *et al*., 1989; Darvill *et al*., 1992; Hadwiger *et al*., 1994). Chitin oligomers were active as elicitors of defense reactions in higher plants, whereas chitosan oligomers had almost no eliciting activity (Yamada *et al*., 1993; Vander *et al*, 1998). Hadwiger *et al*., (1994) reported, however, that higher chitosan oligomers like octamer were efficient elicitors by inducing pisatin accumulation and inhibiting of fungal growth. These results suggest that elicitor activities of chitin and chitosan oligomers are highly dependent on their polymerization and presence of *N*-acetylglucosamine in the structures. The above definition was supported by the study of Vander *et al*., (1998) who reported that chitin oligomers with DP7 or more elicited

peroxidase activity while chitosans with intermediate degrees of acetylation elicited both phenylalanine ammonia-lyase and peroxidase activities.

Antitumor effect by immuno-enhancing function

In the studies of an antitumor agent, Suzuki, (1996) demonstrated that chitin and chitosan oligomers inhibit the growth of tumor cells by immuno-enhancing effects. Suzuki *et al*., (1985) also revealed that chitin oligomers from $(GlcNAc)_4$ to $(GlcNAc)_7$ display strong attracting responses to peritoneal exudate cells in BALB/c mice, whereas chitosan oligomers from $(GlcN)_2$ to $(GlcN)_6$ did not show this effect. For the antitumor effect of chitin and chitosan oligomers with hexamers, $(GlcNAc)_6$ and $(GlcN)_6$, respectively, Suzuki *et al*., (1986) found that the growth-inhibitory effect of both oligomers against allogenic and syngeneic mouse system, including sarcoma 180 solid tumor and MM46 solid tumor, respectively, were pronounced. These results indicated that the effect was not by direct cytocidal action on the tumor cells and was indeed host-mediated. Tokoro *et al*., (1988) showed that the two oligosaccharides $(GlcNAc)_6$ and $(GlcN)_6$ had growth-inhibitory effect against Meth-A solid tumor transplanted into BALB/c mice. The antitumor mechanism was assumed to involve increased production of lymphokines including interleukins 1 and 2, sequentially, leading to the manifestation of antitumor affect through proliferation of cytolytic T lymphocytes. Tsukada *et al*., (Tsukada *et al*., 1990) reported significant antimetastatic effects of $(GlcNAc)_6$ in mice bearing Lewis lung carcinoma. Suzuki *et al*., (1992) analyzed the change of the spleen cells from tumor-bearing mice administered with chitooligosaccharide such as $(GlcNAc)_6$ to unravel the mechanism of inhibition of tumor cell growth by immuno-enhancing effects. It was demonstrated that increase of cytotoxic T-lymphocytes activity by accelerating the differentiation of helper T-cells was remarkable and accompanied a decrease of suppressor T-cell activity.

Protective effect on bacterial infection *in vivo*

Partially deacetylated chitin, especially 70% deacetylated chitin (DAC-70), has been shown to stimulate nonspecific host resistance against *E. coli* and Sendai virus infections in mice (Nidhimura *et al*., 1984; Iida *et al*., 1987). The effect in mice administered with DAC-70 could be explained as activities of an immune adjuvant of DAC-70 which could activate peritoneal macrophages and natural killer cells, and

enhance antibody production and delayed-type hypersensitivity in guinea pigs and cell-mediated cytotoxicity in mice, and induce cytokines such as interleukin-1, colony-stimulating factor and interferon in mice (Nidhimura *et al*., 1984; Nidhimura *et al*., 1985; Nidhimura *et al*., 1986). Chitin and chitosan also showed protecting effects on *Candida albicans* infection by elevating the number of mouse peritoneal exudate cells which generated reactive oxygen intermediates and then displayed candidacidal activities (Suzuki *et al*., 1984). Suzuki *et al*., (1987) reported, in comparison of candidacidal activity between chitin and chitosan, that the activity in the culture supernatant of macrophage from chitin-treated mice was significantly increased, while that from chitosan treated mice did not show any activity. However, chitin, chitosan, and partially deacetylated chitin are water insoluble and their nature is disadvantageous for their use as immunotherapeutic agents in vivo. On the effects of water-soluble chitin and chitosan oligomers, Suzuki *et al*., (1986) demonstrated that chitin hexamer, $(GlcNAc)_6$, possessed a strong candidacidal activity. Tokoro *et al*., (1989) found that chitin hexamer exerted strong growth-inhibitory effect on *Listeria monocytogenes* by elevating the function of cellular immunity. However, there is no report on protective effect of chitosan oligomers on bacterial infection *in vivo*. As described in the antimicrobial effect section of this review, chitosan oligomers were superior to chitin oligomers in their antibacterial activity, while chitin oligomers were better in their protective effect on bacterial infection *in vivo* because of their immunopotentiating activity. These facts suggest that *N*-acetylglucosamine units of the polysaccharides deeply influence and stimulate the immune system.

Hypocholesterolemic effect

It is well-known that chitosan has a significant hypocholesterolemic activity in various experimental animals (Murano *et al*., 1978 Kobayahi *et al*., 1979; Sugano *et al*., 1980; Sugano *et al*., 1988; Hirano *et al*., 1990) while high-cost hydrolyzed chitosan oligomers did not exhibit a cholesterol-lowering activity (Sugano *et al*., 1988). However, chitosan is too highly viscous to be used in physiological and functional foods. Therefore, the relationship between the cholesterol-lowering effect and the average molecular weight of chitosan needs to be established. Sugano *et al*., (1988) studied rat groups fed on a cholesterol-enriched diet in order to study the hypocholesterolemic activity of chitosan hydrolysates with different molecular

weights and viscosity. They demonstrated that the cholesterol-lowering potential of the partial hydrolysates with molecular weights of 8,000 and 20,000 Da was equal to or greater than that of the parent chitosan with a molecular weight of approximately 300,000 Da.

Calcium absorption acceleration effect *in vivo*

Jeon and Kim, (1997) studied the *in vivo* calcium absorption acceleration effect by chitosan oligomers and revealed that chitsoan oligomers from trimer to heptamer lowered fecal calcium excretion and enhanced the breaking force of femur in rats. In the case of chitosan intake for mineral absorption *in vivo*, Wada *et al*., (1997) reported that whole-body retention of radioactive calcium (^{47}Ca) by rats fed a 1-5% chitosan diet was significantly decreased when compared with that of rats fed a cellulose diet. Consequently, dietary chitosan affected calcium metabolism in animals. Deuichi *et al*., (1995) also noted that chitosan feeding for 2 weeks caused a decrease in mineral absorption and bone mineral content in rats. Therefore, while chitosan decreases calcium absorption, chitosan oligomers enhance the absorption of calcium and other minerals *in vivo*.

The aim of this study

Chitinases from different bacteria have different functional domains and domain rearrangements. These functional domains may influence the mechanism of how different chitinases hydrolyzes chitin and also the size of the product these enzymes produce. The chitinase from *Serratia liquefaciens* TU09, Chi60, contains an *N*-terminal domain which can interact with chitin, produces dimer of GlcNAc as a major product (Kamonthip, 2001). However, chitinase from *Bacillus licheniformis* SK-1, ChiA1, which has a chitin binding domain at *C*-terminal, produces a mixture of monomer and dimer of NAG (Sanya, 2001). These functional domains have been shown to be involved in concentrating the enzyme on the chitin substrate. It has also been shown to have a role in guiding of the substrate into the catalytic cleft (Watanabe *et al*., 2001). Previous results from our laboratory and results from Watanabe *et al*., (2001) implies that the product size resulting from the hydrolysis of chitin by chitinase was determined by how far the chitin chain slides through the catalytic site and the minimum number of GlcNAc residues proceeding the catalytic

center necessary for hydrolysis. The functional domains play a role in guiding of the substrate into the catalytic cleft.

In the study, I proposed to production of *N*-acetylglucosamine and/or *N*-acetylchitooligosaccharide. Firstly, I used the enzymes cloned in our laboratory such as chitinase 60 and chitinase B from *Serratia* sp. and chitinase A1 from *Bacillus licheniformis* SK-1 for screening for *N*-acetylchitooligosaccharide production. The second, I used genetic engineering and protein engineering techniques to modified bacterial chitinase such as chitinase A1 from *Bacillus licheniformis* SK-1 to produce the truncated derivatives and modified enzymes by addition some functional domain. Lastly, I used homologous recombination techniques by using recombination bacteria host to construct the homologous recombinant chitinase between chitinase 60 and chitinase B from *Serratia* sp. Finally, I used some modified chitinases to produce *N*-acetylglucosamine and/or *N*-acetylchitooligosaccharide.

CHAPTER II

MATERIALS AND METHODS

Equipments

Autoclave: Model H-88LL, Kokusan Emsinki Co., Ltd., Japan

Autopipette: Pipetteman, Gilson, France

Automated laser fluorescence DNA sequencer, Model CEQ8000, Beckman

Centrifuge, refrigerated centrifuge: Model J2-21, Beckman Instrument, Inc., U.S.A.

Centrifuge, microcentrifuge: Model MC-15A, Tomy Seiko Co., Ltd., Japan

Electrophoresis unit: 2050 MIDGET, LKB, Sweden

Mini protein, Bio-Red, U.S.A.; Submarine Agarose Gel Electrophoresis unit

Incubator: Model 1H-100, Gallenkamp, England

Incubator shaker: Model G-76, New Brunswick Scientific Co.,I nc., U.S.A.

High performance liquid chromatography: Shimadzu, Japan

Incubator, waterbath: Model M20S, Lauda, Germany

Magnetic stirrer: Model Fisherbrand, Fisher Scientific, U.S.A.

pH meter: Model PHM95, Radiometer Copenhegen, Denmark

Spectrophotometer: Spectronic 2000, Bausch&Lomb, U.S.A.

Spectrophotometer UV-240, Shimadsu, Japan, and DU Series 650, Beckman, U.S.A.

Sequencer: Model CEQ^{TM}8000 Genetic Analysis system, Beckman Coulter, U.S.A.

Thermolyne dri-bath: Sybron corporation, U.S.A.

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

Water bath: Charles Hearson Co. Ltd., England

Chemicals

Acetonitrile (HPLC grade), Merck, Germany

Acetone: Merck, Germany

Acrylamide: Merck, U.S.A.

Agarose: GIBCOBRL, U.S.A.

Aqua sorb: Fluka, Switzerland

Ammonium persulphate: Sigma, U.S.A.

Ampicilin: Sigma, U.S.A.

Aniline: Merck, Germany

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

β-mercaptoethanol: Fluka, Switzerland

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

Bovine serum albumin: Sigma, U.S.A.

Bromophenol blue: Merck, U.S.A.

CEQ^{TM} DTCS-Quick Start Kit: Beckman Coulter^{TM}, U.S.A.

Charcoal, activated: Sigma, U.S.A.

Chloroform: BDH, EnglandCongo Red: Sigma, U.S.A.

Coomasie brilliant blue R-250: Sigma, U.S.A.

Dialysis Tube: Sigma, U.S.A.

Diphenylamine: BDH, England

di-Potassium hydrogen phosphate anhydrous: Carlo Erba Reagenti, Italy

di-Sodium ethylenediaminetetra acetate: M&B, England

DNA marker: Lamda (λ) DNA digest with *Hind* III: GIBCOBRL, U.S.A.

85% Phosphoric acid: Mallinckrodt, U.S.A.

Ethidium bromide: Sigma, U.S.A.

Ethyl alcohol absolute: Carlo Erba Reagenti, Italy

Fluorescent Brightener 28: Sigma, U.S.A.

Glacial acetic acid: Carlo Erba Reagenti, Italy

Glycine: Sigma, U.S.A.

Isopropyl-1-thio-β-D-galactopyranoside (IPTG): Sigma, U.S.A.

Magnesium sulphate-7-hydrate: BDH, England

Methanol: Merck, Germany

N-acetyl-D-glucosamine: Sigma, U.S.A.

N, *N*′-methylene-bis-acrylamide: Sigma, U.S.A.

NNN′N′- Tetramethyl-1, 2-diaminoethane: Carlo Erba Reagenti, Italy

Phenol: BHD, England

85%Phosphoric acid: Lab Scan, Ireland

Potassium acetate: Merck, Germany

Potassium ferricyanide: BDH, England

Potassium phosphate monobasic: Carlo Erba Reagenti, Italy

Qiaquick Gel Extraction Kit: Qiagen, Germany

Shrimp shell Chitin and squid pen chitin: Ta Ming Enterprises Co., Ltd, Samutsakon, Thailand

Silica gel plate (Kieselgel 60): Merck, Germany

Sodium carbonate anhydrous: Carlo Erba Reagenti, Italy

Sodium citrate: Carlo Erba Reagenti, Italy

Sodium chloride: Carlo Erba Reagenti, Italy

Sodium dodecyl sulfate: Boehringer Mannheim Gmbtt, Germany

Sodium hydroxide: Merck, Germany

Standard molecular weight marker protein: New England BioLabs, Inc., U.S.A.

ThermoSequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-dGTP, Amersham Pharmacia Biotech, England

Tris (hydroxymethyl)-aminomethane: Carlo Erba Reagenti, Italy

TritonX-100: Merck, Germany

Tryptone: Scharlau, Spain

2, 7-Diamino-10-ethyl-9-phenyl-phenanthridiniumbromide: Sigma, U.S.A.

Yeast extract: Scharlau, Spain

Enzymes and restriction enzymes

Lysozyme: Sigma, U.S.A.

Proteinase K: Sigma, U.S.A.

Restriction Enzymes: GIBCOBRL, U.S.A. and New England BioLabs,Inc., U.S.A.

RNase: Sigma, U.S.A.

T4 DNA ligase: New England BioLabs, Inc., U.S.A.

List of DNA oligonucleotides

Chi60

5’-CTCGGATCCAATCTACAACCGGCT-3’ cloning

5’-TTTGCCCATGGCTGATTCCTTTATTC-3’ cloning

5’-GCTCCGATCCAATCTACAACCG-3’ cloning

5’-CAGGCTGGCGTTCATGCTGT-3’ cloning

ChiA1

5’-GTTTTCCCTTGTTGTCTTC-3’cloning

5’-CTCTTTATCGTTTTCTATCC-3’cloning

5’-GTAAAGGCCATGGAAATCGTGTTGAT-3’ cloning

5’-CGTTTTGGATCCTATTGACTTTCTCTT-3’ cloning

5’-CGGGATCCGTCCGGTGCAAAATCTTAATCG-3’ FnIII&ChB deletion

5’-CGGGATCCGGCATGGAGCTTTCAATCC-3’ FnIII deletion

5’-CATGCCATGGAAATCGTGTTGATCAAC-3’ expression

5’-CGGGATCCGTAGCGATACATTTCAC-3’ expression

5’-GTTTTGGATCCGTTTGACTTTCTC-3’ FnIII&ChB addition

5’-GATTTTGCACCGGACGGAGG-3’ FnIII&ChB addition

ChiB

5’-CAAGAAAAACAGCACCAGCAG-3’ cloning

5’-GCGGCACGGCGACTCCAC-3’ cloning

5’-CATGCCATGGCCACACGCAAAGCCG-3’ expression

5’-CCGCTCGAGTTTACGCTACGCGGCC-3’ expression

5’-GCCCAACGTTATTGGGTATTATTTTATTC-3’ subcloning

5’-CCGCTCGAGTTTACGCTACGCGGCC-3’ subcloning

Bacterial strains

Escherichia coli BL-21 (DE3) with genotype *F- ompT hsdS(rBmB) gal dcm (DE3)* was used for ChiA1 expression.

E.coli BL-21 (DE3) with genotype *F- ompT hsdS(rBmB) gal dcm (DE3) pLysS (CmR)* was used for ChiB expression.

E.coli Top10 with genotype *F′, ∅80δ lacZΔM15, Δ(lacZYA-argV169), endA1, recA1, hsdR17 (rK-mK+), deoR, thi-1, supE44, λ-gyrA96, relA1* (Dower, 1990) was used to construction and production of plasmid carrying the wild-type *chiA* gene and the *chiA* gene with various mutations.

E. coli JC8679 with genotype *F¯, thr-1, leu-6, thi1, lacY1, galK2, ara14, sy15, mtl1, proAZ, jos4, argE3, str31, tsx33, supE44. λ¯, recB21, recC22, abcΔ23* was used for homologous recombination.

Plasmids

pBluescript SK$^-$ (Stratagene) was used as a cloning vector and carrying the *chi60* gene (pKKchi60) and expression of ChiA1 and derivatives (see Appendix A).

pGEM® -T Easy (Promega) was used for cloning PCR fragments into *E. coli* (see Appendix A).

pET19b (+) was used as overexpression for chitinase genes (see Appendix A).

Plasmid constructions

pGSKchiA1

pGSKchiB

pETSKchiA1

pETSKchiB

pP60SKchiA1

pP60SKchiA1ΔChBD

pP60SKchiA1ΔFnIIIDΔChBD

pSKchiA1-60N

pSKchiA1-60NΔFnIIIDΔChBD

pSKchi60-A1FnIIID&ChBD

pSKchi60B

pRecSK-1

Media preparation

Luria-Bertani (LB) medium (Maniatis *et al*., 2001)

LB consisted of 1.0% tryptone, 0.5% yeast extract and 0.5% NaCl; pH was adjusted to pH 7.2 with NaOH. For solid medium, 2% agar was added. Medium was steriled by autoclaving at 121°C for 15 min.

Colloidal chitin minimum medium (CCMM)

Medium for cultivation and screening of enzyme production contained 0.2% colloidal chitin (wet weight), 0.05% yeast extract, 0.1% $(NH_4)_2SO_4$, 0.03%

$MgSO_4.7H_2O$, 0.6% KH_2PO_4 and 1% K_2HPO_4 with pH 7.2. For solid medium, 1.5 - 2% agar was added. Medium was sterilized as described above.

Cultivation of bacteria

Starter inoculum

A colony of ChiA1 and its derivatives were grown in 2 mL of LB medium at 37°C and a colony of *E. coli* carrying pBSSK$^-$ and its derivatives were grown of LB medium containing 100 μg/mL ampicillin at 37°C for 12-16 hr.

Culture conditions

ChiA1 and its mutants were produced in *E. coli* Top10 cells with carrying the plasmid pP60SKchiA1 or its derivatives. Starter culture condition of *E. coli* contained chitinase gene (*chiA*) was diluted to 1: 100 into 100 mL of LB containing 100 μg/mL ampicillin in 250 mL Erlenmeyer flask. The culture was incubated at 37°C for chitinase production and chromosomal DNA extraction. Cells were collected by centrifugation at 8,000 g for 10 minutes at 4°C. For chitinase production, culture broth with crude chitinase enzyme was concentrated by VIVA (50) flow (10,000 cut off). Concentrated crude chitinase enzyme was kept at 4°C for further characterization.

Enzyme assay

In chitinase assay, reducing end groups generated by the degradation of various chitinous substrate such as soluble chitin, colloidal chitin and crystalline chitin (see Appendix B) were measured colorimetrically with ferric cyanide reagent by a modification of the Schales' procedure (Imoto and Yagishita, 1971) using GlcNAc as the standard.

Chitinase activity

Chitinase activity was assayed by measuring reducing sugar produced from a mixture containing 1 mg/mL of colloidal chitin and the desired amount of enzyme in 0.1 M citrate buffer pH 6.0 (750 μL). The mixture was incubated at 37°C for 30 min.

The reaction was stopped by adding 1.0 mL color reagent (made by dissolving 0.5 g of potassium ferric cyanide in 1 L of 1.5 M Na_2CO_3) and heated to 100°C for 15 min. Small particles were removed from the mixture by centrifugation at 3,000g for 10 min. The absorbance of the sample (A1) at 420 nm was measured by a spectrophotometer versus water. A blank value (A0) was obtained when denatured enzyme was used instead of the enzyme in the reaction. The different between A0 and A1 was used to estimate the amount of *N*- acetylglucosamine from standard curve. One unit (U) of enzyme activity was defined as the amount of an enzyme able to liberate 1 μmol product (as *N*-acetylglucosamine equivalent) per minute (see Appendix C).

Chitobiase or *N*-acetylglucosaminidase activity

Chitobiase or *N*-acetylglucosaminidase activity was quantitatively measured by detecting the amount of p-nitrophenol, a product of enzymatic hydrolysis when p-nitrophenol-*N*-acetylglucosamine was used as substrate. The enzyme assay was performed as described in the following. A 100 μl of appropriate diluted enzyme solution was added to 100 μl of 2.5 mM p-nitrophenol-*N*-acetylglucosamine, 50 μL of 1 M citrate buffer pH 5.0 (final 0.05 M) and was adjusted volume to 0.5 mL with distilled water. After incubation at 50 °C for 30 min, one milliliter of Na_2CO_3 was added, stand for 5 min at room temperature then measured at 420 nm. The standard curve for p-nitrophenol was showed (see Appendix C).

Protein concentration determination

Protein concentration was determined by dye binding method (Bradford, 1976), using bovine serum albumin as a standard. Eight hundred microliter of sample was mixed with 200 μL of Bradford working solution (5x) and left for 20 minutes before measuring the absorbance at 595 nm. Bradford working solution (5x) contains 100 mg Coomassie Brilliant Blue G-250, 50 mL of 95% ethanol, 100 mL of 85% phosphoric acid and 50 mL of distilled water (see Appendix D).

Recombinant DNA techniques

All basic recombinant DNA techniques such as, plasmid preparation, ligation, transformation and dideoxynucleotide sequencing were carried out using standard protocols (Sambrook and Russell, 2001).

Selection of domain for study

Construction of three-dimensional structure

Three-dimensional structure (3D-structure) was constructed by homology modeling program from SWISS-MODEL (Schwede *et al*., 2003, http://swissmodel.expasy.org) that is a server for automate comparative modeling of three-dimensional protein structure. Protein sequence of chitinase A1 from *B. licheniformis* SK-1 was submitted to the SWISS MODEL using protein of chitinase A1 from *B. circulans* WL-12 deposited in protein data bank (accession number 1IXT) as template. The resulting protein structure model can be visualized and analyzed using the integrated tool (Raswin program).

Sequencing

Sequencing was done by using an automated laser fluorescence DNA sequencer (Model CEQ8000; Beckman) and the CEQ^{TM} DTCS-Quick Start Kit (Beckman Coulter^{TM}) for sequencing reaction. The sequence data were analyzed with GENETYX computer software.

The plasmids were sequenced by PCR, using CEQ^{TM} DTCS-Quick Start Kit and plasmid as a template. The template was heated at 96 °C for 1 min. The PCR step was performed with 3.2 pmole of primer, 8 μL of DTCS Quick Start Master mix, 200 ng of plasmid DNA template at total volume of 20 μl. The PCR condition was cycles of 96 °C, 20 sec; 50 °C, 20 sec; 60 °C, 4 min and followed by holding at 4 °C. After that, the PCR reaction was stopped by add 4 μL of stop solution (1.5 M NaOAc and 50 mM EDTA and 20 μg glycogen). The reaction was transferred to microfuge tube and mixed thoroughly. The cold 95% ethanol was added and mixed thoroughly. The reaction was centrifuged at 14,000 rmp at 4 °C for 15 min. The pellet was rinsed with

200 μL 70% ethanol 2 times. After removing the supernatant, the pellet was dried at 50 °C for 30 min. The pellet was resuspended in 40 μL of the sample loading solution (SLS) and transferred the resuspended samples to the appropriate well of the CEQ sample plate. The samples were overlaid with one drop of light mineral oil and loaded the sample plate into the CEQ.

Construction and expression of chitinase 60 and chitinase B

The *chi60* gene was constructed and expression by Kamolthip, 2001. The *chiB* gene was amplified from *Serratia marcescens* genomic by PCR, using *Pfu* DNA polymerase. The full-length PCR products were cloned into the pGEM-Teasy vector. The *chiB* gene was subcloned into the expression vector pET19b (+), by PCR using *Pfu* DNA polymerase and plasmid pGSKchiB as a template, to yield plasmid pETSKchiB. The PCR step was performed with 10 pmole of each primers, 0.2 mM dNTP mix, 200 ng of pGSKchiB DNA template, 1.0 U of *Pfu* DNA polymerase in 1X reaction buffer at total volume of 25 μL. The PCR condition was 95 °C for 5 min and 30 cycles of 95 °C, 1 min; 50 °C, 1 min; 72 °C, 5 min. The *Taq* DNA polymerase was added to A-tail addition before cloned to pGEM-Teasy vector. DNA was introduced into *E. coli* BL21 (DE3) pLysS cells by electrophoration and colonies were screened with colloidal chitin minimum medium agar plate. Chitinase gene was confirmed by DNA sequencing.

Construction and expression of chitinase A1

The *chiA* gene was subcloned into the expression vector pET19b (+), by PCR, using *Pfu* DNA polymerase and plasmid pGSKchiA1 as a template, to yield plasmid pETSKchiA1. The PCR step was performed with 10 pmole of each primers, 0.2 mM dNTP mix, 200 ng of pGSKchiA1 DNA template, 1.0 U of *Pfu* DNA polymerase in 1X reaction buffer at total volume of 25 μL. The PCR condition was 95 °C for 5 min and 30 cycles of 95 °C, 1 min; 50 °C, 1 min; 72 °C, 5 min. DNA was introduced into *E. coli* BL21 (DE3) cells by electrophoration and colonies were screened with colloidal chitin minimum medium agar plate. Chitinase gene was confirmed by DNA sequencing.

Construction and expression of chitinase A1 mutants

Deletion mutations were introduced by PCR. The full-length PCR products of wide type ChiA1 and deletion derivatives were digested by *Nco*I and *Bam*HI restriction endonucleases and cloned into these sites of the pKKchi60 plasmid to give pP60SKchiA1 wide type and mutant clones. The deletion mutation of *chiA* gene with deletion of chitin binding domain, ChiA1ΔChBD, and deletion of both fibronectin type III like domain and chitin binding domain, ChiA1ΔFnIIIDΔChBD, were constructed (Figure 2.1).

Addition mutations were introduced by PCR. The *N*-terminal domain of Chi60 (residues 1-157) and *chiA* gene were amplified. The *N*-terminal domain was fused it onto ChiA1 at L42 in Figure 2.2A (ChiA1-60N). The deletion mutation of *chiA-60N* gene with of both fibronectin type III like domain and chitin binding domain, ChiA1-60NΔFnIIIDΔChBD was constructed by using *Xba*I deletion. The FnIIID&ChBD of ChiA1 and *chi60* gene were amplified. The FnIIID&ChBD was fused it onto Chi60 (Chi60-A1FnIII&ChBD) in Figure 2.2B.

Transformation of the ligated DNA was performed by electroporation into *E. coli* Top10 competent cells. Mutant clones were grown on LB agar supplemented with 100 μg/mL amplicillin and colloidal chitin at 37 °C. Mutant colonies were recognized by their inability to degrade chitin. Candidate clones were grown overnight in LB containing 100 μg/mL amplicillin and assayed for chitinase expression by using the substrate colloidal chitin and by running crude protein extracts of each clone on SDS-PAGE. Wild type and mutant clones were confirmed by DNA sequencing.

Construction of chitinase from pKKChi60 and pGSKChiB

The pKKchi60 was previously constructed by Kattiyawong, 2001. In pSKChi60B, the chitinase 60 gene is located on the 5' side of the chitinase B gene in Figure 2.3.

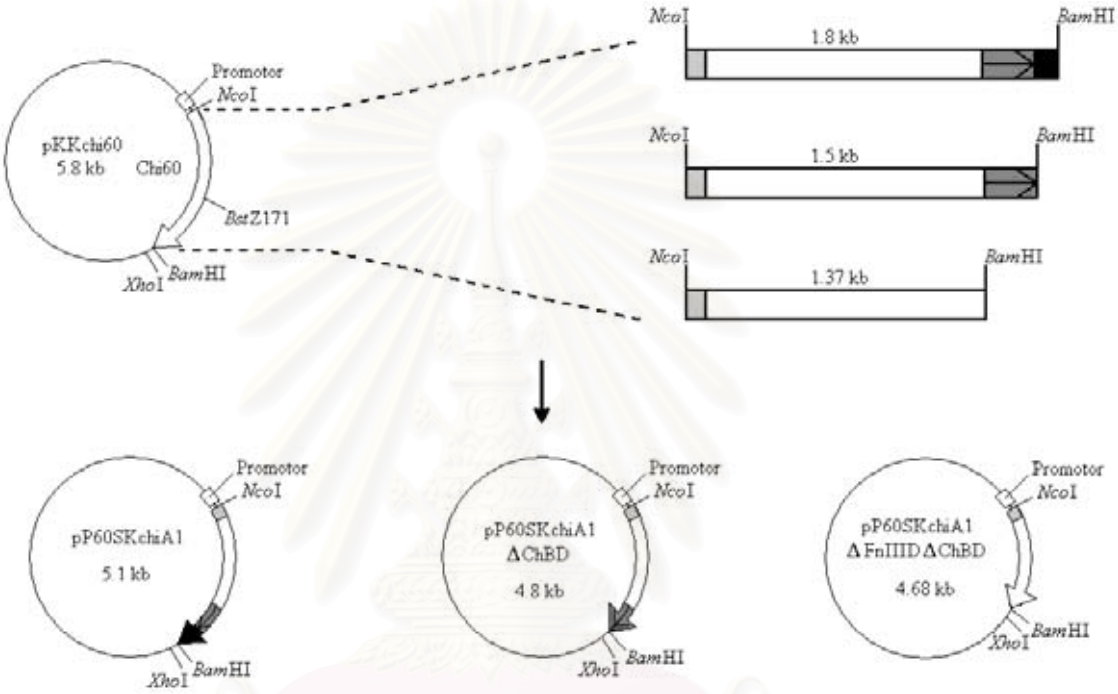


Figure 2.1 Schematic structures of ChiA1 and deletion derivatives. Domain organizations of deletion derivatives of chitinase A1 by modified *chiA* gene.

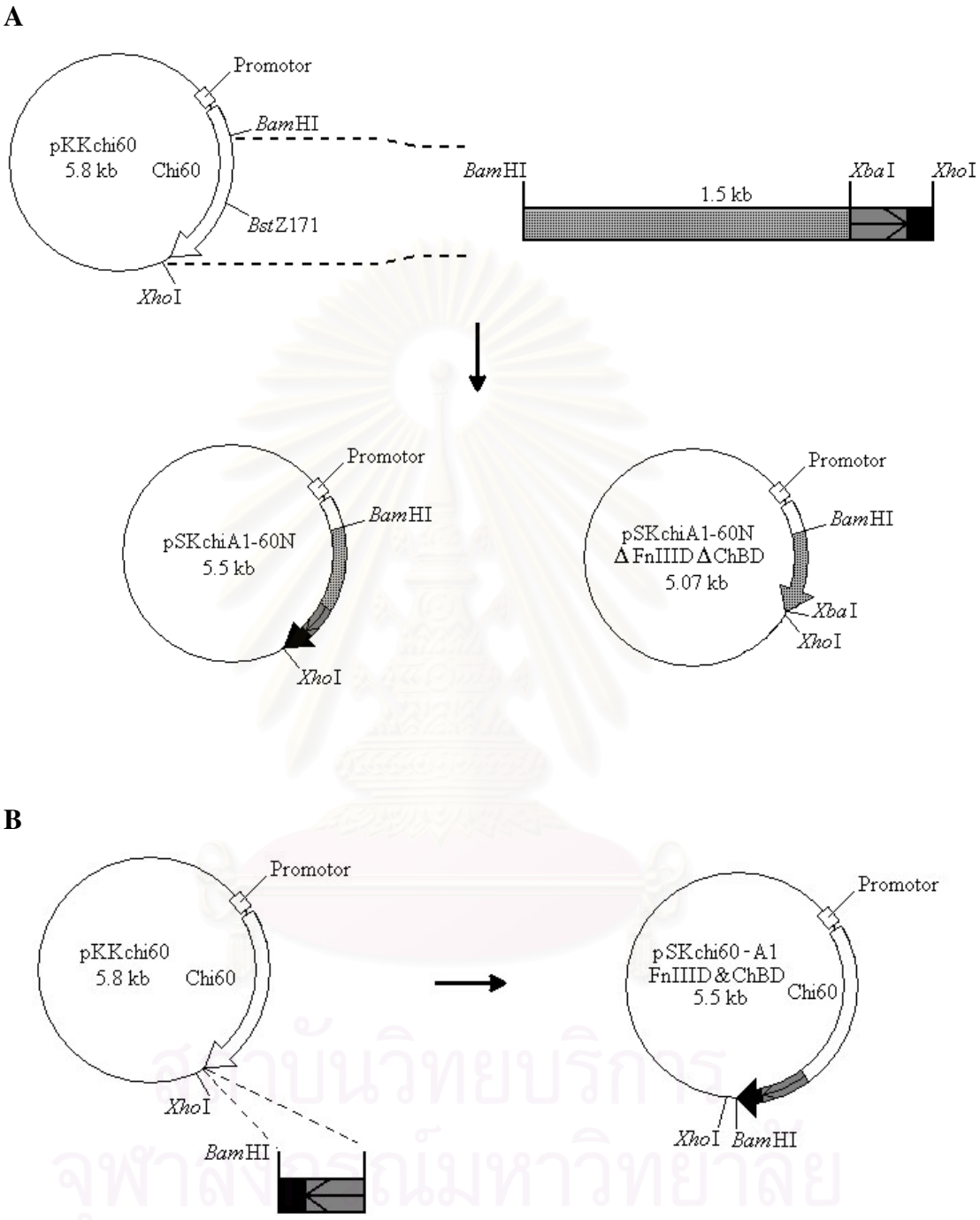


Figure 2.2 Schematic structures of Chi60 and derivatives. (A) Domain organizations of addition derivatives of chitinase A1 by modified *chiA* gene (ChiA1-60N and ChiA1-60ΔFnIIIDΔChBD). (B) Domain organizations of addition derivatives of chitinase 60 by modified *chi60* genes (Chi60-FnIIID&ChBD).

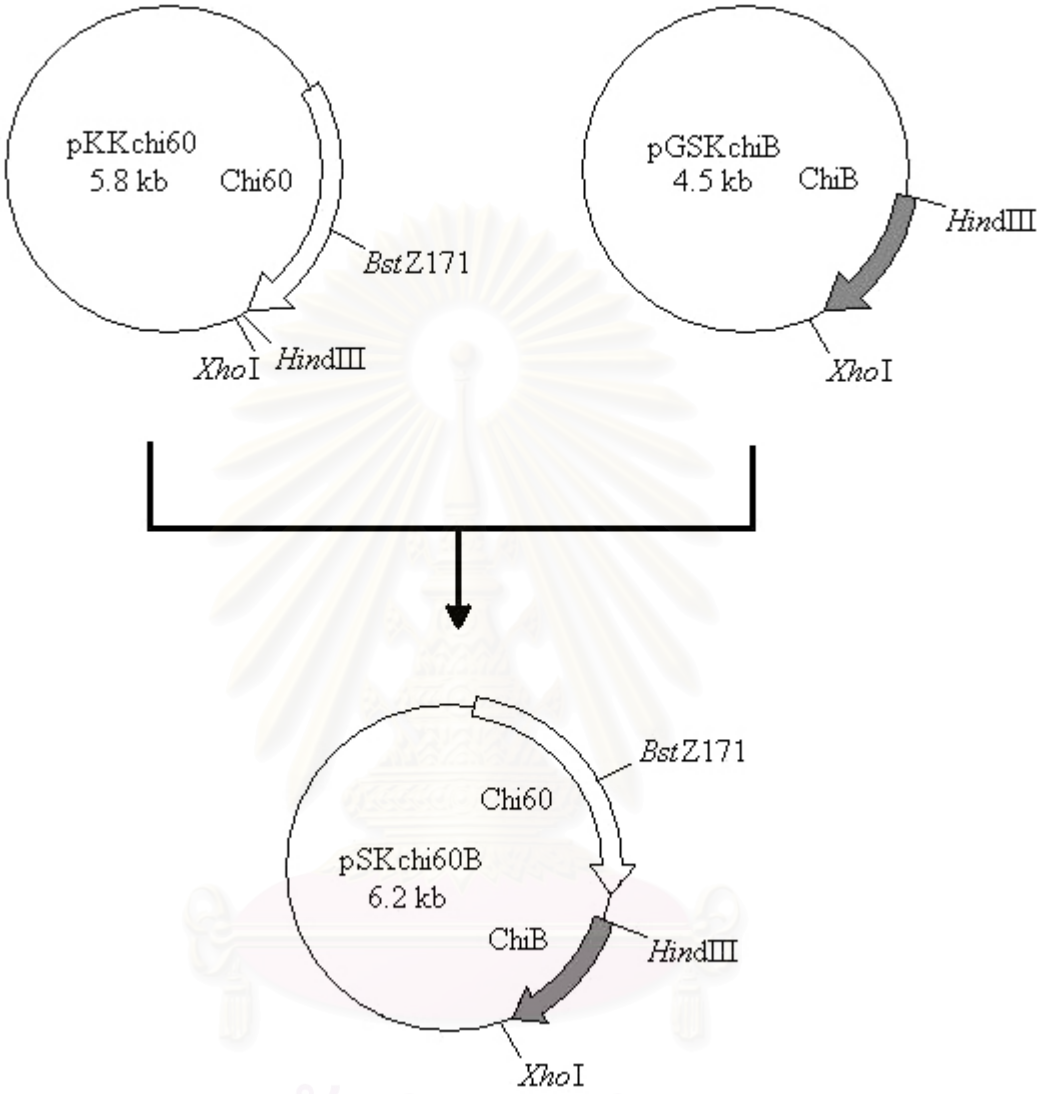


Figure 2.3 The construction of pSKchi60B (bottom) from pKKchi60 (top left) and pGSKchiB (top right). The restriction sites for construction are indicated.

Construction of chimeric chitinase from pKKChi60 and pSKChiB

The pSKchi60B was digested with *Hind*III and *Bst*Z171 (Figure 2.4). The *Hind*III and *Bst*Z171 large fragments were gel purified with Qiaquick gel extraction kit. The eluted fragment was transformed into *E. coli* JC8679 using electroporation. The transformed cells were grown in LB agar containing 100 μg/mL of amplicillin at 37 °C for overnight.

Partially purification of ChiB

Induced culture (400 mL) was harvested by centrifuge. Cells were resuspended in 20 mL of 20 mM potassium phosphate buffer pH 6.0 (KPB) and then were sonicated on ice for 10 min. After centrifugation, the supernatant was stirred gently with fresh colloidal chitin (10x protein) at 0 °C for 4-6 hours. The colloidal chitin was then washed two times with 0.5 M KPB and collected by centrifugation. The precipitated colloidal chitin was resuspended in KPB and incubated at 50 °C for digestion. Chi60 was partially purified by DEAE ion exchanger column.

Purification of ChiA1, ChiA2 and ChiA3

The culture (800 mL) was harvested by centrifuge. After centrifugation, the supernatant was concentrated through VIVA (50) flow (10 kDa cut off). The ChiA1 was partially purified by colloidal chitin adsorption following above. The ChiA2 and ChiA3 were purified by FPLC (Phamacia) with DEAE exchanger and gel filtration G-200. The column was equilibrated with 25 mM Tris-HCl buffer pH 7.0. After washing with the same buffer, protein was eluted by NaCl gradient (0-1 M). The volume of the solution containing the chitinase activity was reduced using VIVA (50) flow (30 kDa cut off) and applied to gel filtration G-200.

Partially purification of ChiA1 and derivatives

Induced culture (800 mL) and uninduced were harvested by centrifuge. After centrifugation, the supernatant was concentrated through VIVA (50) flow (10 kDa cut off). The ChiA1 and derivatives (except ChiA1 with deletion fibronectin type-III like domain and chitin binding domain and ChiA1-60N) were partially purified by

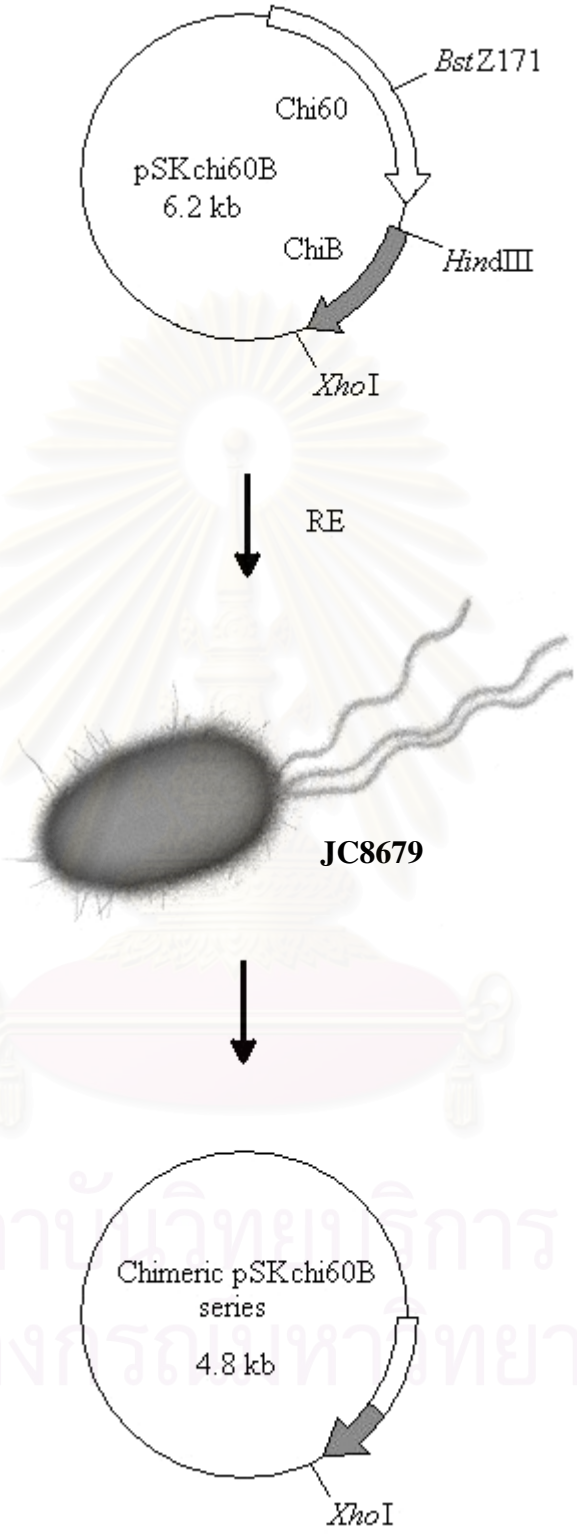


Figure 2.4 The construction of chimeric chitinase by homologous recombination process.

colloidal chitin adsorption following above. The ChiA1ΔFnIIIDΔChBD and ChiA1-60N were applied to DEAE-cellulose column. The column was equilibrated with 25 mM Tris-HCl buffer pH 7.0. After washing with the same buffer, protein was eluted by NaCl gradient (0-1 M). The volume of the solution containing the chitinase activity was reduced using VIVA (50) flow (30 kDa cut off).

Characterization of chitinase enzyme

The chitinase activity was assayed by the colorimetric method as previously described for its properties as the follows:

Estimation of molecular weight and chitinase activity staining

The molecular weight of chitinase was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by activity staining of chitinase. SDS-PAGE was performed by the method of Trudel and Asselin, (1989) using a 10% acrylamide gel containing 0.01% (w/v) glycol chitin. Sample solution of enzymes were denatured by heating at 100°C for 5 min in 1x sample loading dye containing 15% (w/v) sucrose, 2.5 % (w/v) SDS, 125 mM Tris-HCl (pH 6.7), 15% (v/v) β-mercaptoethanol and 0.01% (w/v) Bromophenol blue. Proteins marker, ranging 97-14 kDa, was used. After electrophoresis, the protein band containing chitinase activity after SDS-PAGE was detected by incubating gel overnight at 37°C with reciprocal shaking in 100 mM phosphate buffer (pH 6.0) containing 1% (v/v) Triton X-100. The gel was then stained with 0.01% (w/v) Fluorescent Brightener 28 in 500 mM Tris-HCl (pH 8.9) and destained with distilled water. Lytic zones in the gel were visualized under UV light. After that, the gels were destained with against water and the proteins were stained with 0.25 % Coomassie Brilliant Blue R-250 at room temperature for one hr. The gels were destained with a mixture of 10% (v/v) acetic acid and 25 % (v/v) methanol.

Optimum pH for chitinase activity

The optimum pH for chitinase activity was determined by incubating the enzyme (0.1 mg protein/mL) in appropriate buffers at different pHs (3-10) using 0.1% colloidal chitin or 0.01% PNAC as substrate, and incubated at 50 °C for 30 min.

Citrate buffer (100 mM) and Tris-HCl (100 mM) Glycine-NaOH (100 mM) were used for pH 3-6, pH 7-8 and pH 9-12, respectively. The chitinase activity was assayed as previously described.

Optimum temperature for chitinase activity

The optimum temperature for the chitinase activity was assayed by incubating the reaction mixture, consisted of 75 μL of 1 M phosphate buffer pH 6.0, 75 μL of 1 % colloidal chitin and 100 μL of chitinase (0.1 mg protein/mL) at temperature range of 40-70 °C. Then the release of *N*-acetylglucosamine reducing property at 30 min of the incubation time was measured under standard assay conditions.

The chitinase activity on different substrates (substrate specificity)

The enzyme was incubated with each of the following substrate; 1 mg/mL of partially *N*-acetylated chitin (PNAC) 10 mg/mL of colloidal chitin (CC) and β-chitin (BC) at 50 °C for 30 min. The release of *N*-acetylglucosamine hydrolysis of the substrate was measured by the colorimetric method for determination of its activity.

Chitin binding assay

The standard binding-assay was carried out as follows. Fifty miliunits of ChiA1 and mutants were mixed thoroughly with 10 mg of colloidal chitin in 100 mM sodium citrate buffer, pH 6.0 (total volume, 500 μl). The mixtures were kept on ice at various times. After centrifugation at 10,000 rmp for 5 min, the supernatants containing unadsorbed chitinase was collected, and the chitinase activity was determined by Schales method. The amounts of the bound chitinase were calculated by determining the difference between the amount of the total bound activity and the amount of the unbound activity. The percentages of bound enzyme were calculated. All tests were performed in triplicate.

The kinetic of enzyme

The enzyme was incubated with varies concentration of colloidal chitin (0.5, 1, 2, 4 mg/mL) in 0.1 M citrate buffer at 50 °C for 30 min. The release of *N*-

acetylglucosamine hydrolysis of the substrate was measured by the colorimetric method for determination of its activity.

Effect of ionic strength on the hydrolyzing activity of ChiA1 and its derivative

The effect of ionic strength on the hydrolyzing activity of ChiA1 was first examined by incubating the reaction mixtures at various sodium chloride concentrations ranging from 0-600 mM and various substrates such as PNAC, BC and CC. The second colloidal chitin was selected to study the effect of ionic strength at various ChiA1 and derivatives. The reaction mixture (total 750 µL) contained partial purified chitinase and 0.1 mg/mL of PNAC, 1 mg/mL of colloidal chitin, and 10 mg/mL of β-crystalline chitin of each substrate in 0.1 M sodium citrate buffer, pH 6.0. The enzyme was assayed at 50°C for 30 min.

Analysis of the degradation products by enzyme and its derivatives

The 50 mU/mL partially purified chitinases (the amount of enzyme which yields equal activity on PNAC) were used to digest 10 mg/mL colloidal chitin and β-crystalline chitin. The mixture was incubated in 50 mM sodium citrate buffer, pH 5.0 at 50°C, 12 hr. The product was boiled for 15 min and centrifuge at 8,000 g in a bench-top centrifuge for 10 min.

Purification of hydrolytic product by activated charcoal

The hydrolytic products were applied to activated charcoal column ratio (1: 20 w/w) to salt elimination in Figure 2.5. The sample was washed with water twice and eluted with 60% EtOH. The sample was dried in hot air oven. After drying, the sample was resuspended with distilled water.

Analysis of the degradation products of chitinase by thin layer chromatography (TLC)

The reaction products for *N*-acetyl-chitooligosaccharides were analyzed by silica-thin layer chromatography (TLC). Aliquots (10 µl) of the reactions mixtures were spotted on silica gel plate with isopropanol-ethanol-water (5: 2: 1[v/v/v]) and the

products were detected by dipping the plate in the aniline-diphenylamine reagent (4mL of aniline, 4 g of diphenylamine, 200 mL of acetone and 30 mL of 85% phosphoric acid) and baking it at 130 °C for 3 min (Tanaka *et al*., 1999).

Analysis of the degradation products of chitinase by HPLC

The sample was purified by activated charcoal. Then 300 μL of the purified product was mixed with 700 μL of acetonitrile, and then filtered through a 0.45 μm filter. The product was then analyzed by HPLC (Water system). The used condition was; carbohydrate cartilage column, mobile phase 300 mL ultrapure water: 700 mL acetonitrile, flow rate 1.0 mL/min.

Preparation of *N*- *N′* diacetylchitobiose and *N*-acetylchitooligosaccharide production

Preparation of *N*- *N′* diacetylchitobiose

Beta chitin (1 g) was hydrolyzed with partially purified Chi60W33F/W245F (1 units) in 0.1 M citrate buffer pH 5.0 at 50 °C for 6 days by using dialysis tubing (Figure 2.6). The buffer was circulated continuously through the system and chitinase was on the dialysis tubing for the hydrolysis reaction. The reaction mixtures were applied to an activated carbon spin column (1.5 cm.x 5 cm). Oligosaccharides were eluted with a stepwise of 10% and 20% EtOH. Concentration of GlcNAc was determined reducing sugar, pooled and identified.

Preparation of *N*-acetylchitooligosaccharide by ChiΔChBD

ChiA1ΔChBD (1 units) was incubated with 500 mg of colloidal chitin in 25 mM citrate buffer pH 6.0. The system was kept at 40 °C for 2 hr. Samples (20 μL) were taken at various time intervals. Each sample was heated at 100 °C for 5 min and made up to 100 μL with deionized water. After centrifugation, 5μL aliquots were spotted to TLC.

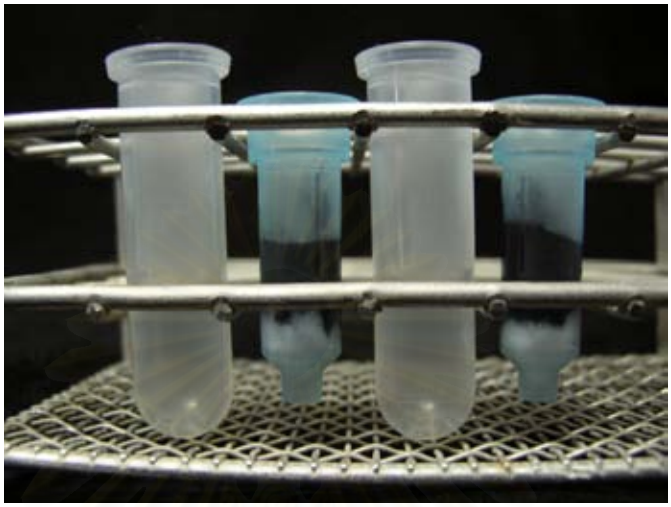


Figure 2.5 The activated charcoal spin column.

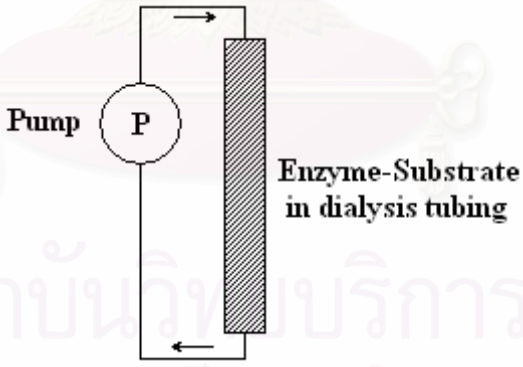


Figure 2.6 The diagram shows the column system reaction. The buffer was circulated continuously through the system and chitinase was on the dialysis tubing for the hydrolysis reaction.

CHAPTER III

RESULTS

Part I: Construction and characterization of bacterial chitinase and screening of chitooligosaccharide production.

Construction of *chi60* and *chiB* genes

The three-dimentional structures of Chi60 and ChiB with their active site are shown in Figure 3.1. *Serratia* sp. TU09 Chi60 comprises three domains: an *N*-terminal domain, a catalytic $(\beta/\alpha)_8$ barrel domain and a small $(\alpha+\beta)$ domain, which is inserted into the $(\beta/\alpha)_8$ barrel domain. *S. marcescens* ChiB comprises a catalytic $(\beta/\alpha)_8$ barrel domain and a *C*-terminal chitin binding domain. The position of the carbohydrate binding modules relative to the enzyme active site is different in Chi60 and ChiB. The support loop and the chitin binding domain extend the substrate binding cleft of ChiB on the reducing side of the active site, whereas the chitin binding domain of Chi60 extends the substrate binding cleft at the non reducing side, (Mastumoto *et al*, 1999). The co-crystallized chitooligosaccharide is inserted into the catalytic cleft either from the reducing end digested by Chi60, or from the non reducing end digested by ChiB (Figure 3.1).

Production and partial purification of the Chi60 and ChiB

Plasmids carrying intact *chi60* and *chiB* genes were introduced into *E. coli* XL1-Blue and BL21 (DE3) pLys for chitinase production, respectively. After *E.coli* cell harbouring plasmid containing *chi60* and *chiB* genes were grown in LB medium, the supernatant was collected. Purification of chitinase was performed by colloidal chitin adsorption. SDS-PAGE analysis of the partial purified chitinase is shown in Figure 3.2. The molecular mass of Chi60 and ChiB were determined by SDS-PAGE. The molecular weights of the chitinases were found to be 60 kDa for Chi60 and 54 kDa for ChiB.

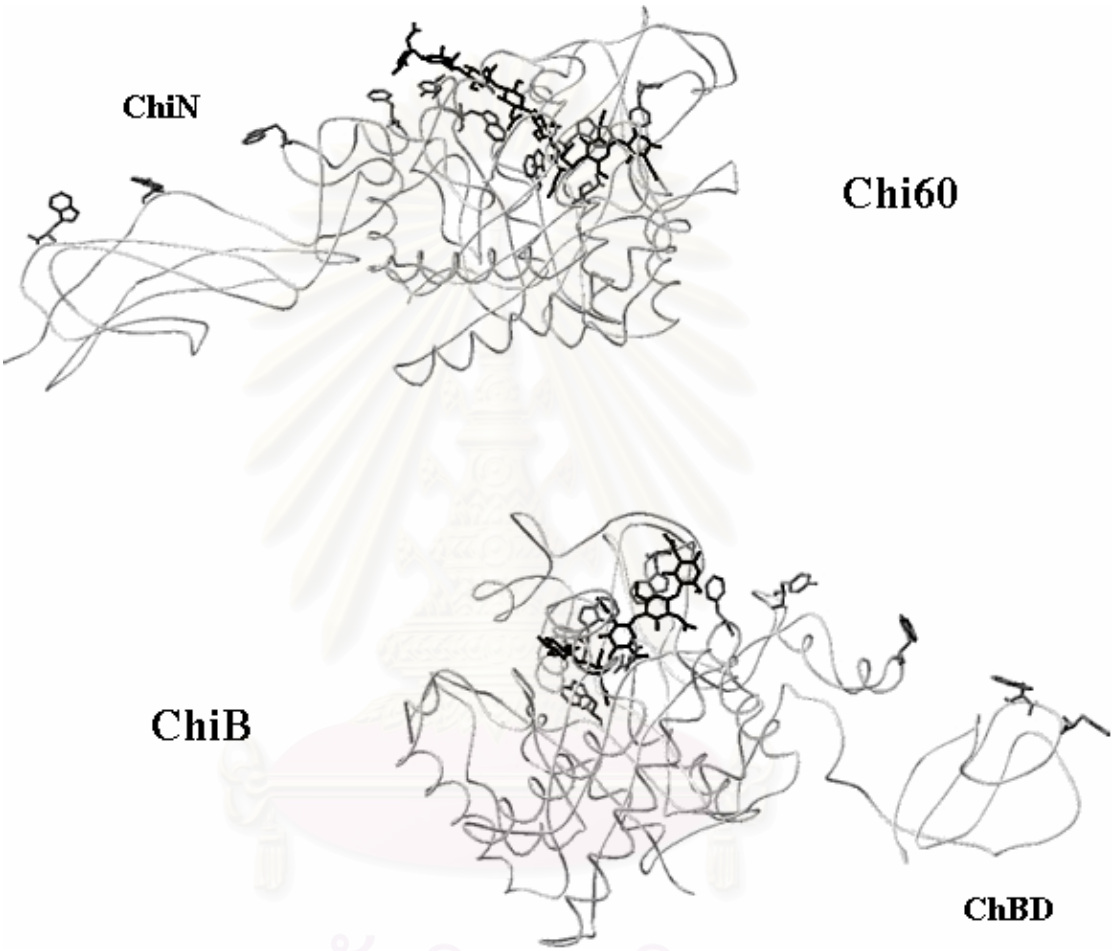


Figure 3.1 Crystallographic two-dimentional structures of Chi60 (top) and ChiB 1E6N (bottom). The figure shows the catalytic domain co-crystallized with an oligosaccharide and the position of the aromatic residues (modified from Eva-Lena HULT et al, 2005).

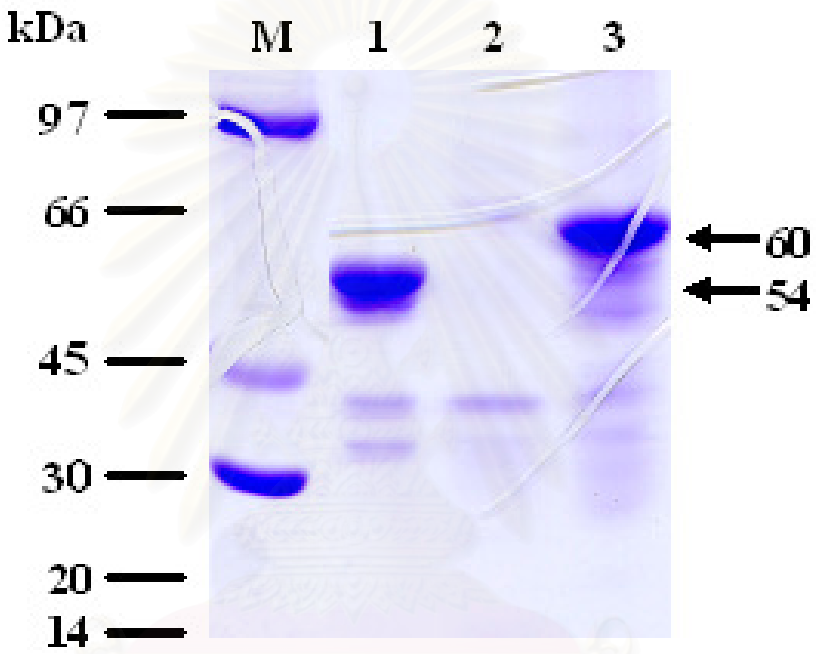


Figure 3.2 SDS-PAGE of partial purified chitinase of Chi60 and ChiB

Lane M = standard protein marker

Lane 1 = partial purified ChiB

Lane 2 = control plasmid

Lane 3 = partial purified Chi60

Construction of *chiA* gene

Bacillus licheniformis SK-1 chitinase A1 (ChiA1) was PCR and cloned from *B. licheniformis* SK-1 into pGEMT-easy. Derivatives of the cloned genes were constructed by subcloning the chitinase gene into pET system and other bactereial promoters. It consists of *N*-terminal catalytic domain (CatD, residues 33-451), fibronectin type III like domain (FnIIID, residues 464-549), and a *C*-terminal chitin binding domain (ChBD, residues, 554-598) as shown in Figure 3.3. CatD belongs to glycosyl hydrolase (GH) family 18 and ChBD belongs to carbohydrate binding modules (CBM) family.

Production and purification of the chitinases A1

Plasmids carrying intact *chiA* gene was introduced into *E. coli* BL21 (DE3) for chitinase production. Chitinase was collected from cells suspension by centrifugation. The supernatant was concentrated by VIVA (50) flow (10,000 Da cut off). When expressed in *E. coli*, *chiA* gene gave 3 peptides with hydrolytic activity, with the molecular weight of 70, 65 and 58 kDa. When concentrated crude enzyme was adsorbed onto colloidal chitin, chitinase A1 was found, molecular weight of 70 kDa, could bind with colloidal chitin, while the protein with the molecular weight of 65 and 58 kDa could not bind to colloidal chitin (Figure 3.4). All three chitinases in *E. coli* transformants were expressed from a single gene. The molecular weight of intact ChiA1 (70 kDa) can be match to that of the fullenght protein predicted from a *chiA* sequence. This indicated that ChiA1 was the original form the enzyme which possibly converted into ChiA2 (65 kDa) and ChiA3 (58 kDa) by proteolysis. ChiA1 was the predominant form of chitinase in *E. coli* transformants and *B. licheniformis* SK-1, whereas ChiA2 and ChiA3 were produced in small amount in transformants and *B. licheniformis* SK-1 (Kudan, 2001).

Optimum pH and optimum temperature of Chi60, ChiB and ChiA1

The optimum pH of partial purified enzyme was determined. Chitinase activity was assayed at 50 °C in buffer with pH ranging from 3-12. The activity at different pH was shown in Figure 3.5. Partially purified enzymes showed a broad pH range, from pH 4-12, for Chi60. While the other two enzyme shows peaks with

>ChiA1 599 aa 1797 bp

MKIVLINKSKKFFVFSFIFVMMLSLSFVNGEVAKADSGKNYKIIGYYPSWGAYGRDFQVWDMDVSKVSH
INYAFADICWEGRHGNPDPTGPNPQTWSCQDENGVIDAPNGTIVMGDPWIDAQKSNPGDVWDEPIRGNF
KQLLKLKKSHPHLKTFISVGGWTWSNRFSDVAADPAARENFAASAVEFLRKYGFDGVDLDWEYPVSGGL
PGNSTRPEDKRNYTLLLQEVRKKLDAAEAKDGKEYLLTIASGASPDYVSNTELDKIAQTVDWINIMTYD
FNGGWQSISAHNAPLFYDPKAKEAGVPNAETYNIENTVKRYKEAGVKGDKLVLGTPFYGRGWSGCEPGG
HGEYQKCGPAKEGTWEKGVFDFSDLERNYVNQNGYKRYWNDQAKVPFLYNAENGNFITYDDEQSFGHKT
DFIKANGLSGAMFWDFSGDSNRTLLNKLAADLDFAPDGGNPEPPSSAPVNVRVTGKTATSVSLAWDAPS
SGANIAEYVVSFENRSISVKETSAEIGGLKPGTAYSFTVSAKDADGKLHAGPTVEVTTNSDQACSYDEW
KETSAYTGGERVAFNGKVYEAKWWTKGDRPDQSGEWGVWRLIGGCE

Figure 3.3 Amino acid sequence and domain mapping of ChiA1. The amino acid sequence was translated by Swissprot program, whilst the domain mapping was predicted by BLASTP program (green: catalytic domain; red: fibronectin type III like domain; blue: chitin binding domain 3).

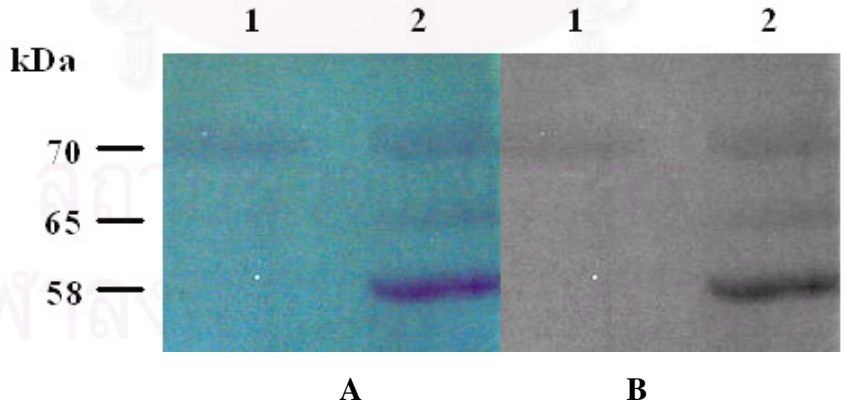


Figure 3.4 SDS-PAGE of chitinase A1 and deletion truncated chitinase (A2 and A3). protein staining (A), chitinase activity staining (B).

Lane 1 = adsorbed ChiA1

Lane 2 = unadsorbed Chi A1

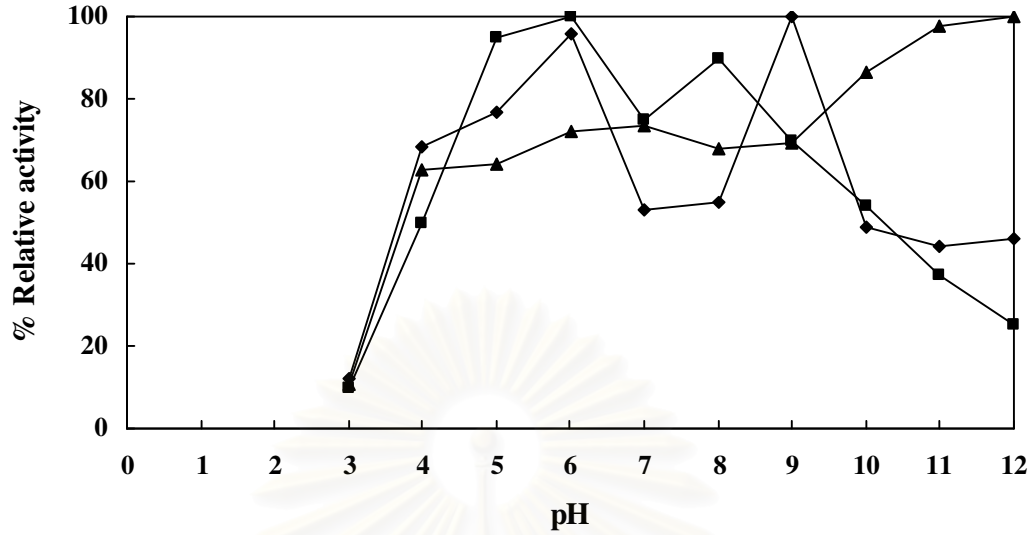


Figure 3.5 The optimum pH of ChiA1, Chi60 and ChiB. Chitinolytic activity was measured in pH range of 3-12 at 50 °C for 30 min by colorimetric method used PNAC as substrate (◆ , ChiA1; ▲, Chi60; ■, ChiB).

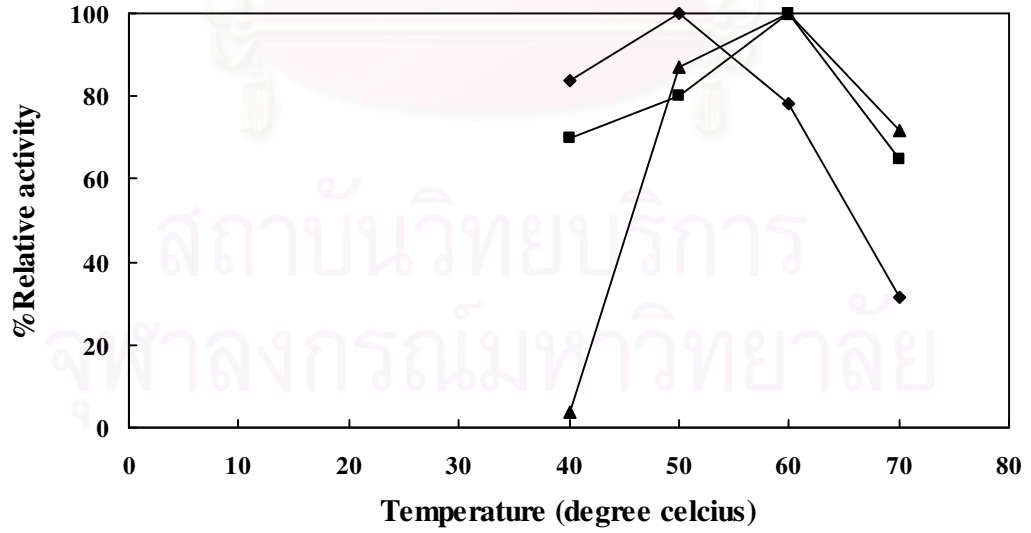


Figure 3.6 The optimum temperature of ChiA1, Chi60 and ChiB. Chitinolytic activity was measured in pH 6 at various temperature 40-70 °C, 30 min by colorimetric method used PNAC as substrate (◆ , ChiA1; ▲, Chi60; ■, ChiB).

optimum pH at 6 and 9 for ChiB, and at pH 6 and 8 for ChiA1.

The optimum temperature of partial purified enzyme was determined at pH 5.0, in the range of 40-80 °C. Chi60 and ChiA1 exhibited the optimum temperature at 60 °C for, while ChiB at 50 °C (Figure 3.6).

Chitin binding activities of chitinase A1, chitinase 60 and chitinase B

The importance of the *C*-terminal region in chitin binding activity of chitinases has been suggested by previous experiments. Chitinases (50 mU) was mixed with colloidal chitin in citrated buffer pH 5.0 at 0° C to provide adsorption, the mixtures were centrifuged and the activity of chitinases remaining in the supernatant was determined. The amounts of bound chitinase was calculated by subtracting the chitinase activity added initially with the activity remaining in the supernatant after binding. Approximate 40% of ChiA1 was bound at 30 min, more than 80% absorption was found after 3 hr of incubation (Figure 3.7).

Although chitinase A1 degrades partially acetylated chitosan quite well (unpublished data), the level of binding of chitinase A1 to chitosan was unexpectedly low. More than 80% of the chitinase A1 remained unadsorbed, suggesting that the binding activity of the chitinase A1 is quite specific and the acetyl group is essential for its binding.

Effect of ionic strength on activity of chitinase A1

Ionic strength effects were also monitored by using different substrates at various NaCl concentrations (Figure 3.8). When β-chitin was used as a substrate, the activity of ChiA1 increased with increasing NaCl concentration from 0-50 mM, while on colloidal chitin, the activity of ChiA1 increased with increasing NaCl concentration from 0-100 mM, but slightly decreased at levels over 200 mM. The ChiA1 activity was rapidly decreased at 400 mM NaCl and remained constant. For PNAC, the activity of ChiA1 did not change by ionic strength.

The product hydrolysis by ChiA1, Chi60 and ChiB from chitinous substrate

For hydrolysis, short chitooligosaccharide [$(GlcNAc)_n$, n = 3, 4, 6] and β-chitin and colloidal chitin were used as substrate.

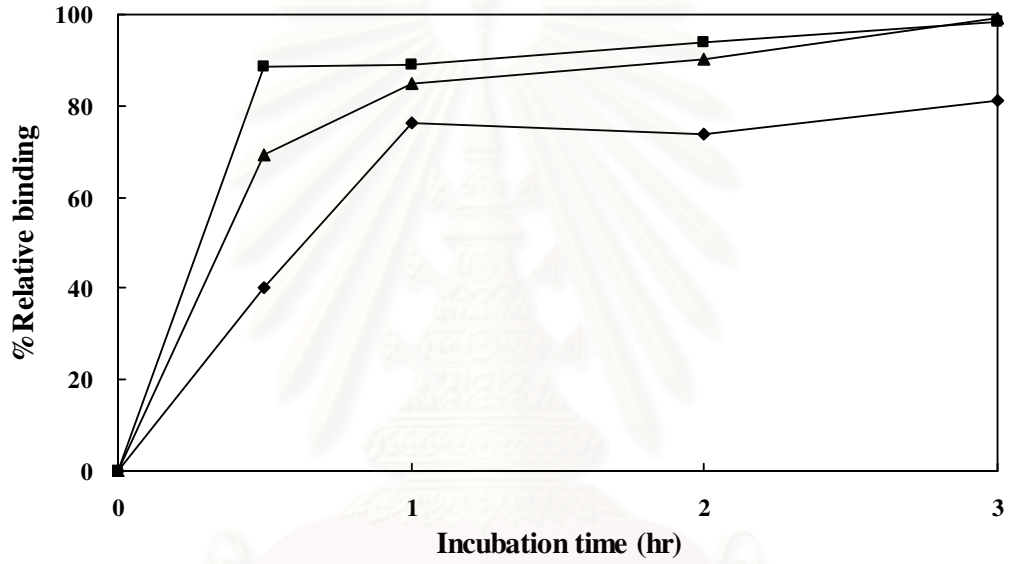


Figure 3.7 The chitin binding assays for ChiA1, Chi60 and ChiB by colloidal chitin. The reaction mixtures were incubated on ice in 0.1 M citrate buffer pH 5.0 containing 20 mg/mL colloidal chitin and 100 mU/mL of each enzyme. Each mixture was taken out at different time and assayed activity (◆ , ChiA1; ▲, Chi60; ■, ChiB).

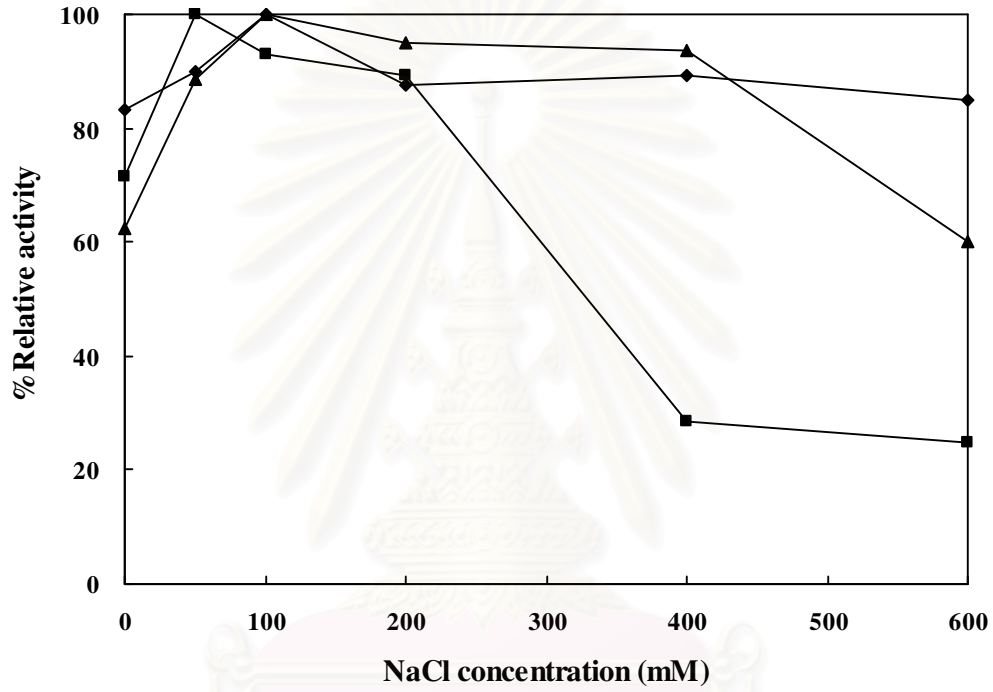


Figure 3.8 Hydrolysis of various substrates by wild-type ChiA1. Reaction mixtures contained of 1.0 mg/mL PNAC or 10 mg/mL colloidal chitin and β-chitin. Reactions were performed at 50 °C, 30 min, and the amount of reducing sugar generated was monitored (◆, PNAC; ■, colloidal chitin; ▲, β-chitin).

β- chitin was hydrolysed by ChiA1, Chi60 and Chi B and provided a major product of dimer as a major product (Figure 3.9), whereas when using colloidal chitin as a substrate, ChiA1 produced dimer as a major product (Figure 3.12), while ChiB and Chi60 produced a mixture of monomer and dimer (Figure 3.10). Accordingly, these enzymes could hydrolyze trimer, tetramer and pentamer and produced dimer and monomer of NAG as well (Figure 3.11).

The hydrolytic product and ratio by adsorbed ChiA1 and unadsorbed ChiA1

The adsorbed ChiA1 (intact ChiA1) and unadsorbed ChiA1 from colloidal chitin were used to hydrolysis colloidal chitin and β-chitin as the substrate. As intact ChiA1 hydrolyzes colloidal chitin and β-chitin to produce *N*-acetylchitobiose as a major product, while unadsorbed ChiA1 produce a mixture of monomer and dimer (Figure 3.12 and Figure 3.13).

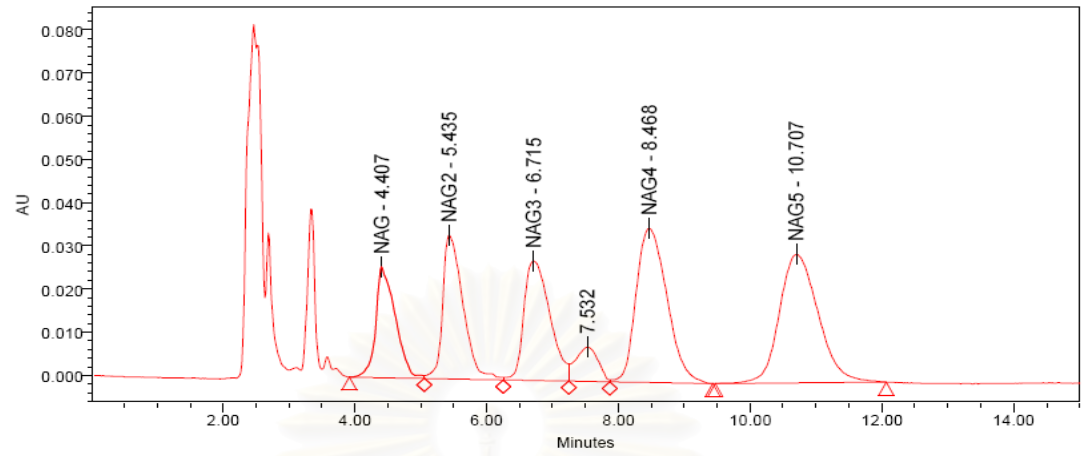
NAG/NAG_2 ratio can give information on substrate-binding mode and: or processivity. The high M/D (≅ 1) indicates the high processive binding, endo-type mode of catalysis, while low M/D (< 1) ratio indicates the high processivity exo-type mode of catalysis.

NAG_2/NAG_3 ratio can give information on substrate-binding mode and /or processivity (Teeri et al., 1998; Medve et al., 1998). The high D/M ratio indicates the high processivity, exo-type mode of catalysis, while low D/M ratio indicates the low processivity endo-type mode of catalysis. Interestingly, intact ChiA1 and truncated derivatives shown clear differences in their NAG_2/NAG_3 ratio, intact ChiA1 producing higher ratio suggesting the high processivity, exo-type mode of catalysis (Table 3.1).

Purification of ChiA1, ChiA2 and ChiA3

Plasmids carrying intact *chiA* gene was introduced into *E. coli* BL21 (DE3) for chitinase production. After culture, chitinases in the medium were separated from cells by centrifugation. The supernatant was concentrated by VIVA (50) flow (10,000 Da cut off), the concentrated enzymes were subjected to FPLC using DEAE and gel filtration G-200 column. Interestingly, chitinase A1 auto-degrades to produce the truncated chitinases, A2 and A3 which can be purified from the enzyme mixture.

A



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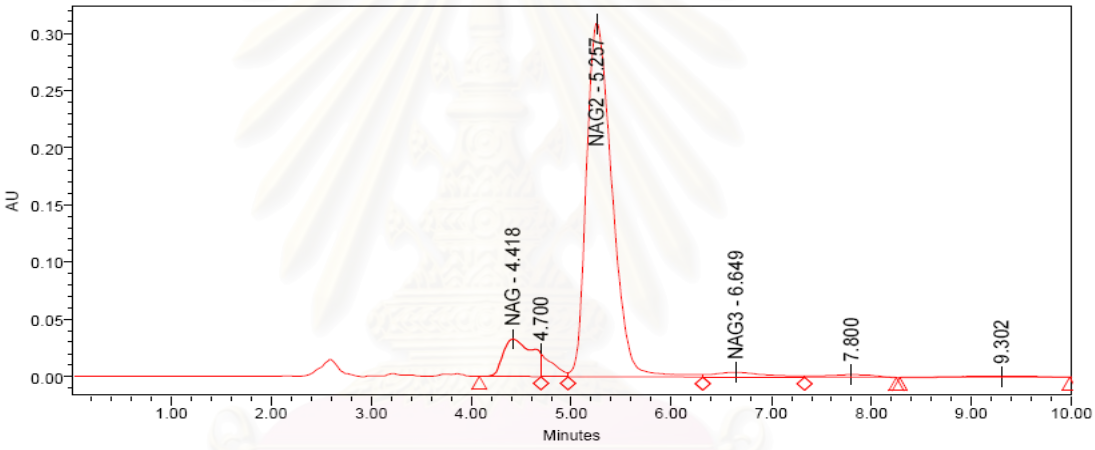

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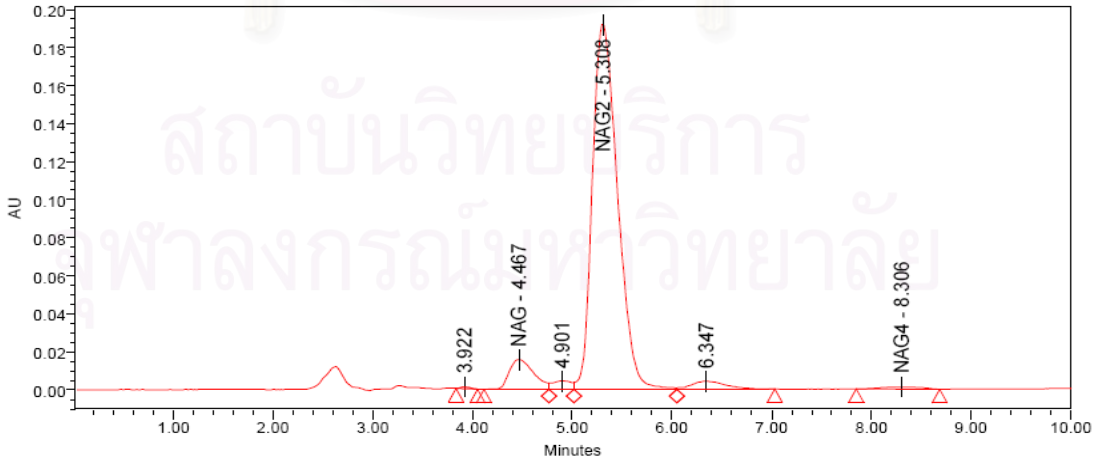
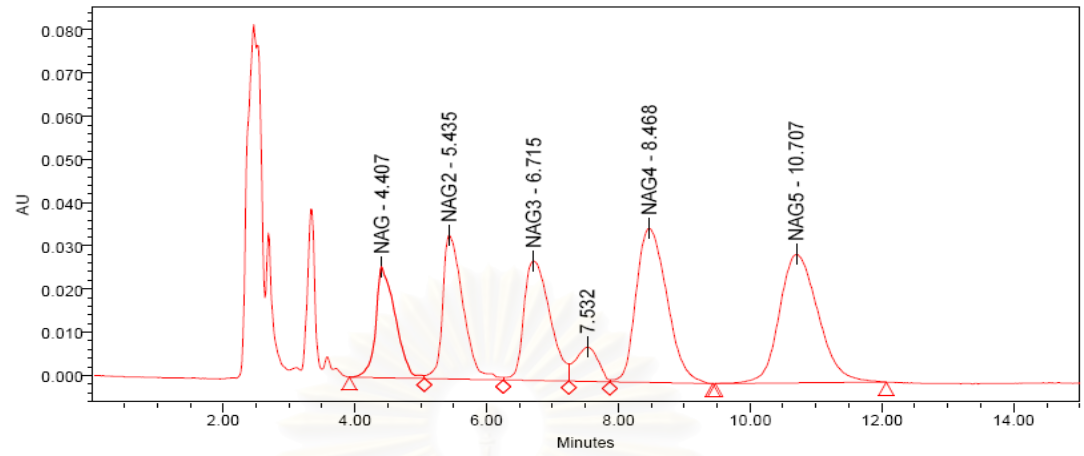


Figure 3.9 The HPLC chromatogram of the hydrolytic product from β-chitin by enzymes. *N*-acetylchitooligosaccharide standard (A), chitinase B (B) and chitinase 60 (C). The reactions were incubated at 50 °C for 12 hr.

A



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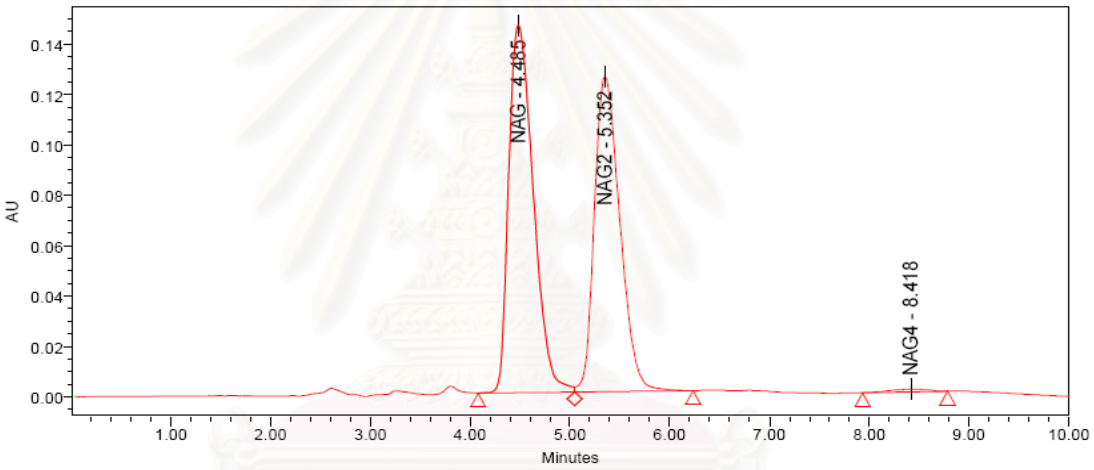

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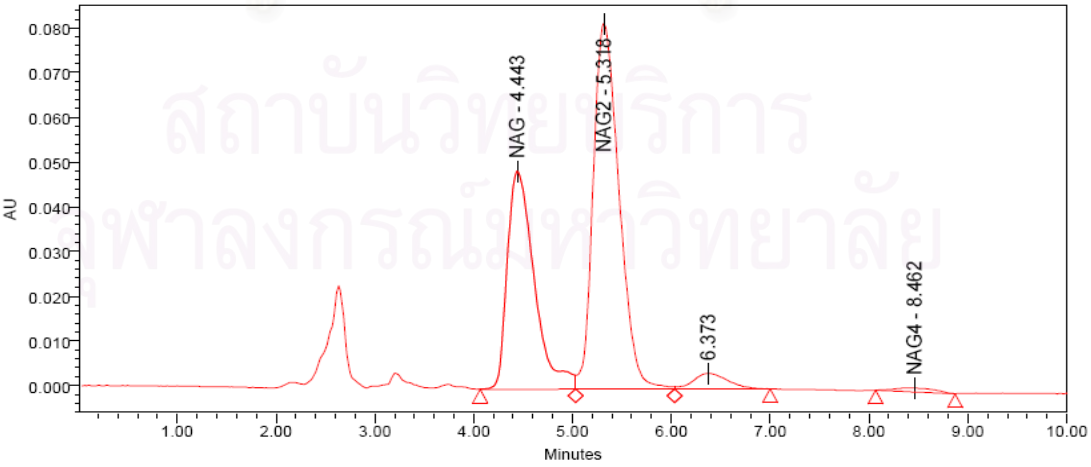


Figure 3.10 The HPLC chromatogram of the hydrolytic product from colloidal chitin by enzymes. *N*-acetylchitooligosaccharide standard (A), ChiB (B) and Chi60 (C). The reactions were incubated at 50 °C for 12 hr.

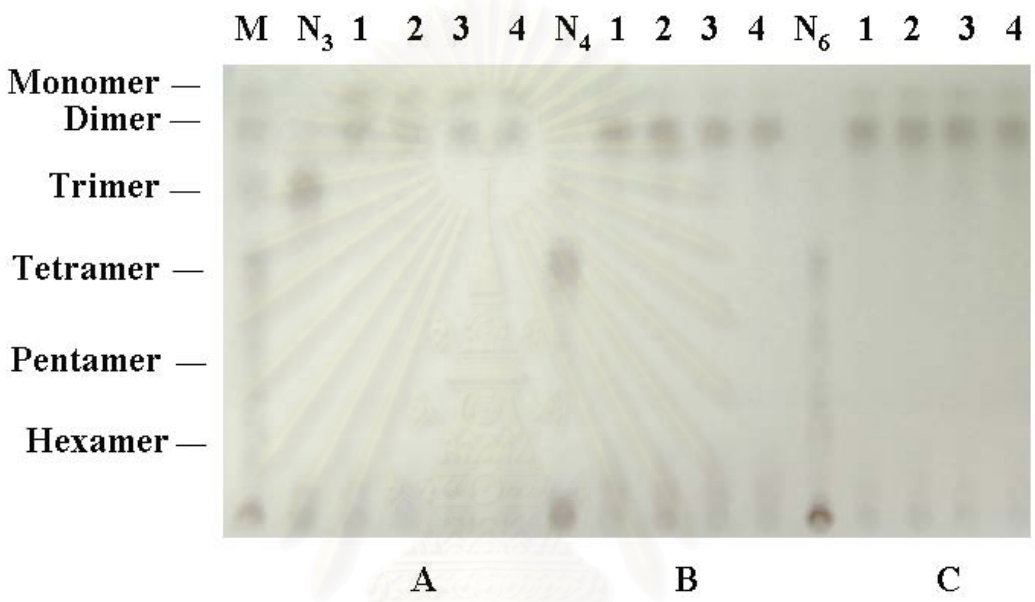


Figure 3.11 The TLC chromatogram of the hydrolytic product from various chitooligosaccharide. Trimer: N_3 (A), tetramer: N_4 (B) and hexamer: N_6 (C). The reactions were incubated at 50 °C for 12 hr.

Lane M = *N*-acetylchitooligosacchaide standard

Lane 1 = the product hydrolyzate from ChiA1

Lane 2 = the product hydrolyzate from ChiB

Lane 3 = the product hydrolyzate from Chi60

Lane 4 = the product hydrolyzate from Chi65

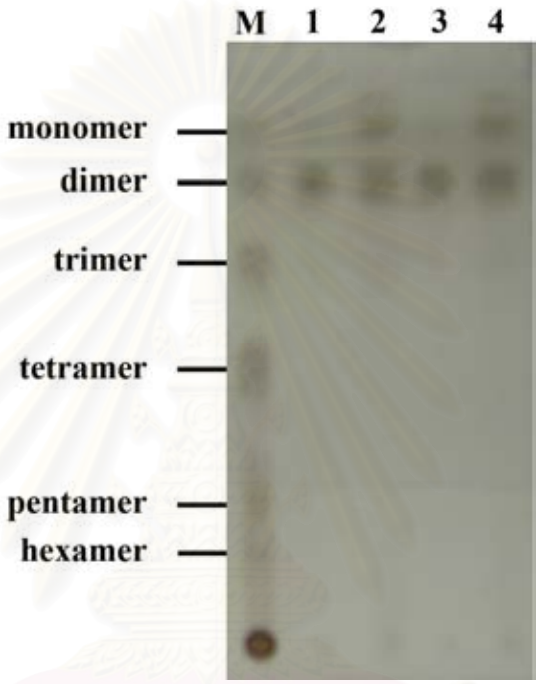


Figure 3.12 TLC of the hydrolytic product from adsorbed chitinase A1 and unadsorbed chitinase A1. The reactions were incubated at 50 °C for 12 hr.

Lane M = *N*-acetylchitooligosaccharide standard

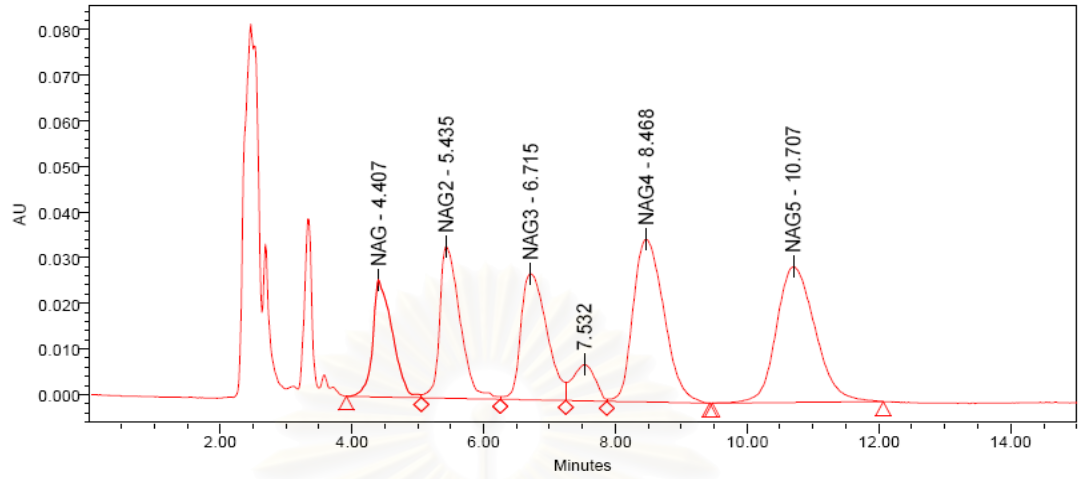
Lane 1 = the hydrolysate from colloidal chitin by adsorbed ChiA1

Lane 2 = the hydrolysate from colloidal chitin by unadsorbed ChiA1

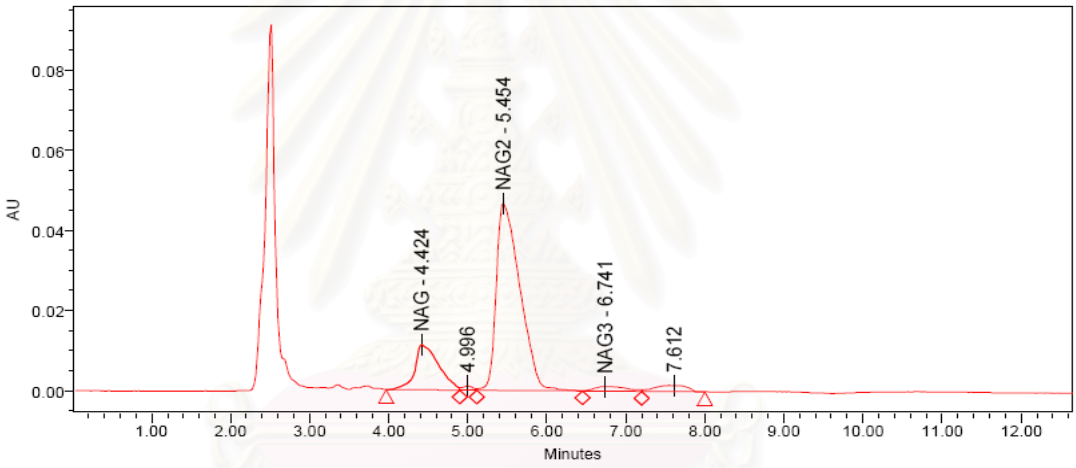
Lane 3 = the hydrolysate from β-chitin by adsorbed ChiA1

Lane 4 = the hydrolysate from β-chitin by unadsorbed ChiA1

A



B



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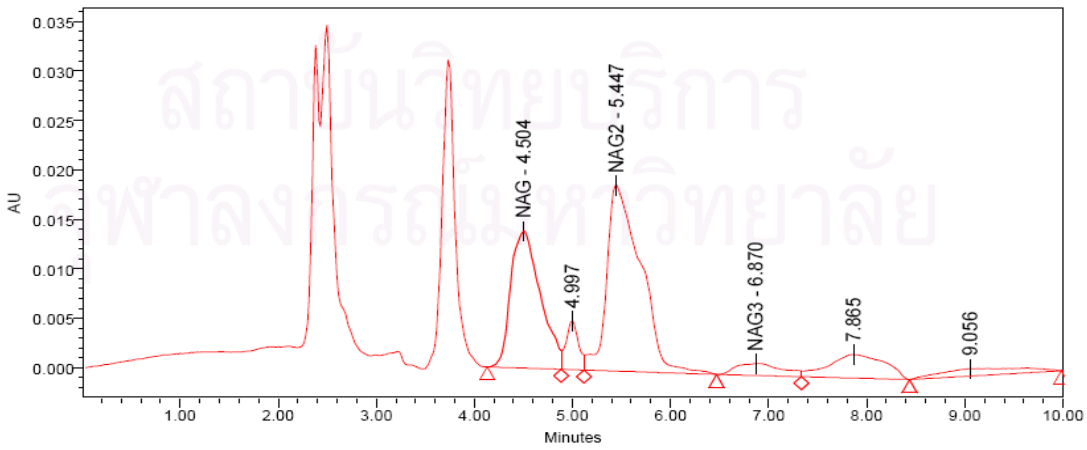

Figure 3.13 The HPLC chromatogram of the hydrolytic product from colloidal chitin by enzymes. *N*-acetylchitooligosaccharide standard (A), adsorbed ChiA1 (B) and unadsorbed ChiA1 (C). The reactions were incubated at 50 °C for 12 hr.

Table 3.1 The hydrolytic product of adsorbed ChiA1 and unadsorbed ChiA1.

Enzyme	Product (mM)			Ratio	
	Monomer (M)	Dimer (D)	Trimer (T)	M/D	D/M
Adsorbed ChiA1	0.81	2.24	0.07	0.36	2.76
Un-adsorbed ChiA1	1.00	1.04	0.08	0.96	1.04

SDS-PAGE analyses of the purified chitinases A1, A2 and A3 are shown in Figure 3.14. After purification ChiA1 continues to degrade. The molecular weights of ChiA1, ChiA2 and ChiA3 were determined by SDS-PAGE. Molecular weights of the chitinases were 70 kDa for ChiAl, 65 kDa for ChiA2, and 58 kDa for ChiA3. Intact ChiAl contains all 3 domains (catalytic domain, fibronectin type III like domain and chitin binding domain), while ChiA2 (lacking chitin-binding domain) and ChiA3 (lacking both of fibronectin type III like domain and chitin binding domain).

Optimum pH and temperature of ChiA1, ChiA2 and ChiA3

The optimum pH of purified enzymes was determined. Chitinases activities were examined at 50 °C in 0.1 M citrate buffers with pH range from 3-12. An activity at different pH was shown in Figure 3.15. ChiA1 showed optimum pH at 6 and 8. ChiA2 and ChiA3 showed slightly alkaline pH than ChiA1, with optimum at pH 7 and 9.

The optimum temperature of purified enzyme was determined at pH 6.0, in the range of 40-80 °C. ChiA1 showed optimum temperature at 60 °C. ChiA2 and ChiA3 showed lower optimum temperature, at 50 °C, than ChiA1 (Figure 3.16).

The kinetic of ChiA1, ChiA2 and ChiA3

The enzyme kinetic parameters such as Michaelis's constant (K_m) and maximum velocity (V_m) of chitinase A1, A2 and A3 were determined by using various substrate concentrations such as 0.5, 1.0, 2.0, 4.0 mg/ml (w/v, dry weight) colloidal chitin in 100 mM citrate buffer pH 6.0 at 50 °C. Lineweaver- Burk's equation and protein concentration of enzymes that used in the reaction, the Michaelis's constant (K_m) and maximum velocity (V_m) of enzymes were measured. From these results, K_m were obtained from reciprocal absolute value of intersection point of 1/[S] on X-axis and were calculated to be 0.62, 4.05 and 1.62 mg/ml while V_m was obtained from reciprocal value of intersection point of 1/ [V] on Y-axis and were calculated to be 0.16, 0.32, 0.22 U/mL (Figure 3.17). Since the 160 x 10^{-3}, 320 x 10^{-3}, 220 x 10^{-3} μmoles of product was produced by enzyme solutions which contained 5 μg of protein.

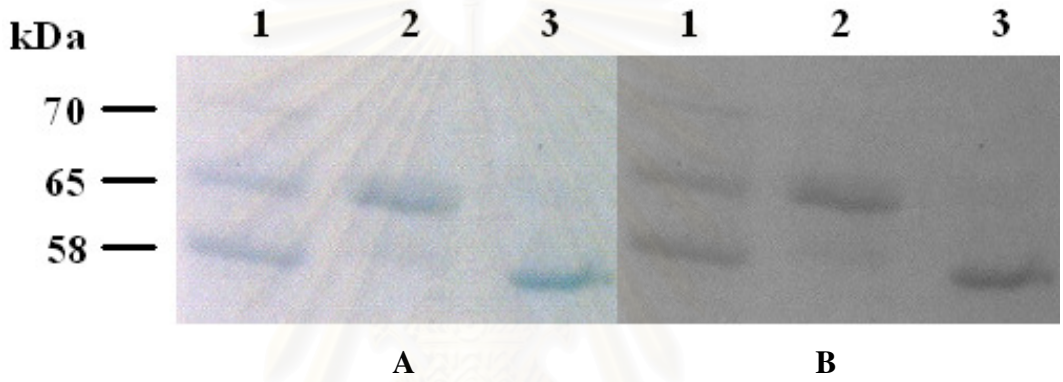

Figure 3.14 SDS-PAGE of chitinase A1, A2 and A3. protein staining (A); chitinase activity staining (B).

Lane 1 = ChiA1

Lane 2 = ChiA2

Lane 3 = ChiA3

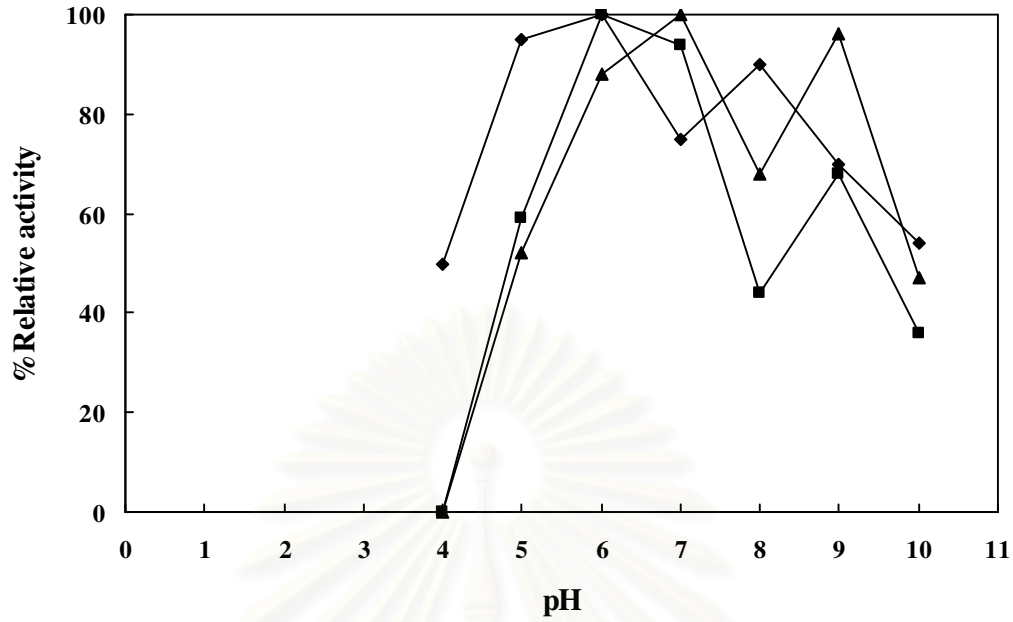

Figure 3.15 The optimum pH of ChiA1, ChiA2 and ChiA3. Chitinolytic activity was measured in pH range of 3-10 at 50 °C for 30 min by colorimetric method when used PNAC as substrate (◆= ChiA1, ■= ChiA2, ▲= ChiA3).

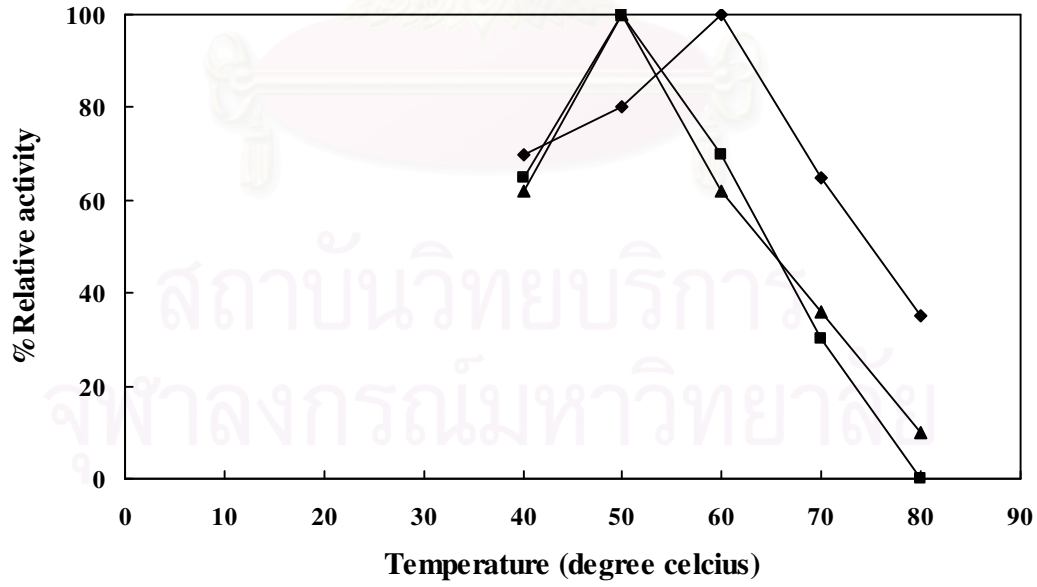

Figure 3.16 The optimum temperature of ChiA1, ChiA2 and ChiA3. Chitinolytic activity was measured in pH 6 at various temperature 40-80 °C for 30 min by colorimetric method when used PNAC as substrate (◆= ChiA1, ■= ChiA2, ▲= ChiA3).

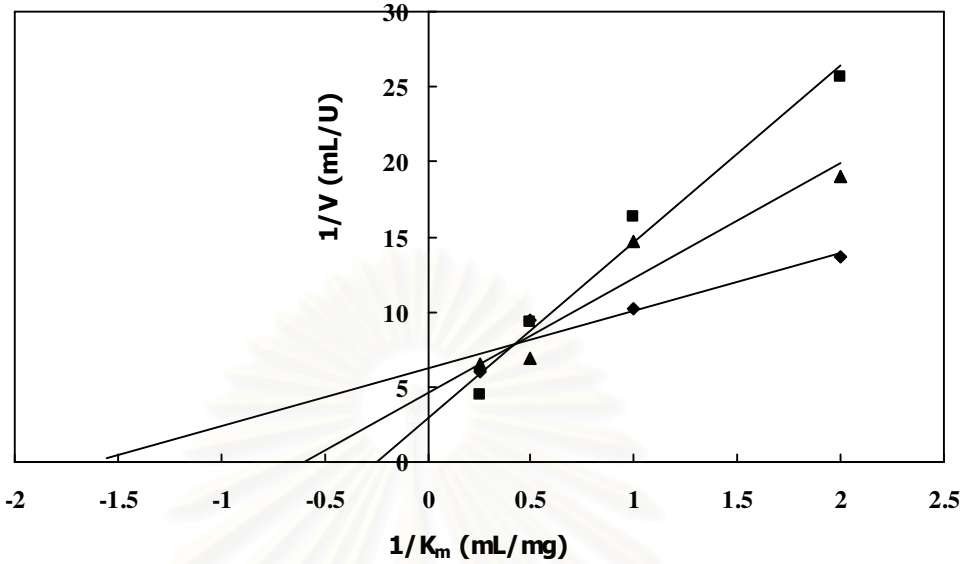

Figure 3.17 The kinetic of ChiA1, ChiA2 and ChiA3. The enzyme kinetic parameters such as Michaelis's constant (K_m) and maximum velocity (V_m) of chitinase A1, A2 and A3 were determined by using various substrate concentrations such as 0.5, 1.0, 2.0, 4.0 mg/ml (w/v, dry weight) colloidal chitin in 100 mM citrate buffer pH 6.0 at 50 °C (◆= ChiA1, ■= ChiA2, ▲ = ChiA3).

As show in Table 3.2, kinetic properties of chitinases are determined and kinetic constants are estimated. ChiA1 had the lowest Km, Vmax and Kcat, following ChiA2 and ChiA3, respectively. In contrast, ChiA1 had the highest k_{cat}/K_m, following ChiA3 and ChiA2, respectively.

The hydrolytic products and ratio by ChiA1, ChiA2 and ChiA3

The ChiA1, ChiA2 and ChiA3 were used to hydrolysis colloidal chitin as the substrate. All enzyme were hydrolyzes colloidal chitin to produce *N*-acetylchitobiose as a major product.

NAG/NAG_2 ratio can give information on substrate binding mode and: or processivity. The high M/D (≅ 1) indicates the low processivity, exo-type mode of catalysis, while low M/D (< 1) ratio indicates the high processivity exo-type mode of catalysis. ChiA2 shown the highest M/D and lowest D/M followed by ChiA3 and ChiA1 (Table 3.3).

Table 3.2 Kinetic constants of chitinase A1, A2 and A3.

Enzyme	K_m (mg/mL)	K_{cat} $(s^{-1})x10^{-3}$	K_{cat}/K_m $(s^{-1}x\ mg^{-1})x10^{-3}$
ChiA1	0.62	3.83	6.2
ChiA2	4.05	8.82	2.2
ChiA3	1.65	6.18	3.7

Kinetic assays were performed with colloidal chitin as described in the Materials and Methods. The results above are averages of two independent assays.

Table 3.3 The hydrolytic product from colloidal chitin by ChiA1, A2 and A3.

Enzyme	Product (mM)			Ratio	
	Monomer (M)	Dimer (D)	Trimer (T)	M/D	D/M
ChiA1	9.29	57.40	1.00	0.16	6.17
ChiA2	35.25	126.00	2.74	0.28	3.58
ChiA3	11.79	61.57	1.29	0.19	5.22

The products were analyzed by HPLC as described in the Materials and Methods. The results above are averages of two independent assays.

Part II: Construction and characterization of modified chitinase A1 and modified chitinase 60

Construction of modified *chiA* gene

In order to study the roles of the domains which constitute chitinase A1, various deletion derivatives encoded by modified *chiA* gene were constructed as summarized in Figure 2.1. The modified chitinases were used promoter of Chi60. A modified *chiA* gene encoding the chitinase A1 with only fibronectin type III like domain (ChiA1ΔChBD) and the modified *chiA* gene lacking both of *C*-terminal domain and fibronectin type III like domain (ChiA1ΔFnIIIDΔChBD) were constructed by PCR deletion mutagenesis. The ChiA1-60N and Chi60-A1FnIIIDChBD were constructed by domain insertion as summarized in Figure 2.2A and Figure 2.2B. ChiA1-60NΔFnIIIDΔChBD was constructed for deletion of ChiA1-60N (Figure 2.2A). The amino acid sequence and mapping domain were shown in Figure 3.18.

The clear zone formation of the modified chitinases on CCMM

Clearing zones formed by these modified chitinases on agar plates containing colloidal chitin are different. Modified chitinases lacking *C*-terminal chitin binding domain but containing fibronectin type III like domain the formed transparent clearing zones with clear edges similar to those formed by intact chitinase Al on plates containing colloidal chitin. On the other hand, modified chitinase A1 containing *N*-terminal domain of Chi60 and chitinase 60 containing fibronectin type III like domain and *C*-terminal chitin binding domain of ChiA1 were formed transparent clear zones slower than intact chitinase A1. Moreover, clearing zones formed by modified chitinases lacking of fibronectin type III like domain and *C*-terminal domain were faint and fuzzy.

Production and partial purification of the modified chitinases

Plasmids carrying intact and truncated *chiA* gene was introduced into *E. coli* Top10 for chitinase production. After culture, chitinases were separated from the

>ChiA1ΔChBD 529 aa 1587 bp

MKIVLINKSKKFFVFSFIFVMMLSLSFVNGEVAKADSGKNYKIIGYYPSWGAYGRDFQVWDMDVSKVSH
INYAFADICWEGRHGNPDPTGPNPQTWSCQDENGVIDAPNGTIVMGDPWIDAQKSNPGDVWDEPIRGNF
KQLLKLKKSHPHLKTFISVGGWTWSNRFSDVAADPAARENFAASAVEFLRKYGFDGVDLDWEYPVSGGL
PGNSTRPEDKRNYTLLLQEVRKKLDAAEAKDGKEYLLTIASGASPDYVSNTELDKIAQTVDWINIMTYD
FNGGWQSISAHNAPLFYDPKAKEAGVPNAETYNIENTVKRYKEAGVKGDKLVLGTPFYGRGWSGCEPGG
HGEYQKCGPAKEGTWEKGVFDFSDLERNYVNQNGYKRYWNDQAKVPFLYNAENGNFITYDDEQSFGHKT
DFIKANGLSGAMFWDFSGDSNRTLLNKLAADLDFAPDGGNPEPPSSAPVNVRVTGKTATSVSLAWDAPS
SGANIAEYVVSFENRSISVKETSAEIGGLKPGTAYSFTVSAKDAD

>ChiA1ΔFnIIIDΔChBD 446 aa 1338 bp

MKIVLINKSKKFFVFSFIFVMMLSLSFVNGEVAKADSGKNYKIIGYYPSWGAYGRDFQVWDMDVSKVSH
INYAFADICWEGRHGNPDPTGPNPQTWSCQDENGVIDAPNGTIVMGDPWIDAQKSNPGDVWDEPIRGNF
KQLLKLKKSHPHLKTFISVGGWTWSNRFSDVAADPAARENFAASAVEFLRKYGFDGVDLDWEYPVSGGL
PGNSTRPEDKRNYTLLLQEVRKKLDAAEAKDGKEYLLTIASGASPDYVSNTELDKIAQTVDWINIMTYD
FNGGWQSISAHNAPLFYDPKAKEAGVPNAETYNIENTVKRYKEAGVKGDKLVLGTPFYGRGWSGCEPGG
HGEYQKCGPAKEGTWEKGVFDFSDLERNYVNQNGYKRYWNDQAKVPFLYNAENGNFITYDDEQSFGHKT
DFIKANGLSGAMFWDFSGDSNRTLLNKLAAD

>ChiA1-60N 716 aa 2148 bp

MRKFNKPLLALLIGSTLCSAAQAAAPGKPTIAWGNTKFAIVEVDQAATAYNSLVKVKDAADVSVSWNLW
NGDTGTTAKVLLNGKEAWSGPSTGSSGTANFKVNKGGRYQMQVALCNADGCSASDATEIVVADTDGSHL
APLKEPLLEKNKPYKQNSGSKIIGYYPSWGAYGRDFQVWDMDVSKVSHINYAFADICWEGRHGNPDPTG
PNPQTWSCQDENGVIDAPNGTIVMGDPWIDAQKSNPGDVWDEPIRGNFKQLLKLKKSHPHLKTFISVGG
WTWSNRFSDVAADPAARENFAASAVEFLRKYGFDGVDLDWEYPVSGGLPGNSTRPEDKRNYTLLLQEVR
KKLDAAEAKDGKEYLLTIASGASPDYVSNTELDKIAQTVDWINIMTYDFNGGWQSISAHNAPLFYDPKA
KEAGVPNAETYNIENTVKRYKEAGVKGDKLVLGTPFYGRGWSGCEPGGHGEYQKCGPAKEGTWEKGVFD
FSDLERNYVNQNGYKRYWNDQAKVPFLYNAENGNFITYDDEQSFGHKTDFIKANGLSGAMFWDFSGDSN
RTLLNKLAADLDFAPDGGNPEPPSSAPVNVRVTGKTATSVSLAWDAPSSGANIAEYVVSFENRSISVKE
TSAEIGGLKPGTAYSFTVSAKDADGKLHAGPTVEVTTNSDQACSYDEWKETSAYTGGERVAFNGKVYEA
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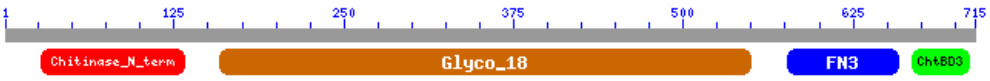

>ChiA1-60ΔFnIIIDΔChBD 568 aa 1694 bp

MRKFNKPLLALLIGSTLCSAAQAAAPGKPTIAWGNTKFAIVEVDQAATAYNSLVKVKDAADVSVSWNLW
NGDTGTTAKVLLNGKEAWSGPSTGSSGTANFKVNKGGRYQMQVALCNADGCSASDATEIVVADTDGSHL
APLKEPLLEKNKPYKQNSGSKIIGYYPSWGAYGRDFQVWDMDVSKVSHINYAFADICWEGRHGNPDPTG
PNPQTWSCQDENGVIDAPNGTIVMGDPWIDAQKSNPGDVWDEPIRGNFKQLLKLKKSHPHLKTFISVGG
WTWSNRFSDVAADPAARENFAASAVEFLRKYGFDGVDLDWEYPVSGGLPGNSTRPEDKRNYTLLLQEVR
KKLDAAEAKDGKEYLLTIASGASPDYVSNTELDKIAQTVDWINIMTYDFNGGWQSISAHNAPLFYDPKA
KEAGVPNAETYNIENTVKRYKEAGVKGDKLVLGTPFYGRGWSGCEPGGHGEYQKCGPAKEGTWEKGVFD
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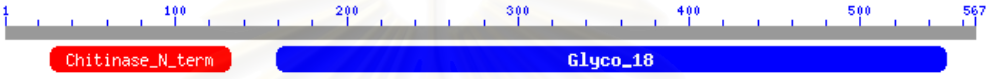
RTLLNKLAADLDFAP



>Chi60-A1FnIIID&ChBD 709 aa 2118 bp

MRKFNKPLLALLIGSTLCSAAQAAAPGKPTIAWGNTKFAIVEVDQAATAYNNLVKVKNAADVSVSWNLW
NGDAGTTAKILLNGKEAWSGPSTGSSGTANFKVNKGGRYQMQVALCNADGCTASDATEIVVADTDGSHL
APLKEPLLEKNKPYKQNSGKVVGSYFVEWGVYGRNFTVDKIPAQNLTHLLYGFIPICGGNGINDSLKEI
EGSFQALQRSCQGREDFKVSIHDPFAALQKAQKGVTAWDDPYKGNFGQLMALKQAHPDLKILPSIGGWT
LSDPFFFMGDKVKRDRFVGSVKEFLQTWKFFDGVDIDWEFPGGKGANPNLGSPQDGETYVLLMKELRAM
LDQLSAETGRKYELTSAISAGKDKIDKVAYNVAQNSMDHIFLMSYDFYGAFDLKNLGHQTALNAPAWKP
DTAYTTVNGVNALLAQGVKPGKIVVGTAMYGRGWTGVNGYQNNIPFTGTATGPVKGTWENGIVDYRQIA
GQFMSGEWQYTYDATAEAPYVFKPSTGDLITFDDARSVQAKGKYVLDKQLGGLFSWEIDADNGDILNSM
NASLLDFAPDGGNPEPPSSAPVNVRVTGKTATSVSLAWDAPSSGANIAEYVVSFENRSISVKETSAEIG
GLKPGTAYSFTVSAKDADGKLHAGPTVEVTTNSDQACSYDEWKETSAYTGGERVAFNGKVYEAKWWTKG
DRPDQSGEWGVWRLIGGCE

Figure 3.18 Amino acid sequence and domain mapping of modified chitinase A1 and chitinase 60. The amino acid sequence was translated by Swissprot program and the domain mapping was predicted by BLASTP program.

supernatant by centrifugation. Partial purification of chitinases was performed by colloidal chitin adsorption, except for ChiA1-60N and ChiA1-60NΔFnIIIΔChBD, lacking the chitin binding ability, was performed by DEAE ion exchange column chromatography. Crude enzymes were concentrated by ultrafiltration with VIVA (50) flow (10 kDa cut off) and adsorbed onto colloidal chitin, the purified enzymes are shown in Figure 3.19 and Figure 3.20. The molecular mass of ChiA1 and modified A1 were determined by SDS-PAGE. Molecular weights of the chitinase derivatives were 70 kDa for ChiAl, 62 kDa for ChiA1ΔChBD, 52 kDa for ChiA1ΔFnIIIDΔChBD, 77.8 kDa for ChiA1-60N, 59.8 kDa for ChiA1-60NΔFnIIIΔChBD, and 78 kDa for Chi60-A1FnIIID&ChBD. In addition, the modified enzymes were also found to auto-degrade producing truncated derivative(s).

Optimum pH and optimum temperature of ChiA1 and derivatives

The optimum pH of partial purified enzymes was determined. Chitinase activity was assayed at 50 °C in buffer with pH ranging from 3-12. An activity at different pH was shown in Figure 3.21. The truncated enzymes showed the optimum pH towards the alkaline pH comparing to wild type ChiA1 and showed optimum pH at two pH, 5-7 and 9.

Chitin binding activities of chitinase A1 and derivatives

Chitinases (50 mU) were mixed with colloidal chitin at various time in buffer, and left on ice to allow adsorption, the mixtures were centrifuged and the activity of chitinase Al and derivatives remaining in the supernatant were determined. The amount of bound chitinase was calculated from the difference between the amounts of chitinase activity initially added subtracted with the remaining activity in the supernatant. The amount of chitinase Al and modified chitinases remaining in the supernatant was decreased with time (Figure 3.22).

Effect of ionic strength on activity of ChiA1, ChiA1ΔChBD ChiA1ΔFnIIIDΔChBD and ChiA1-60N

Ionic strength effects were assayed on colloidal chitin at various buffer concentrations (Figure 3.23). The acitivity of ChiA1 increased when the ionic strength

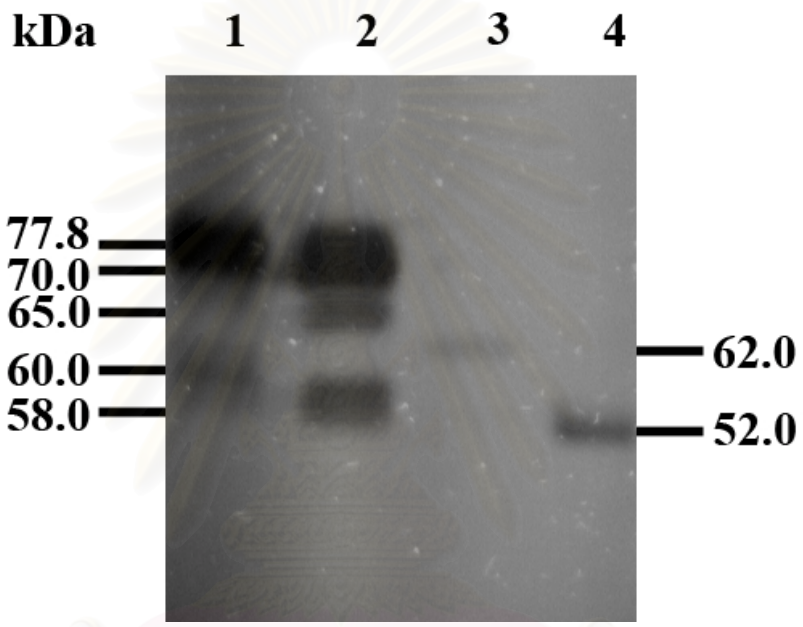


Figure 3.19 Activity staining of ChiA1 and modified chitinase.

Lane 1 = ChiA1-60N

Lane 2 = WT ChiA1

Lane 3 = ChiA1ΔChBD

Lane 4 = ChiA1ΔFnIIIDΔChBD

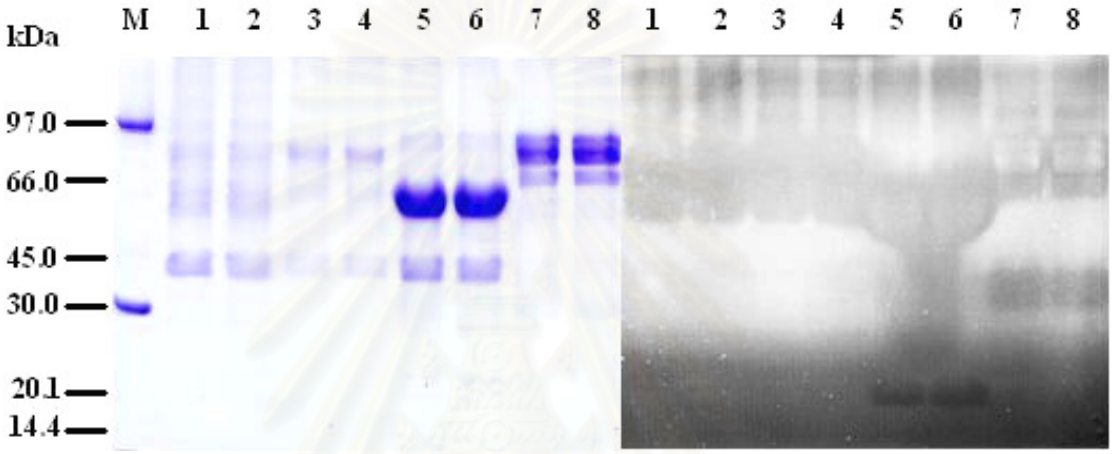


Figure 3.20 SDS-PAGE of ChiA1 derivative chitinases. protein staining (A); activity staining (B).

Lane M = protein marker standard

Lane 1 = ChiA1

Lane 2 = ChiA1 + 1 mM PMSF

Lane 3 = ChiA1-60N

Lane 4 = ChiA1-60N + 1 mM PMSF

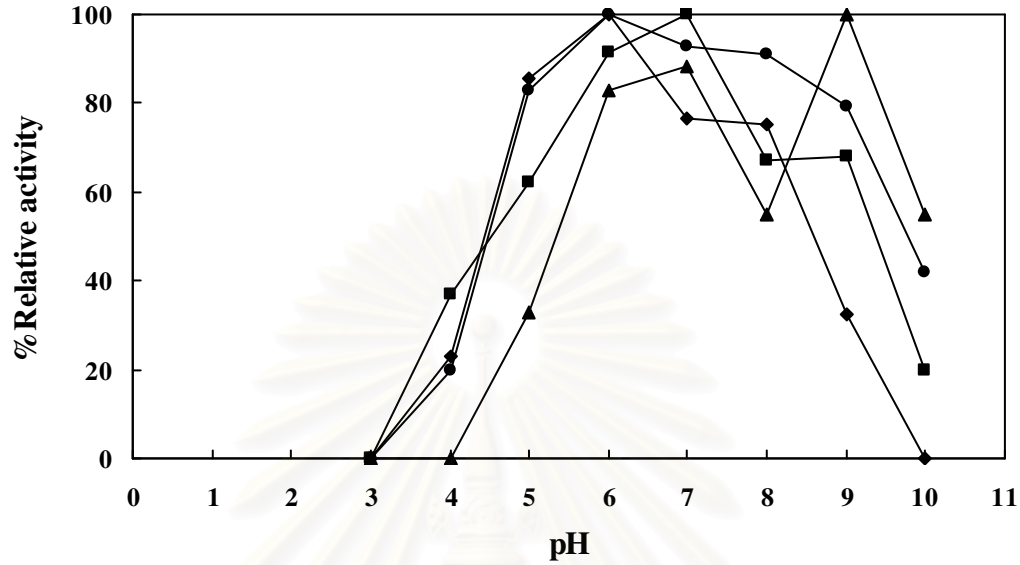
Lane 5 = ChiA1-60NΔFnIIIDΔChBD

Lane 6 = ChiA1-60NΔFnIIIDΔChBD + 1 mM PMSF

Lane 7 = Chi60-A1FnIID&ChBD

Lane 8 = Chi60-A1FnIID&ChBD + 1 mM PMSF

A



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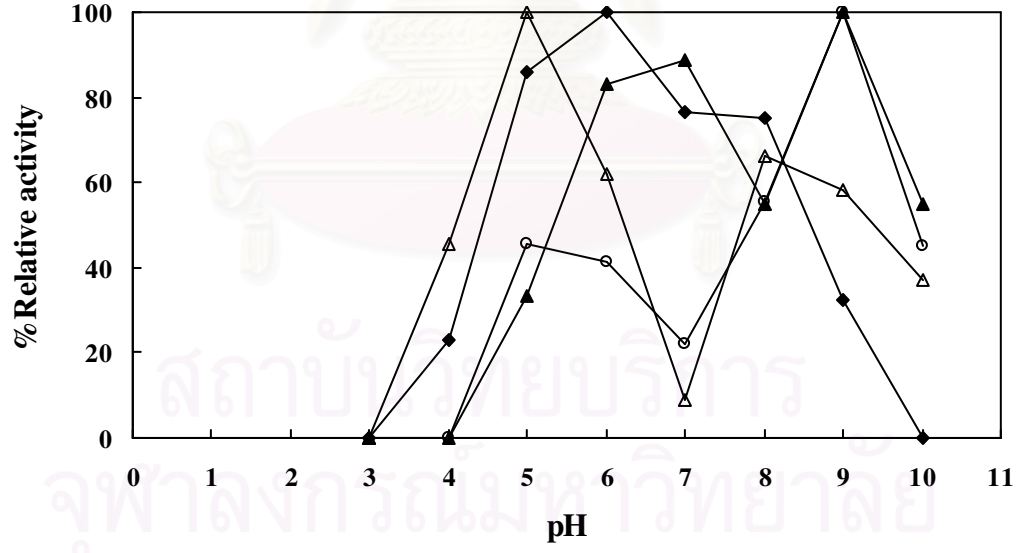


Figure 3.21 The optimum pH of WT ChiA1 and derivative chitinases.

◆, ChiA1; ■, ChiA1ΔChBD; ●, ChiA1ΔFnIIIDΔChBD; ▲,ChiA1-60N: (A)

▲, ChiA1-60N; △,ChiA-60NΔFnIIIDΔChB; ○, Chi60-A1FnIIID&ChBD: (B)

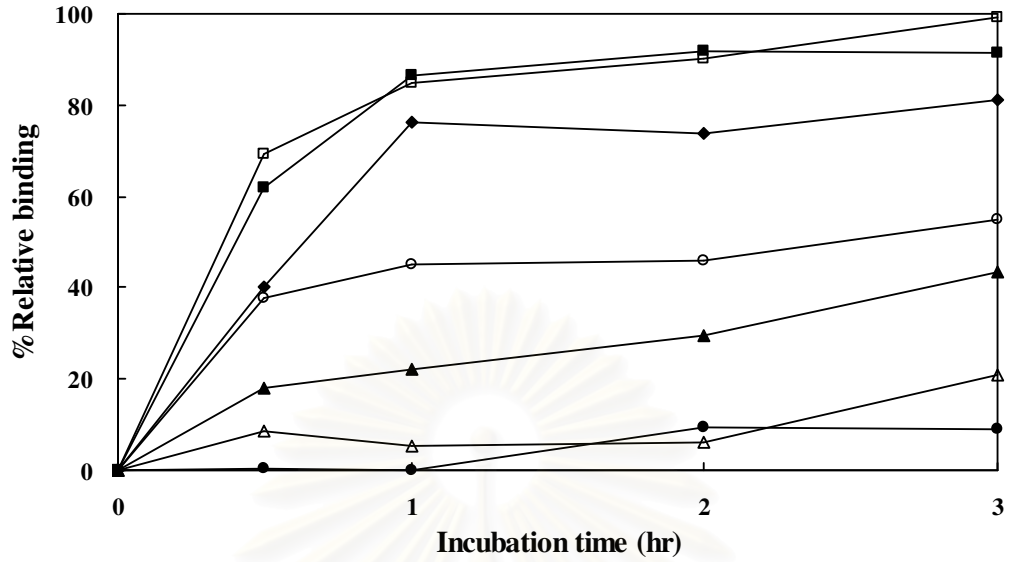


Figure 3.22 Binding of ChiA1 and modified chitinases. The modified enzyme was incubated in 0.1 M citrate buffer pH 6.0 on ice at various times, and taken to assay activity. ChiA1 (◆), ChiA1ΔChBD (■), ChiA1ΔFnIIIDΔChBD (●), ChiA-60N (▲), ChiA-60NΔFnIIIDΔChB (△), Chi60-A1FnIIID&ChBD (○), Chi 60 (□)

Table 3.4 Binding assay of modified chitinases

Enzyme/% Binding	Time (hr)			
	0.5	**1**	**2**	**3**
ChiA1	40.1	76.4	74.0	81.1
ChiA1ΔChBD	61.9	86.5	91.6	91.2
ChiA1ΔFnIIIDΔChBD	0.4	0	9.6	9.1
ChiA1-60N	18.2	22.0	29.5	43.3
ChiA1-60NΔFnIIIDΔChBD	8.7	5.5	6.2	20.7
Chi60-A1FnIIID&ChBD	37.6	45.2	46.0	54.8
Chi60	69.2	84.7	90.3	99.1

The amounts of the bound chitinase were calculated by determining the difference between the amount of the total bound activity and the amount of the unbound activity.

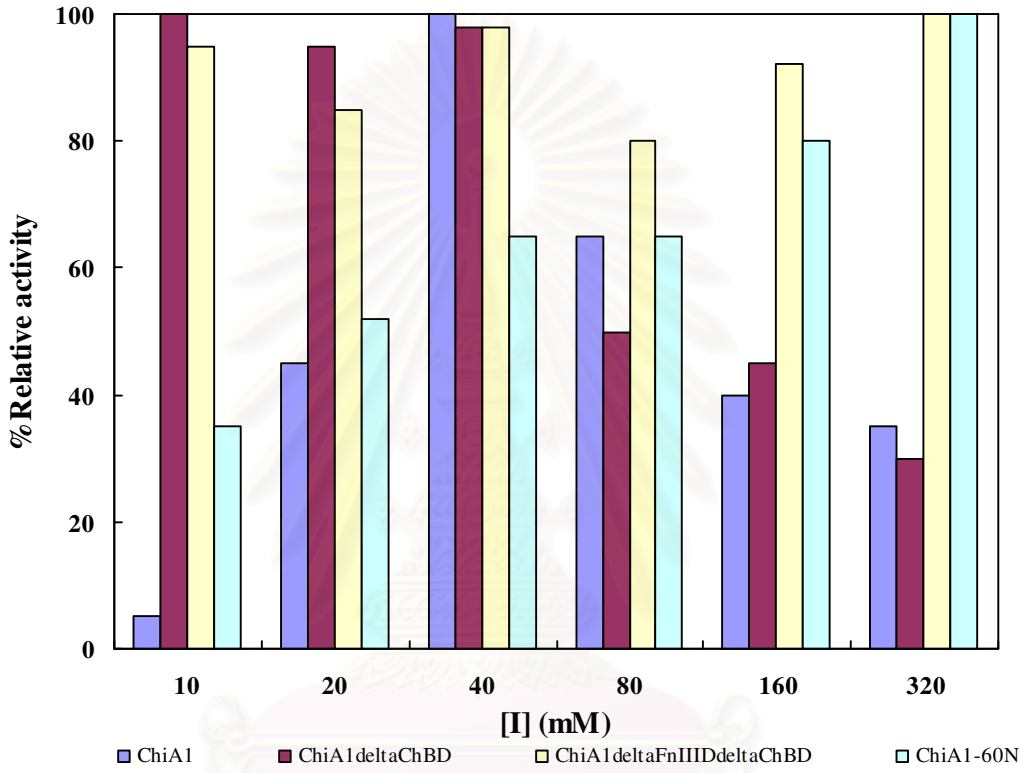


Figure 3.23 Effect of ionic strength on various chitinases. A reaction mixture was contained 10 mg/mL of each chitinase at various ionic strengths. Reactions were performed at 50 °C, and the amount of reducing sugar generated was monitored.

was increased from 0-40 mM NaCl, and decreased at ionic strength higher than 320 mM NaCl. The activity of ChiA1ΔChBD decreased at 80 mM NaCl and above. On the other hand, the ionic strength did not effect ChiA1ΔFnIIIDΔChBD activity. ChiA1-60N activity slightly increased when the ionic strength was increased and further increase in ionic strength did not have any futher effect.

Hydrolysis of chitin by modified chitinases

The effect of various deletions on the hydrolysis of insoluble substrates was examined by using colloidal chitin and beta chitin as the substrate. Wild-type ChiA1 and derivatives were first studied for their substrate specificity. The enzymes were assayed on soluble substrate, partially *N*-acetylated chitin (PNAC). The amount of enzyme which yields equal activity on PNAC was used to hydrolyze crystalline substrate, β chitin, and amorphous substrate, colloidal chitin. This is to ensure equal quantity of active catalytic domain present in each reaction. The differences in the activity observed would be a result from the change in substrate specificity of the ChiA1 derivatives. On β-chitin we observed that the activity of the deletion mutants ChiA1ΔChBD and ChiA1ΔIIIΔChBD reduces as more of the *C*-terminal was deleted, Table 3.5. Interestingly, when colloidal chitin was use as substrate we observed the highest activity with ChiA1ΔChBD followed by ChiA1ΔIIIΔChBD and wild-type ChiA1, Table 3.6.

Hydrolysis of various chitinase substrates by modified chitinases

The effect of deletions on the hydrolysis of various chitinase substrates was examined (Figure 3.24). The substrates tested included partially *N*-acetylated chitin as soluble substrate, colloidal chitin and β-chitin as insoluble substrate. As the size of the deleted region of the modified chitinase increased, the activity of the enzymes toward all substrates tested tended to decrease.

Table 3.5 The chitinase activity of WT and mutants on crystalline and amorphous substrate.

Wild type and derivative mutants	Chitinase activity (mU/ml)		
	PNAC	CC	BC
ChiA1 (WT)	50	66	47
ChiA1ΔChBD	50	204	45
ChiA1ΔFnIIIDΔChBD	50	110	22
ChiA1-60N	50	46	48

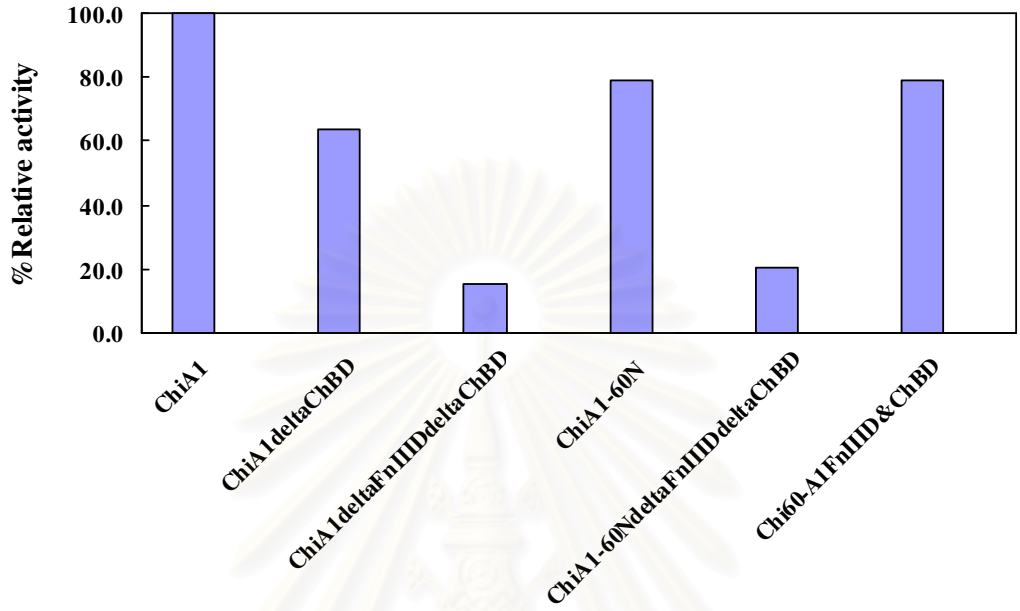
The activity of wild-type and derivatives of ChiA1 was assayed on crystalline (β-chitin, BC) and amorphous (colloidal chitin, CC) substrate. The table presents the activity of wild-type and its derivatives on each substrate. The results above are averages of three independent assays.

Table 3.6 The relative activity of WT and mutants on crystalline and amorphous substrate.

Wild type and derivative mutants	Relative activity	
	CC	BC
ChiA1 (WT)	0.30	0.50
ChiA1ΔChBD	1.00	0.38
ChiA1ΔFnIIIDΔChBD	0.54	0.23
ChiA1-60N	0.21	0.52
ChiA1-60NΔFnIIIDΔChBD	0.29	0.19
Chi60-A1FnIIID&ChBD	0.47	0.32
Chi60	0.49	1.00

The activity of wild-type and derivatives of ChiA1 was assayed on crystalline (β-chitin, BC) and amorphous (colloidal chitin, CC) substrate. The table presents the relative activity of wild-type and its derivatives on each substrate. The results above are averages of three independent assays.

A



B

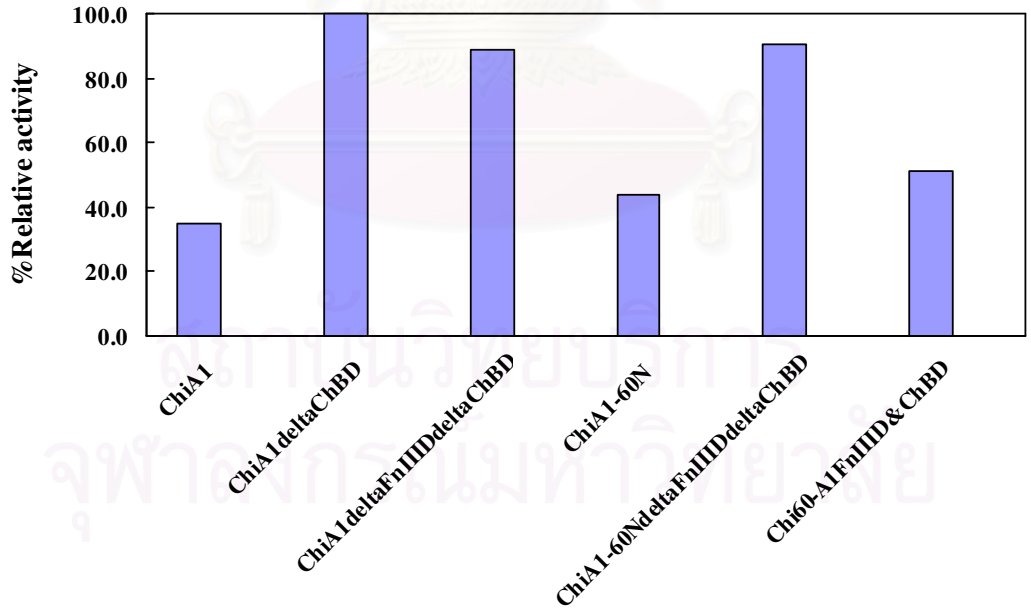


Figure 3.24 The substrate specificity of wild type ChiA1 and derivatives.
β-chitin, (A); colloidal chitin, (B).

The hydrolytic product from chitin by modified chitinases

The products from hydrolysis of colloidal chitin, β-chitin and chitooligosaccharides were also analyzed by silica TLC. Hydrolysis of colloidal chitin in 100 mM citrate pH 6.0 at 50 °C for 24 hr by the chitinase drivatives produced a final mixture of chitobiose, chitotriose and GlcNAc, in which chitobiose was the dominant product (Figure 3.25). As shown in Figure 3.26A, chitotriose was broken down to chitobiose and NAG, whereas chitobiose was not split by the enzyme at all, even with the incubation time prolonged to overnight (data not shown). When chitohexaose was used as substrate (Figure 3.26B), chitobiose, chitotriose, chitotetraose, and chitopentaose were detected in product mixture within 1 hr. of incubation. Chitotriose and chitopentaose could be produced only by the action of endochitinase activity, whereas chitobiose and chitotetraose could be released by either endochitinase or exochitinase activity. These results demonstrated that the truncated of ChiA1 possessed both endo- and exosplitting activities.

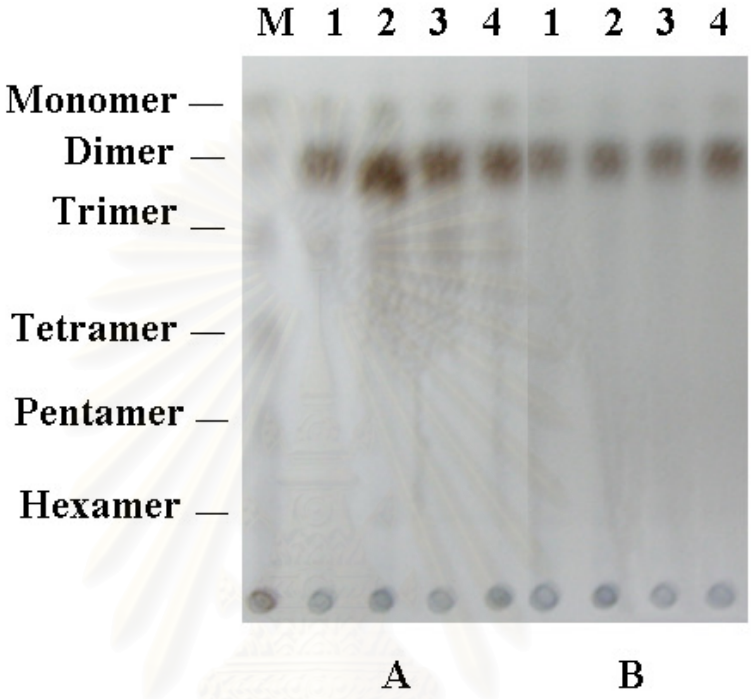


Figure 3.25 The hydrolytic product from colloidal chitin and β-chitin by modified chitinase. colloidal chitin (A); β-chitin(B).

Lane M = *N*-acetylchitooligosaccharide standard

Lane 1 = ChiA1

Lane 2 = ChiA1ΔChBD

Lane 3 = ChiA1ΔFnIIIDΔChBD

Lane 4 = ChiA1-60N

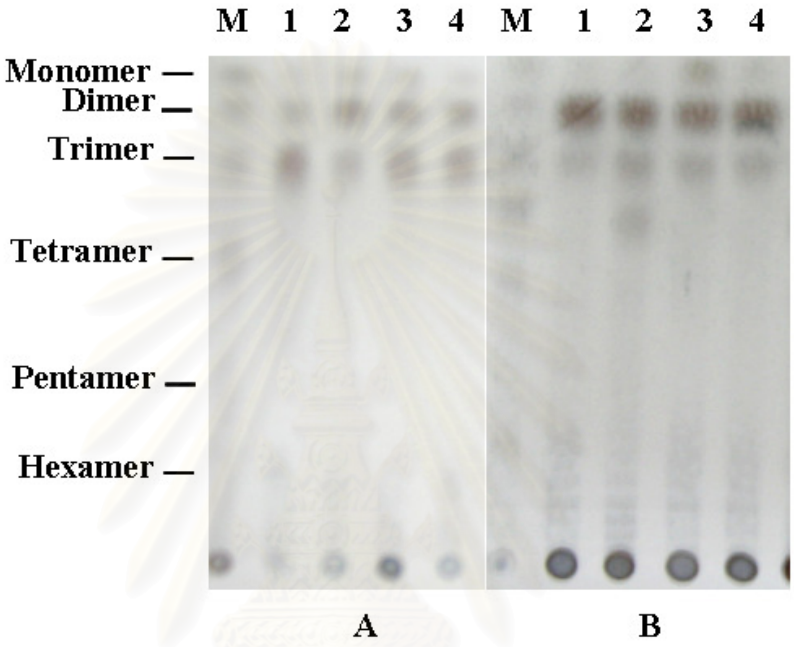


Figure 3.26 The hydrolytic product from colloidal chitin and β-chitin by modified chitinase. NAG_3 (A); NAG_6 (B).

Lane M = *N*-acetylchitooligosaccharide standard

Lane 1 = ChiA1

Lane 2 = ChiA1ΔChBD

Lane 3 = ChiA1ΔFnIIIDΔChBD

Lane 4 = ChiA1-60N

Part III: Construction and characterization of recombination of Chi60 and Chi B (chimeric chitinase) by homologous recombination.

Construction of pSKchi60B

As shown in Figure 2.3, pSKChi60B was constructed by the insertion of a fragment of pSKChiB into site of pKKChi60, resulting in a plasmid that had the chitinase 60 gene on the 5′ end of ChiB gene (Figure 2.3 and Figure 2.4)

Screening for chitinolytic activity

The homologous recombinants were screened on CCMM and incubated 37 °C for several weeks. The result showed that the chimeric chitinase did not form transparent clearing zones (Figure 3.27).

The homologous recombinants were produced enzyme in LB broth supplement with 100 μg/mL amplicillin. The chitinolytic activity was assayed with NAG_4 by TLC. The results showed that the 4 homologous recombinants were hydrolyzed NAG_4 (Figure 3.28).

DNA sequencing

All mutated and chimeric clones were checked by DNA sequencing. The recombination site of the recombinant was showed in Figure 3.29. The corrected nucleotide sequence and amino acid sequence were shown in Figure 3.30 and Figure 3.31.

Protein prediction by SWISS-MODEL

Homology modeling of the structure of RecSK-1 was accomplished by SWISS model protein modeling (SWISS-MODEL version 36.002, Guex and Peitsch, 1995; Peitsch, 1996; Peitsch, 1997). The homology modeling of the structure was created by RasWin version 2.7.3 as shown in Figure 3.32. From the results, the theoretical model of RecSK-1 was homology with Chi60 (residues 1-418) and was deleted the residues 419-563 and inserted the residues 462-499 of ChiB (Figure 3.33).

Production and partial purification of the chimeric chitinase

Plasmids carrying *RecSK-1* gene was introduced into *E. coli* Top10 for chitinase production. After culture, chitinase in the supernatant was separated from cells by centrifugation. The supernatant was concentrated by VIVA (50) flow (10,000 Da cut off) (Figure 3.34).

The optimum pH and temperature of chimeric chitinase

The optimum pH of the chimeric enzyme was pH 4.0. The enzyme activity decreased rapidly from pH 6.0 and totally lost all activity at pH 8.0 and above (Figure 3.35).

The optimum temperature of partial purified enzyme was determined at pH 4.0, in the ranging of 40-80 °C. The partial purified enzyme has the optimum temperature at 60 °C (Figure 3.36).

The chitinase activity on different substrates

An action of crude enzyme from chimeric chitinase on chitin and related compounds was studied in this work. The hydrolysis of chitin and related a compound with chitinase was examined at pH 5.0, 50 °C. Chitinase showed high activity on partially *N*-acetylated chitin, followed by β-chitin, colloidal chitin and pNP-NAG, respectively (Figure 3.37). Interestingly, chimeric chitinase hydrolyzed pNP-NAG and NAG_2 to produce NAG.

The hydrolytic products from colloidal chitin by crude chimeric enzyme

The chimeric chitinase hydrolyzed β-chitin and colloidal chitin to produce a mixture of *N*-acetylglucosamine and *N*, *N′*-diacetylchitobiose (Figure 3.38-Figure 3.40).

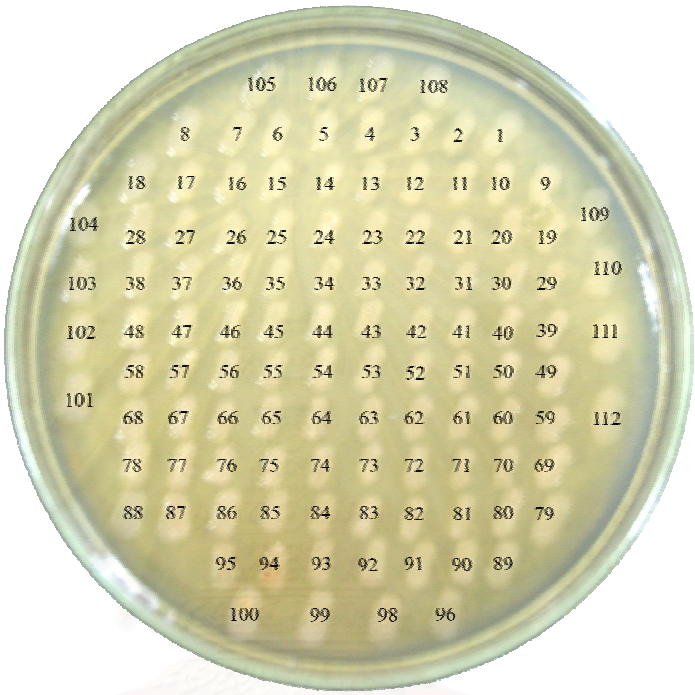
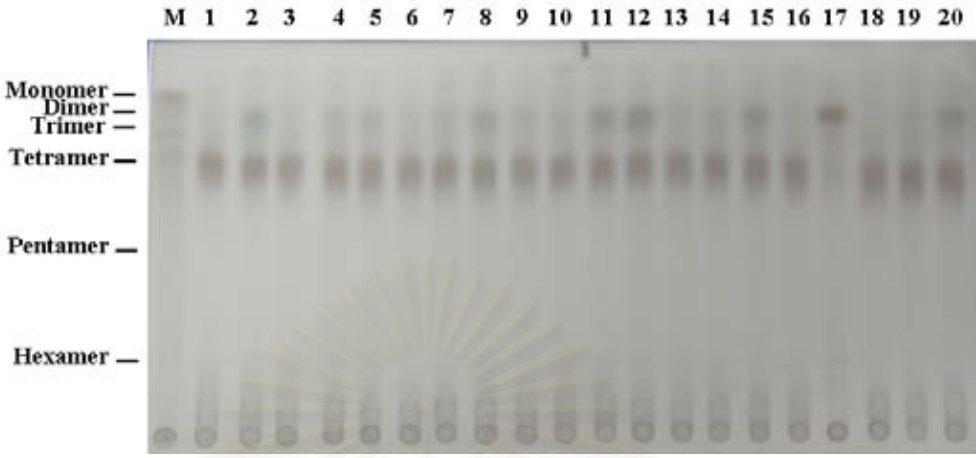
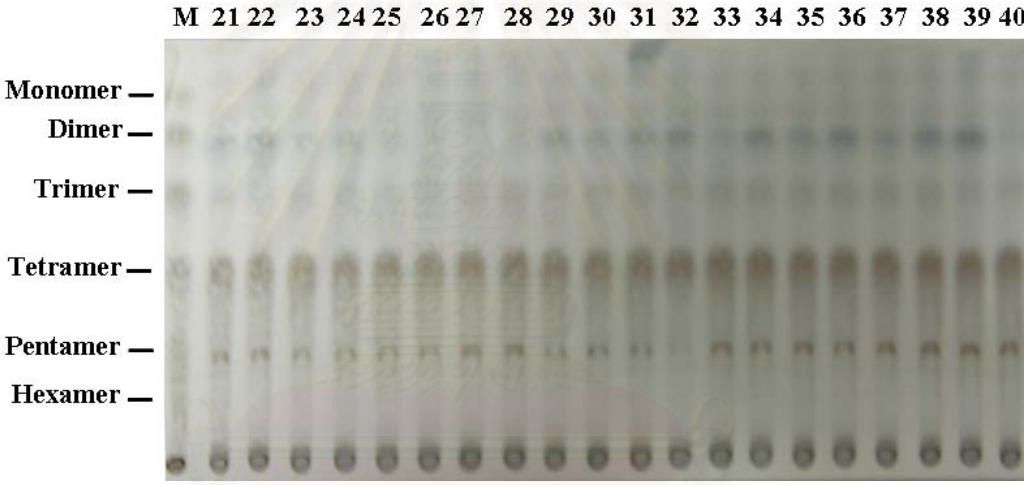


Figure 3.27 The primary screening of chimeric chitinases on colloidal chitin minimum medium agar. The homologous recombination clones were incubated at 37 °C for 7 days.

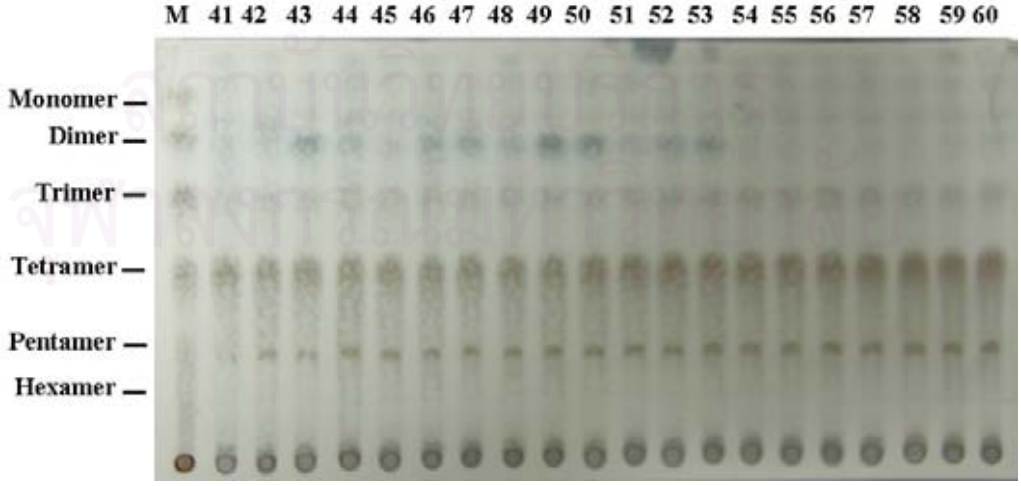
A


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Monomer
Dimer
Trimer
Tetramer
Pentamer
Hexamer

B


M 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
Monomer
Dimer
Trimer
Tetramer
Pentamer
Hexamer

C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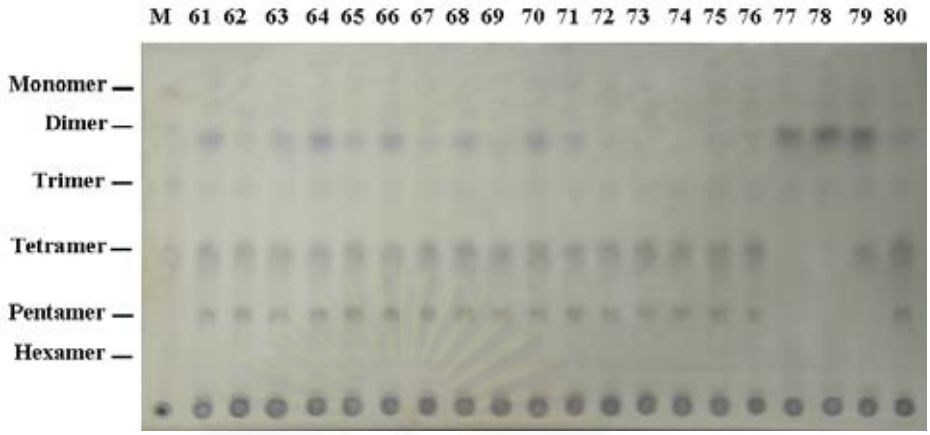
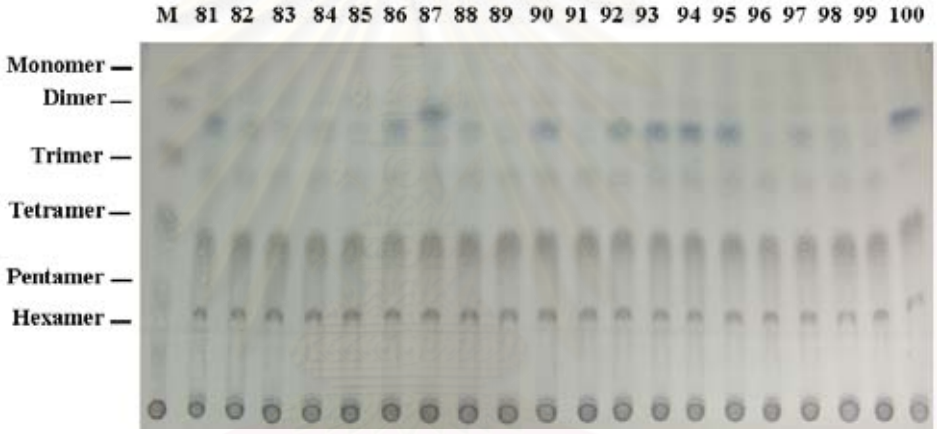
Monomer
Dimer
Trimer
Tetramer
Pentamer
Hexamer

D



E



F

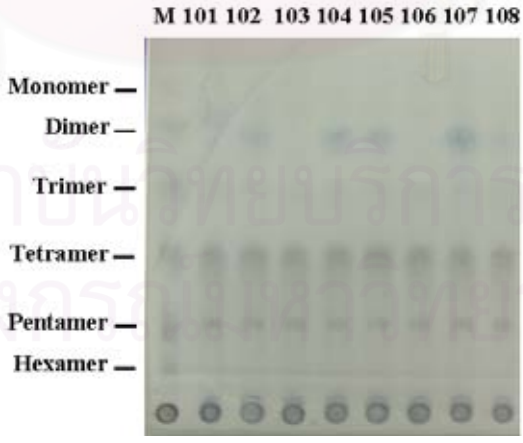


Figure 3.28 The secondary screening of chimeric chitinase on TLC. The crude enzyme from recombinants was used to hydrolyze NAG_4 at 50 °C for 12 hr and spotted on TLC. Recombinant number 1-20 (A); 21-40 (B); 41-60 (C); 61-80 (D); 81-100 (E); 101-108 (F).

```
ChiB     ------------------------------------------------------------
Chi60    ATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCGCG 60

ChiB     ------------------------------------------------------------
Chi60    GCGCAGGCCGCGGCGCCGGGCAAGCCGACCATCGCCTGGGGCAATACCAAGTTCGCCATC 120

ChiB     ------------------------------------------------------------
Chi60    GTTGAAGTTGACCAGGCGGCTACCGCTTATAATAGTTTGGTGAAGGTAAAAGATGCCGCC 180

ChiB     ------------------------------------------------------------
Chi60    GATGTTTCGGTCTCCTGGAATTTATGGAATGGCGACACCGGTACGACGGCAAAAGTTTTA 240

ChiB     ------------------------------------------------------------
Chi60    TTAAATGGCAAAGAGGCGTGGAGCGGCCCGTCAACCGGTTCTTCCGGTACGGCGAATTTT 300

ChiB     ------------------------------------------------------------
Chi60    AAAGTCAATAAAGGCGGCCGTTATCAAATGCAGGTGGCATTGTGCAATGCCGACGGCTGC 360

ChiB     ------------------------------------------------------------
Chi60    AGCGCCAGCGACGCCACCGAAATTGTGGTGGCCGACACCGACGGCAGCCATTTGGCGCCG 420

ChiB     ------------------------------------------------------------
Chi60    TTGAAAGAGCCGCTGCTGGAAAAGAATAAACCGTATAAACAGAACTCCGGCAAAGTCGTC 480

ChiB     ------------------------------------------------------------
Chi60    GGTTCTTATTTCGTCGAGTGGGGCGTTTACGGGCGCAATTTCACCGTCGACAAGATCCCG 540

ChiB     ------------------------------------------------------------
Chi60    GCGCAGAACCTGACCCACCTGCTGTACGGCTTTATCCCGATCTGCGGCGGCAACGGCATC 600

ChiB     ------------------------------------------------------------
Chi60    AACGACAGCCTGAAAGAGATCGAAGGCAGCTTCCAGGCGCTGCAGCGCTCCTGCCAGGGC 660

ChiB     ------------------------------------------------------------
Chi60    CGCGAGGACTTCAAAGTCTCGATCCACGATCCGTTCGCCGCGCTGCAAAAAGCGCAGAAG 720

ChiB     ------------------------------------------------------------
Chi60    GGCGTTACCGCCTGGGATGACCCCTACAAGGGCAACTTCGGCCAGCTGATGGCGCTGAAA 780

ChiB     ------------------------------------------------------------
Chi60    CAGGCGCATCCTGACCTGAAAATTCTGCCGTCGATCGGCGGCTGGACGCTGTCCGACCCG 840

ChiB     ------------------------------------------------------------
Chi60    TTCTTCTTCATGGGCGATAAGGTGAAGCGCGATCGCTTCGTCGGTTCGGTGAAAGAGTTC 900

ChiB     ------------------------------------------------------------
Chi60    CTGCAGACCTGGAAGTTCTTCGATGGCGTGGATATCGACTGGGAGTTCCCGGGCGGCAAA 96

ChiB     ------------------------------------------------------------
Chi60    GGCGCCAACCCGAACCTGGGCAGCCCGCAGGACGGGGAAACCTATGTGCTGCTGATGAAG 1020

ChiB     ------------------------------------------------------------
Chi60    GAGCTGCGGGCGATGCTGGATCAGCTGTCGGCGGAAACCGGCCGCAAATATGAACTGACC 1080

ChiB     ------------------------------------------------------------
Chi60    TCCGCCATCAGCGCCGGCAAGGACAAGATCGATAAGGTGGCTTACAACGTTGCGCAGAAC 1140

ChiB     ------------------------------------------------------------GC 2
Chi60    TCGATGGATCACATCTTCCTGATGAGCTACGACTTCTATGGCGCCTTCGATCTGAAGAAC 1200
                                                                     *

ChiB     CCCGGCAACCTGCCGATCATGACCGCGCCGGCCTATGTGCCGGGCACCACTTACGCCCAG 62
Chi60    CTGGGGCATCAGACCGCGCTGAATGCGCCGGCCTGGAAGCCGGACACCGCTTACACC-AC 1259
         *  **  * * * *     ***  **********    ***** **** ***** ** *
```

```
ChiB     GGCGCGCTGGTGTCCTACCAAGGCTACGTCTGGCAGACCAAGTGGGGTTATATCACC-TC 121
Chi60    GGTGAAC-GGCGTCAATGCGCTGCTG-GCGCAGGGCGTCAAGCCGGGCAAGATCGTGGTC 1317
         ** *  * ** ***    *   ***  *    *     ****  ***  * ***    **

ChiB     GGCGCC--CGGCTCAGACAGCGCCTGGCTGAAGGTGGGCCGC--CTGGCGTAA------- 170
Chi60    GGCACCGCCATGTATGGCCGCGGCTGGACCGGGGTGAACGGCTACCAGAACAACATTCCG 1377
         *** **  *   *  * * *** ****     ****  * **  *  *   **

ChiB     ------------------------------------------------------------
Chi60    TTCACCGGTACCGCCACTGGGCCGGTCAAAGGCACCTGGGAGAACGGCATCGTGGACTAC 1437

ChiB     ------------------------------------------------------------
Chi60    CGCCAAATCGCCGGCCAGTTCATGAGCGGCGAGTGGCAGTATACCTACGACGCCACGGCG 1497

ChiB     ------------------------------------------------------------
Chi60    GAAGCGCCTTACGTGTTCAAACCTTCCACCGGCGATCTGATCACCTTCGACGATGCCCGC 1557

ChiB     ------------------------------------------------------------
Chi60    TCGGTGCAGGCCAAAGGCAAGTACGTGCTGGATAAGCAGCTGGGCGGCCTGTTCTCCTGG 1617

ChiB     ------------------------------------------------------------
Chi60    GAGATCGACGCGGACAACGGCGATATTCTCAACAGCATGAACGCCAGCCTGGGCAACAGC 1677


ChiB     ---------------
Chi60    GCCGGCGTTCAATAA 1692
```

Figure 3.29 Linear alignment of nucleotide sequence of *chiB* and *chi60* genes. This alignment used Clustal W version 1.8.3. A star (*) indicates that the identity of base, a dash (-) indicates that the gap between of two sequences. The red letters show the recombination site of two sequences.

>pRecSK-1

ATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCGCGGCGCAGGCC
GCGGCGCCGGGCAAGCCGACCATCGCCTGGGGCAATACCAAGTTCGCCATCGTTGAAGTTGACCAGGCG
GCTACCGCTTATAATAGTTTGGTGAAGGTAAAAGATGCCGCCGATGTTTCGGTCTCCTGGAATTTATGG
AATGGCGACACCGGTACGACGGCAAAAGTTTTATTAAATGGCAAAGAGGCGTGGAGCGGCCCGTCAACC
GGTTCTTCCGGTACGGCGAATTTTAAAGTCAATAAAGGCGGCCGTTATCAAATGCAGGTGGCATTGTGC
AATGCCGACGGCTGCAGCGCCAGCGACGCCACCGAAATTGTGGTGGCCGACACCGACGGCAGCCATTTG
GCGCCGTTGAAAGAGCCGCTGCTGGAAAAGAATAAACCGTATAAACAGAACTCCGGCAAAGTCGTCGGT
TCTTATTTCGTCGAGTGGGGCGTTTACGGGCGCAATTTCACCGTCGACAAGATCCCGGCGCAGAACCTG
ACCCACCTGCTGTACGGCTTTATCCCGATCTGCGGCGGCAACGGCATCAACGACAGCCTGAAAGAGATC
GAAGGCAGCTTCCAGGCGCTGCAGCGCTCCTGCCAGGGCCGCGAGGACTTCAAAGTCTCGATCCACGAT
CCGTTCGCCGCGCTGCAAAAAGCGCAGAAGGGCGTTACCGCCTGGGATGACCCCTACAAGGGCAACTTC
GGCCAGCTGATGGCGCTGAAACAGGCGCATCCTGACCTGAAAATTCTGCCGTCGATCGGCGGCTGGACG
CTGTCCGACCCGTTCTTCTTCATGGGCGATAAGGTGAAGCGCGATCGCTTCGTCGGTTCGGTGAAAGAG
TTCCTGCAGACCTGGAAGTTCTTCGATGGCGTGGATATCGACTGGGAGTTCCCGGGCGGCAAAGGCGCC
AACCCGAACCTGGGCAGCCCGCAGGACGGGGAAACCTATGTGCTGCTGATGAAGGAGCTGCGGGCGATG
CTGGATCAGCTGTCGGCGGAAACCGGCCGCAAATATGAACTGACCTCCGCCATCAGCGCCGGCAAGGAC
AAGATCGATAAGGTGGCTTACAACGTTGCGCAGAACTCGATGGATCACATCTTCCTGATGAGCTACGAC
TTCTATGGCGCCTTCGATCTGAAGAACCTGGGGCATCAGACCGCGCTGAATGCGCCGGCCTGGAAGCCG
GACACCGCTTACGCCCAGGGCGCGCTGGTGTCCTACCAAGGCTACGTCTGGCAGACCAAGTGGGGTTAT
ATCACCTCGGCGCCCGGCTCAGACAGCGCCTGGCTGAAGGTGGGCCGCCTGGCGTAA

Figure 3.30 The nucleotide sequence of chimeric chitinase. The blue letters show the sequence of *chiB* gene and the black letters show the sequence of *chi60* gene. The red letters show the recombination site between *chi60* and *chiB* genes.

atgcgcaaatttaataaaccgctgttggcgctgttgatcggcagcacgctgtgttccgcg
M R K F N K P L L A L L I G S T L C S A
gcgcaggccgcggcgccgggcaagccgaccatcgcctggggcaataccaagttcgccatc
A Q A A A P G K P T I A W G N T K F A I
gttgaagttgaccaggcggctaccgcttataatagtttggtgaaggtaaaagatgccgcc
V E V D Q A A T A Y N S L V K V K D A A
gatgtttcggtctcctggaatttatggaatggcgacaccggtacgacggcaaaagtttta
D V S V S W N L W N G D T G T T A K V L
ttaaatggcaaagaggcgtggagcggcccgtcaaccggttcttccggtacggcgaatttt
L N G K E A W S G P S T G S S G T A N F
aaagtcaataaaggcggccgttatcaaatgcaggtggcattgtgcaatgccgacggctgc
K V N K G G R Y Q M Q V A L C N A D G C
agcgccagcgacgccaccgaaattgtggtggccgacaccgacggcagccatttggcgccg
S A S D A T E I V V A D T D G S H L A P
ttgaaagagccgctgctggaaaagaataaaccgtataaacagaactccggcaaagtcgtc
L K E P L L E K N K P Y K Q N S G K V V
ggttcttatttcgtcgagtggggcgtttacgggcgcaatttcaccgtcgacaagatcccg
G S Y F V E W G V Y G R N F T V D K I P
gcgcagaacctgacccacctgctgtacggctttatcccgatctgcggcggcaacggcatc
A Q N L T H L L Y G F I P I C G G N G I
aacgacagcctgaaagagatcgaaggcagcttccaggcgctgcagcgctcctgccagggc
N D S L K E I E G S F Q A L Q R S C Q G
cgcgaggacttcaaagtctcgatccacgatccgttcgccgcgctgcaaaaagcgcagaag
R E D F K V S I H D P F A A L Q K A Q K
ggcgttaccgcctgggatgacccctacaagggcaacttcggccagctgatggcgctgaaa
G V T A W D D P Y K G N F G Q L M A L K
caggcgcatcctgacctgaaaattctgccgtcgatcggcggctggacgctgtccgacccg
Q A H P D L K I L P S I G G W T L S D P
ttcttcttcatgggcgataaggtgaagcgcgatcgcttcgtcggttcggtgaaagagttc
F F F M G D K V K R D R F V G S V K E F
ctgcagacctggaagttcttcgatggcgtggatatcgactgggagttcccgggcggcaaa
L Q T W K F F D G V D I D W E F P G G K
ggcgccaacccgaacctgggcagcccgcaggacggggaaacctatgtgctgctgatgaag
G A N P N L G S P Q D G E T Y V L L M K
gagctgcgggcgatgctggatcagctgtcggcggaaaccggccgcaaatatgaactgacc

E L R A M L D Q L S A E T G R K Y E L T

tccgccatcagcgccggcaaggacaagatcgataaggtggcttacaacgttgcgcagaac

S A I S A G K D K I D K V A Y N V A Q N

tcgatggatcacatcttcctgatgagctacgacttctatggcgccttcgatctgaagaac

S M D H I F L M S Y D F Y G A F D L K N

ctggggcatcagaccgcgctgaatgcgccggcctggaagccggacaccgcttacgcccag

L G H Q T A L N A P A W K P D T A Y A Q

ggcgcgctggtgtcctaccaaggctacgtctggcagaccaagtggggttatatcacctcg

G A L V S Y Q G Y V W Q T K W G Y I T S

gcgcccggctcagacagcgcctggctgaaggtgggccgcctggcgtaa

A P G S D S A W L K V G R L A –

Figure 3.31 The amino acid translation of the chimeric chitinase was created by Swissprot. The red letters indicated that the recombination site. The blue letters indicated that the amino acid sequence of ChiB.

>RecSK-1

MRKFNKPLLALLIGSTLCSAAQAAAPGKPTIAWGNTKFAIVEVDQAATAYNSLVKVKDAADVSVSWNLW
NGDTGTTAKVLLNGKEAWSGPSTGSSGTANFKVNKGGRYQMQVALCNADGCSASDATEIVVADTDGSHL
APLKEPLLEKNKPYKQNSGKVVGSYFVEWGVYGRNFTVDKIPAQNLTHLLYGFIPICGGNGINDSLKEI
EGSFQALQRSCQGREDFKVSIHDPFAALQKAQKGVTAWDDPYKGNFGQLMALKQAHPDLKILPSIGGWT
LSDPFFFMGDKVKRDRFVGSVKEFLQTWKFFDGVDIDWEFPGGKGANPNLGSPQDGETYVLLMKELRAM
LDQLSAETGRKYELTSAISAGKDKIDKVAYNVAQNSMDHIFLMSYDFYGAFDLKNLGHQTALNAPAWKP
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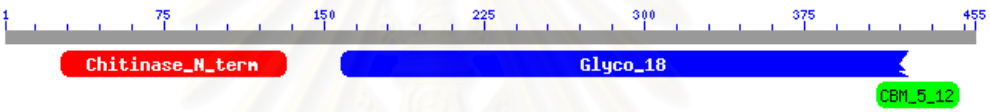


Figure 3.32 Amino acid sequence and domain mapping of chimeric. The amino acid sequence was translated by Swissprot program and the domain mapping was predicted by BLASTP program (red: *N*-terminal domain; blue: catalytic domain; green: chitin binding domain 5).

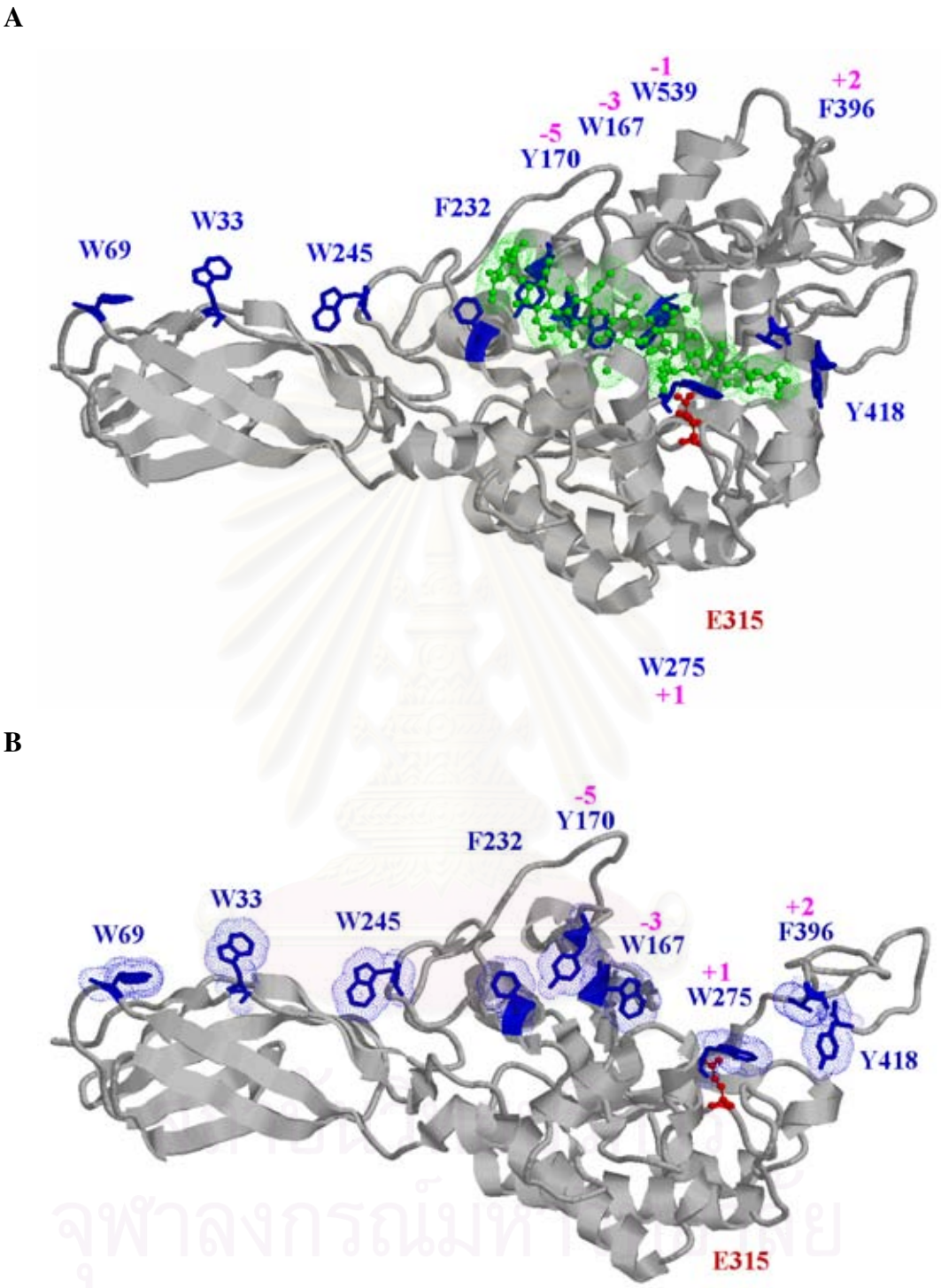


Figure 3.33 The 3D structure of ChiA (pdb id: 1EHN, upper panel) and the theoretical models of chimeric Chi60B (lower panel). The catalytic acids (Glu315 in ChiA) ChiA binds $(GlcNAc)_8$ (colored green) in subsites -4 to +2; Chi60B in subsites -4 to +2. Side chains of aromatic residues involved in substrate binding are shown as blue sticks. This figure was created using RasWin version 2.7.3.

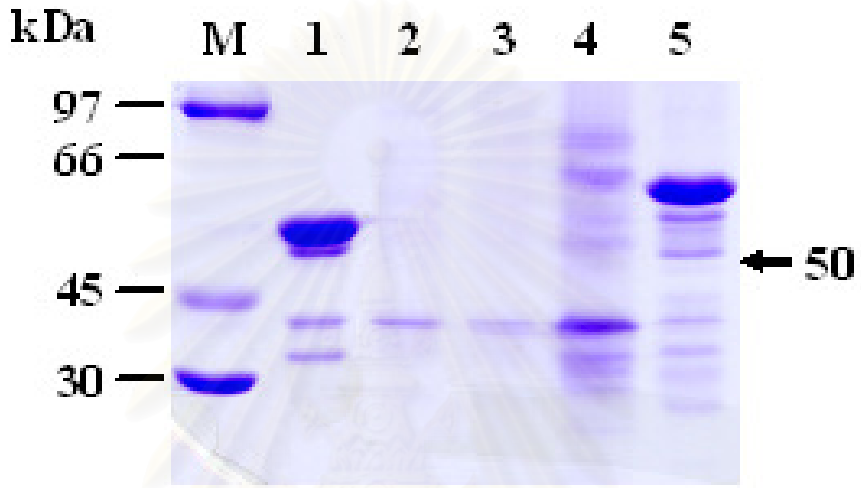


Figure 3.34 SDS-PAGE of homologous recombination chimeric chitinase between Chi60 and ChiB from *Serratia* sp.

Lane M = protein marker standard

Lane 1 = partial purified ChiB

Lane 2 = recombination 1

Lane 3 = recombination 2

Lane 4 = recombination 3 (RecSK-1)

Lane 5 = partial purified Chi60

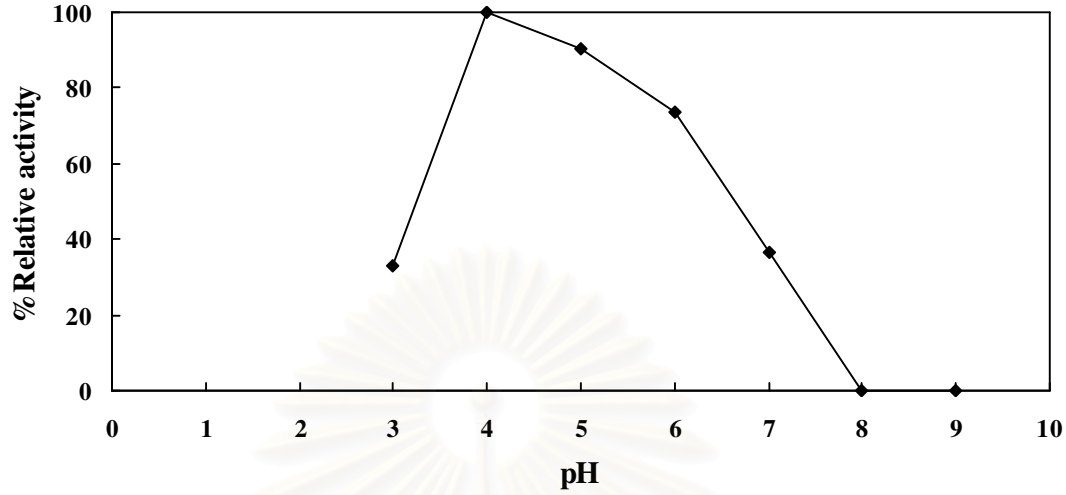


Figure 3.35 The optimum pH of chimeric chitinase. Chitinolytic activity was measured in pH range of 3-10 at 50 °C for 1 hr by colorimetric method when using PNAC as substrate.

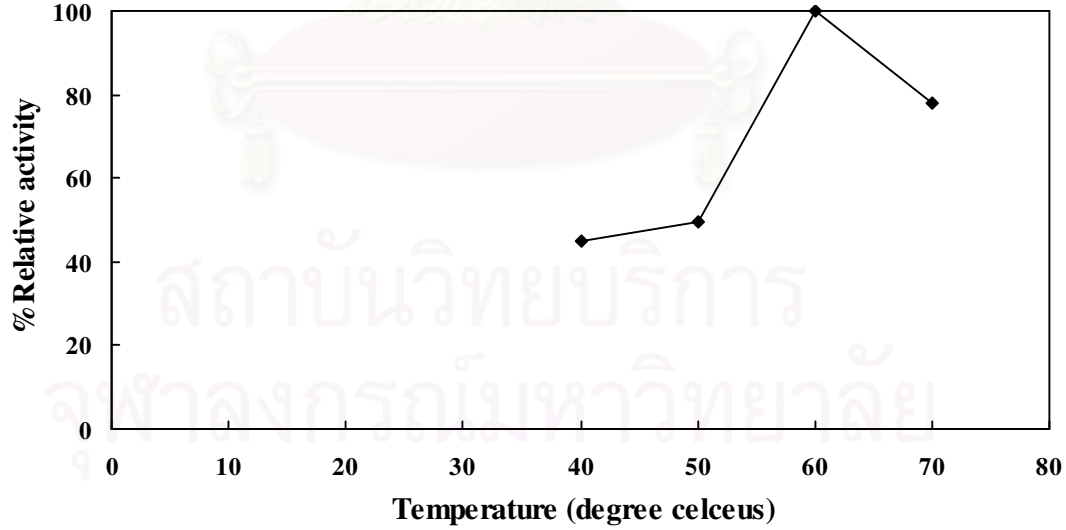


Figure 3.36 The optimum temperature of chimeric chitinase. Chitinolytic activity was measured in pH 4.0 at various temperature 40-80 °C for 1 hr by colorimetric method when using PNAC as substrate.

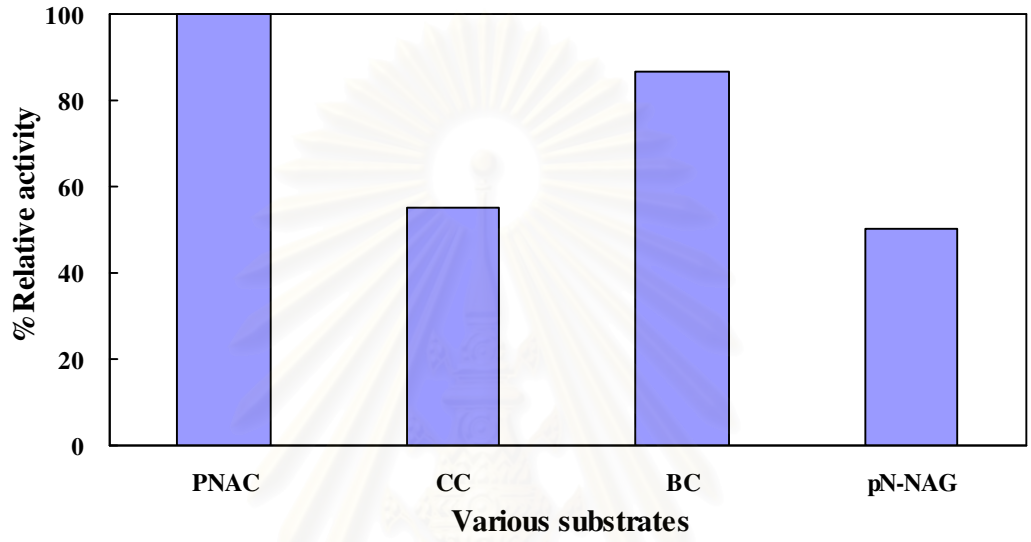


Figure 3.37 Substrate specificity of chimeric chitinase. Chitinolytic activity was measured in 0.1 M citrate buffer pH 4.0 at 50 °C for 1 hr with different substates.

PNAC = Partially *N*-acetylated chitin

CC = Colloidal chitin

BC = β-chitin

pNP-NAG = para-nitrophenol-*N*-acetylglucosamine

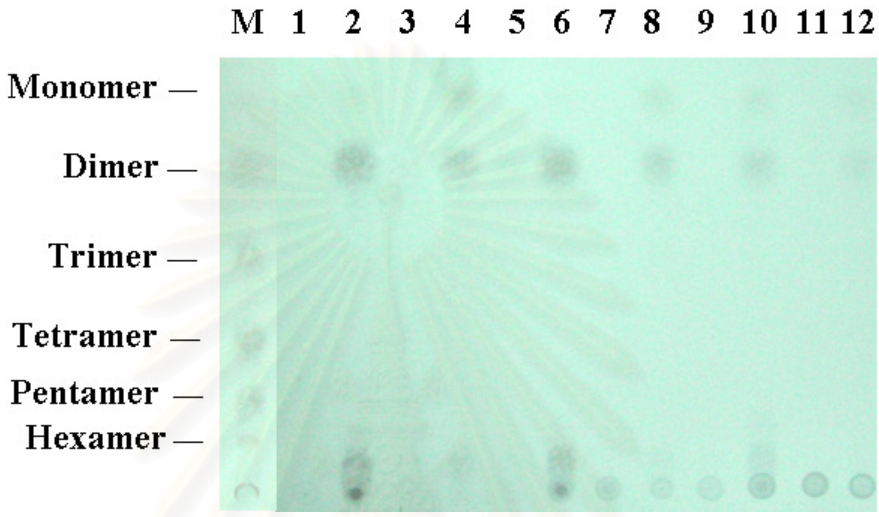


Figure 3.38 The hydrolytic product from colloidal chitin and β-chitin by Chi60, ChiB and chimeric chitinases. Chitinolytic activity was measured in 0.1 M cicrate buffer pH 5.0 at 50 °C.

M = *N*-acetylchitooligosaccharide standard

1 = BC/heated Chi60 2 = BC/Chi60

3 = CC /heated Chi60 4 = CC/Chi60

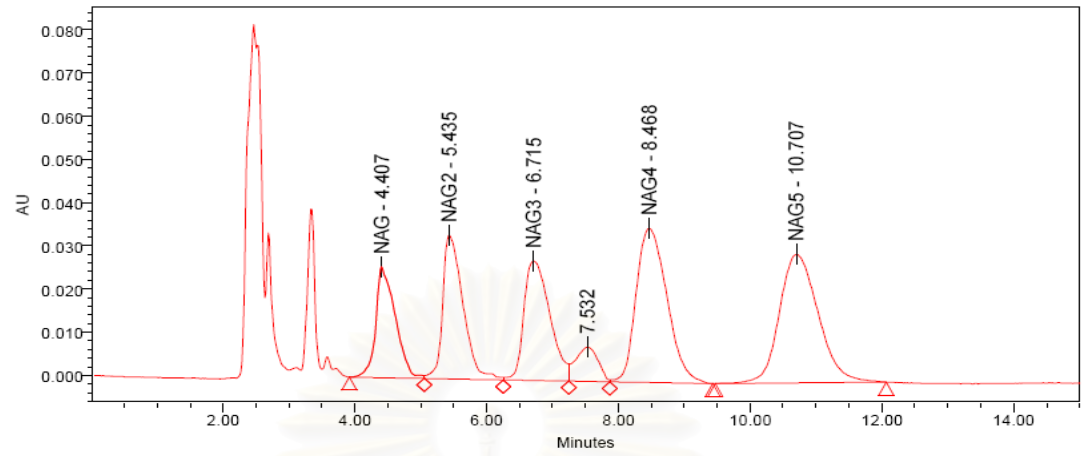
5 = BC/heated ChiB 6 = BC/ChiB

7 = CC/heated ChiB 8 = CC/ChiB

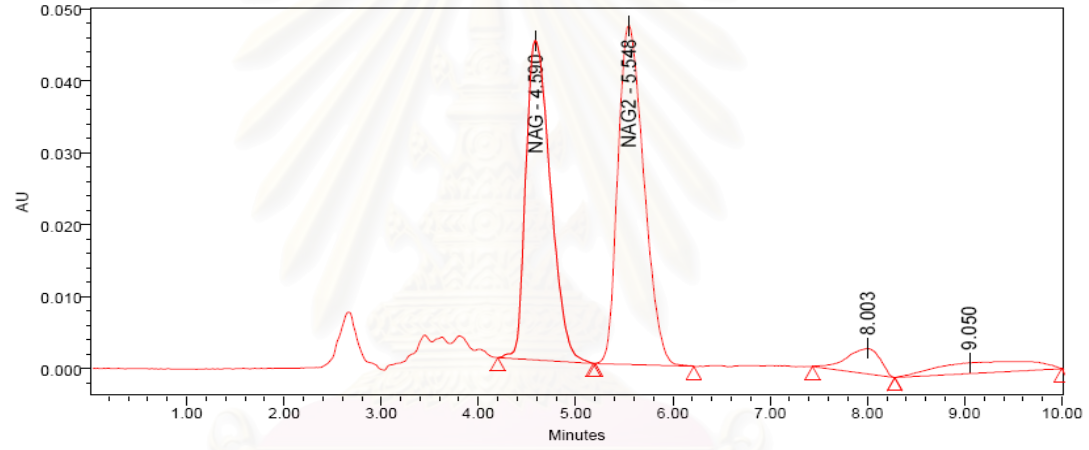
9 = BC/heated RecSK-1 10 = BC/RecSK-1

11 = CC/heated RecSK-1 12 = CC/RecSK-1

A



B



C

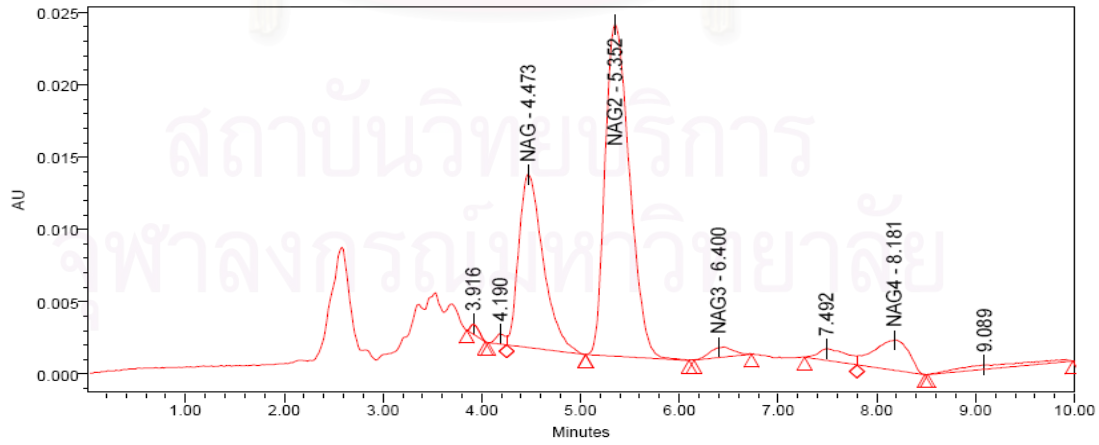


Figure 3.39 The HPLC chromatogram of the hydrolytic product by chimeric chitinase. *N*-acetylchitooligosaccharide standard (A) colloidal chitin (B) β-chitin (C). The reactions were incubated at 50 °C for 12 hr.

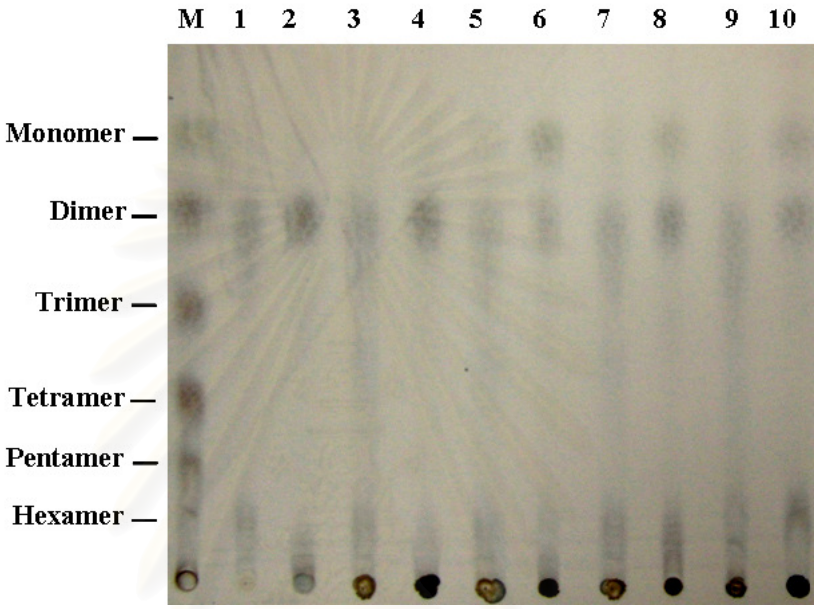


Figure 3.40 The hydrolytic product from NAG_2 by Chi60, ChiB and chimeric chitinases. The reactions were incubated at 50 °C for 12 hr.

M = *N*-acetylchitooligosaccharide standard

1 = NAG_2/heated Chi60 pH 4.0 2 = NAG_2/ Chi60 pH 4.0

3 = NAG_2/ heated RecSK-1 pH 4.0 4 = NAG_2/ RecSK-1 pH 4.0

5 = NAG_2/heated RecSK-1 pH 5.0 6 = NAG_2/ RecSK-1 pH 5.0

7 = NAG_2/heated RecSK-1 pH 6.0 8 = NAG_2/ RecSK-1 pH 6.0

9 = NAG_2/heated RecSK-1 pH 7.0 10 = NAG_2/ RecSK-1 pH 7.0

Table 3.7 The hydrolytic product from chimeric chitinase on β-chitin.

Chitinase	Product from β-chitin (mM)			Product ratio	
	Monomer (M)	Dimer (D)	Timer (T)	M/D	D/M
ChiB	2.70	2.01	0.18	1.34	0.74
Chi60	2.28	12.63	0.32	0.18	5.53
	0.91	7.71	0.43	0.12	8.47

Table 3.8 The hydrolytic product from chimeric chitinase on colloidal chitin.

Chitinase	Product from β-chitin (mM)			Product ratio	
	Monomer (M)	Dimer (D)	Timer (T)	M/D	D/M
RecSK-1	0.71	0.91	0.02	0.78	1.28
ChiB	8.59	5.09	0.43	1.68	0.59
Chi60	3.20	3.41	0.21	0.94	1.06

Part IV: Production of *N*- *N′* diacetylchitobiose and *N*-acetyl-chito-oligosaccharide

Production of *N*- *N′* diacetylchitobiose by dialysis method

Beta chitin (1 g) was hydrolyzed with partially purified Chi60W33F/W245F in 0.1 M citrate buffer pH 5.0 at 50 °C for 6 day by using dialysis tubing. The buffer was circulated continuously through the system and chitinase was on the dialysis tubing for the hydrolysis reaction. The chitobiose level increased as the incubation time increases. After 4 days of the incubation time chitobiose level was stable (Figure 3.41). Using this method, greater than 85% NAG_2 can be removed through the dialysis tubing. The sugar was purified and isolated by activated carbon adsorption. Oligosaccharides were eluted with a stepwise of 10% and 25% EtOH. Concentration of NAG was determined reducing sugar, pooled and identified as a chitobiose as the predominant product. Only trace amounts of *N*-acetylglucosamine and chitotriose were detected. When compare with conventional method, incubating the enzyme with substrate for 6 days using the same conditions. The percent yield of chitobiose was less than a haft of the dialysis method. After 6 days of incubation, the HPLC yields of NAG and NAG_2 from the hydrolysis of β-chitin were 85 mg and 565 mg, respectively, representing 8.5% and 56.5% (w/w).

Production of *N*-acetylchitooligosacchaide by ChiA1ΔChBD

Colloidal chitin was hydrolyzed by partially purified ChiA1ΔChBD in 0.1 M citrate buffer pH 5.0 at 50 °C for 2 hr by using dialysis bag. The chitooligosacchaides increased as the incubation time increases. After 2 hr of the inubation time the level chitooligosacchaides was stable. The results showed a mixtue of oligomers monomer to pentamer (Figure 3.42). The results from TLC were confirmed by HPLC in Figure 3.43. In addition, when ethanol was added in the reaction, the long oligosaccharides were increased. Oligosaccharides were isolated by using charcoal coumn. Each of the oligosaccharide can be eluted by stepwise elution of 35 and 40% EtOH. Chitotetraose can be prepared and purified. Chitotetraose was used as substrate for ChiA1 to further confirming its identity. The results showed that chitotetraose prepared can be hydrolyzed to chitobiose (Figure 3.43).

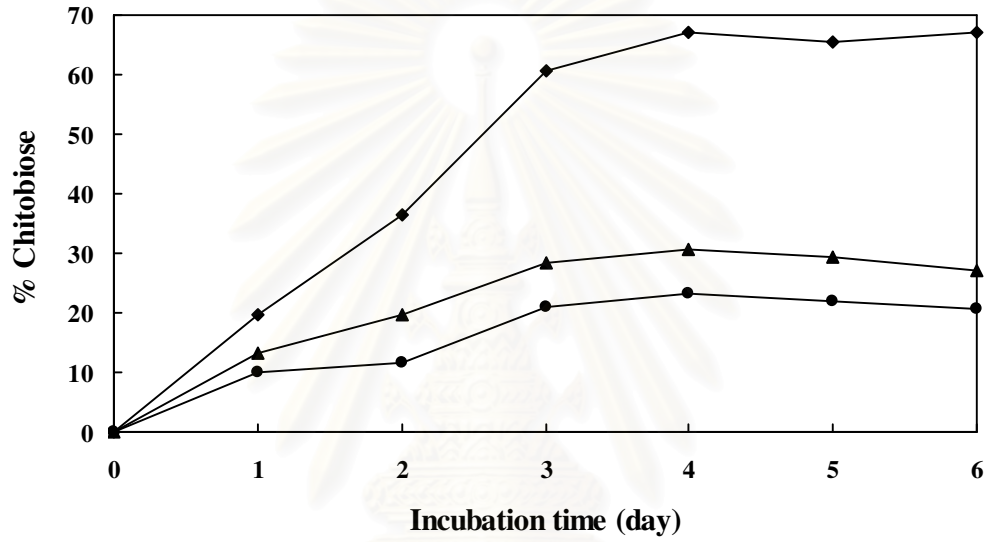


Figure 3.41 Chitobiose productions by Chi60 mutant. The enzyme was incubated with substrate in dialysis tubing and flow the buffer to circulate**.** The system was incubated at 50 °C for 6 days. Samples (1 mL) were taken at various time intervals. Each sample was heated at 100 °C for 5 min and assayed reducing sugar by Schales method. Circuated /2M NaCl (◆), non-circulated (▲), non-circulated/2M NaCl (●)

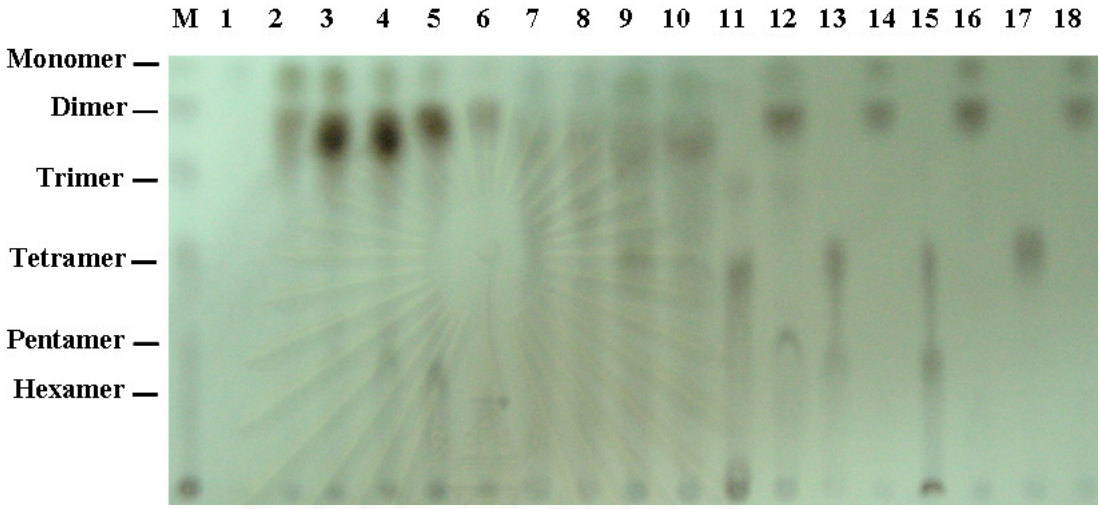
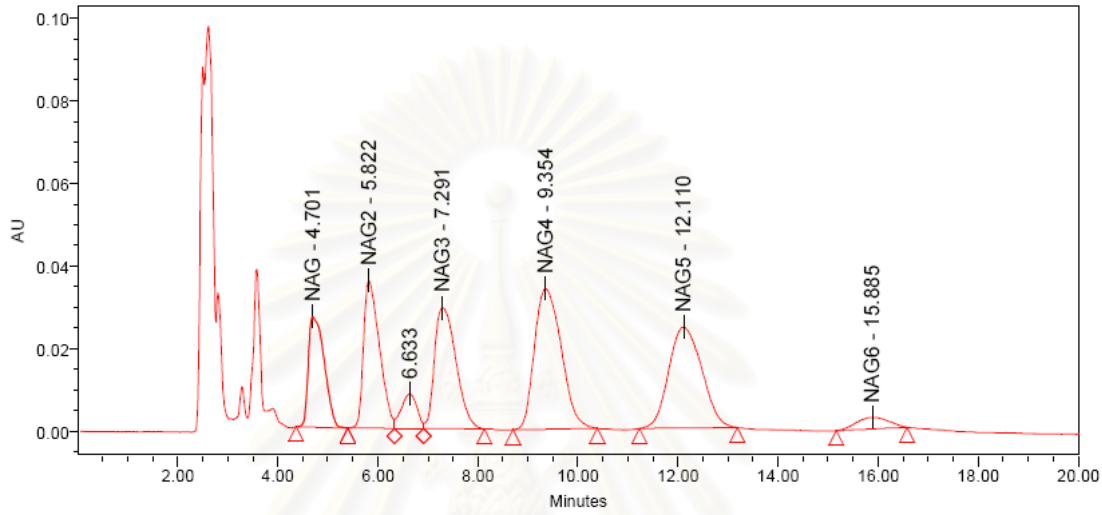


Figure 3.42 The hydrolytic products from colloidal chitin by ChiA1ΔChBD. The system was circulated at 40 °C for 2 hr. Samples (20 μL) were taken at various time intervals. Each sample was heated at 100 °C for 5 min and made up to 100 μL with deionized water. After centrifugation, 5μL aliquots were spotted to TLC.

Lane M = *N*-acetylchitooligosaccharide standard

Lane 1 = 0 min
Lane 2 = 20 min
Lane 3 = 40 min
Lane 4 = 60 min
Lane 5 = 120 min
Lane 6 = 0 min + 10%EtOH
Lane 7 = 20 min + 10%EtOH
Lane 8 = 40 min + 10%EtOH
Lane 9 = 60 min + 10%EtOH
Lane 10 = 120 min + 10%EtOH
Lane 11,17 = std NAG_4
Lane 12,18 = std NAG_4/ChiA1
Lane 13,15 = NAG_4
Lane 14,15 = NAG_4/ChiA1

A



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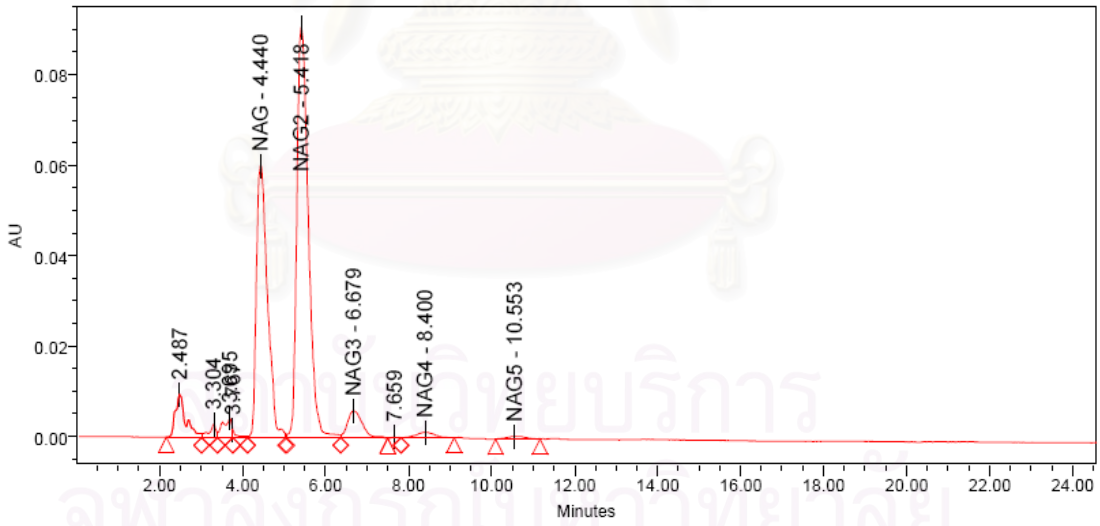

Figure 3.43 The HPLC chromatogram of the hydrolytic product by chimeric chitinase. *N*-acetylchitooligosaccharide standard (A) colloidal chitin (B). The system was circulated at 40 °C for 2 hr.

CHAPTER IV

DISCUSSION

This study explores the possibility of using genetically engineered derivatives of bacterial chitinase for the production of *N*-acetylglucosamine and/or *N*-acetylchitooligosaccharide. Substrates including soluble chitin, colloidal chitin and β-chitin were used to screen the enzymes for oligosaccharide production. Chitinase from *B. licheniformis* and *S. marcescens* were used. Three enzymes from two bacterial were cloned, expressed, partial purified and used to screen for oligosaccharide production.

Chitinase A1

A single chitinase gene from *B. licheniformis chiA* produces three protein with chitinase activity in the medium; ChiA1, which is the fullenght protein, ChiA2, which lack of chitin binding domain, and ChiA3, which lack of both fibronectin and chitin binding domain. ChiA1 hydrolyzed colloidal chitin and produced a mixture of monomer and dimer. This may be caused by the different deletion derivatives of enzyme represented in each of enzyme preparation (Kudan, 2001).

The ChiA1 was purified by using the colloidal chitin adsorbtion affinity technique. The result showed that ChiA1 could bind to colloidal chitin, while the truncated chitinase could not bind. The adsorbed ChiA1 produced an *N*- *N'*-diacetylchitobiose as major products, whereas the unadsorbed ChiA1 composing of truncated derivatives of ChiA1 produced a mixture of *N*-acetylglucosamine and *N*- *N'* diacetylchitobiose when hydrolyzed colloidal chitin. The results suggeste that chitinase A1 works processively on exo-type mode by cleaving *N*-*N'*diacetylchitobiose without dissociation from the substrate. In contrast, the truncated of ChiA1 works on endo-type mode with dissociation from the substrate as indicated by the M/D and D/M ratio.

***chiA* produce three gene products with exo and endo-type activity by auto-degradation of the gene product**

The activity of WT ChiA1 was determined on soluble substrate: partially *N*-acetylated chitin (PNAC), amorphous substrate: colloidal chitin, and crystalline substrate: β-crystalline chitin. The application of different substrate made it possible for distinguishing exo-and endo- type enzyme.

Soluble substrates can freely associate and dissociate from the active site; therefore, the activity determined on this substrate may reflect to the activity of active catalytic domain presenting in the reaction. Amorphous substrate such as colloidal chitin used for endo-type activity assay (Park *et al*., 1997). When the enzyme associates to colloidal chitin, it hydrolyzes and then dissociate from the initial site to the next site since it can processively move along the chitin strands of amorphous chitin. β-crystalline chitin was used to study the processive exo-type mode of enzyme. β-crystalline chitin is arranged in a parallel unidirectional manner of the chitin strands. Moreover, in β-crystalline chitin had shown a very few intermolecular hydrogen bondings between the strands, therefore, it is a good substrate for exo-chitinase.

The ChiA1 can hydrolyze both amorphous and crystalline chitin by using endo- and exo- mode activity; depending on type of substrate (Figure 3.8). Since the interaction of the enzyme and substrate is the hydrophobic interaction between the sugar ring and Trp residues, the activity of ChiA1 was influenced to by changing of the ionic strength of the environments. Therefore, in this study the hydrolysis of ChiA1 under different ionic strength of the different substrates such as soluble, amorphous, and crystalline were analyzed.

Our previous results demonstrated that the mechanism of ChiA1 hydrolysis depend on types of substrate. Soluble chitin substrate can diffuse into catalytic site and did not depend on the chitin binding domain. Therefore, activity of ChiA1 was not affected by ionic strength. ChiA1 associate with insoluble chitin by using *C*-terminal domain. For amorphous chitin, ChiA1 and truncated chitinases could not processively hydrolyze chitin chain. At low ionic strength, ChA1 and truncated derivatives can bind and hydrolyzed and then must dissociate from the chitin chain to

the next cleavage site, and then hydrolyze again. At high ionic strength, the enzymes could not dissociate from the substrate resulted in a reduction in activity. In β-chitin, enzyme can processively hydrolyzed chitin without releasing from the substrate therefore the enzyme could retain its activity under high ionic strength.

The ChiA1 hydrolyzed soluble chitin, PNAC, by using an endo-mode of catalysis. The activity did not depend on the domain involved in substrate binding which can be clearly demonstrated in Figure 3.8. Changing in ionic strength did not affect the enzyme activity. ChiA1 could hydrolyze amorphous chitin by using both endo- and exo-mode. Increasing of ionic strength resulted in the decreasing of endo-type activity. At this elevated ionic strength the enzyme remains bound to the substrate and could not dissociate to a new hydrolytic site. The ChiA1 has higher activity at low ionic strength and lower activity as the ionic strength was increased. In our previous study, ChiA1 could auto-degrade by itself yielding truncated chitinases with higher endo-type activity. On the other hand, the intact ChiA1 has an exo-type activity. On colloidal chitin substrate ChiA1 and its proteolytic derivatives can work cooperatively. ChiA1 uses exo-type mode when it processively hydrolyzed β-crystalline chitin. The enzyme remains active at about 60% at high ionic strength, 600 mM NaCl.

The kinetic parameters of the purified ChiA1, ChiA2 (deletion of chitin binding domain) and ChiA3 (deletion of both fibronectin and chitin binding domain) were determined in Table 3.2. ChiA2 and ChiA3 increased both the K_m and K_{cat} values significantly, suggesting that the two domain of ChiA1 are involved in substrate binding. The K_{cat}/K_m values of ChiA2 and ChiA3 were approximately one-thrid and one-second of that of ChiA1, respectively. These results suggest that the increase in hydrolyzing activity against colloidal chitin by ChiA2 and ChiA3. The result was similarity to Watanabe *et al*., 2003.

The mode of catalysis of ChiA1, Chi60 and ChiB

To determine the modes of cleavage by ChiA1, Chi60 and ChiB, the hydrolytic products formed from colloidal chitin, β-chitin and various *N*-acetyl-chitooligosaccharide (NAG_{3-6}) was analyzed by TLC and HPLC (Figure 3.10-3.13).

The three enzymes produced a NAG_2 as a major product from β-chitin. On the other hand, ChiA1 hydrolyzed colloidal chitin to produce dimer as a major product, while ChiB and Chi60 produced a mixture of monomer and dimer. From this results suggested that the intact ChiA1 hydrolyzed β-chitin and colloidal chitin by using exo-type mode. On the other hand, ChiA1 produces the endo-type truncated derivatives for colloidal chitin hydrolysis. In contrast, Chi60 and ChiB use exo-type mode for β-chtin hydrolysis and use endo-type mode for colloidal chitin hydrolysis. (Figure 4.1)

However, previous study by Horn *et al*., (2006) and Kattiyawong, (2007) have shown that *S. marcescens* produces multiple enzyme, ChiA, ChiB, and Chi60, which favor different mode of catalysis, exo- or endo-type. In addition, the mode of catalysis of ChiA is quite ambiguous and ChiA is a processive chitinase. The proposed mode of catalysis of ChiA suggests that it is an exo-chitinase, hydrolyzing chitin strand from the reducing end (Uchiyama *et al*., 2001), however, ChiA and Chi60 was able to hydrolyze both amorphous and crystalline chitin equally well, indicating that it posses both exo- and endo-type activity (Table 4.1).

In the addition, a plausible model for the mechanism by which Family 18 glycosidases degrade pure β-chitin has been proposed previously (Sugiyama *et al*., 1999, Uchiyama *et al*., 2001 and Imai *et al*., 2002). It appears that Family 18 chitinases first locate and expose the reducing end of a chitin chain on the surface of the insoluble chitin matrix. The reducing end disaccharide at positions +1 and +2 is then hydrolyzes and the enzyme moves symmetrically two NAG residues towards the non-reducing end to degrade the chain processively. New chitin molecules are exposed and hydrolyzed by a procession of reducing-end starts from new enzyme molecules that lag behind those which initiated outer-chain degradation. Such a unidirectional hydrolysis of exterior chitin chains explains the sharpening observed at the reducing end of pure β-chitin fibers that was hydrolyzed with family 18 chitinases (Sugiyama *et al*., 1999, Uchiyama *et al*., 2001, Imai *et al*., 2002 and Lindsay and Gooday, 1985).

The effect of functional domain on enzyme activity

When ChiA1 and modified chtinases were used to hydrolyze amorphous or crystalline, a mixture of monomer and dimer of NAG was observed, which was not

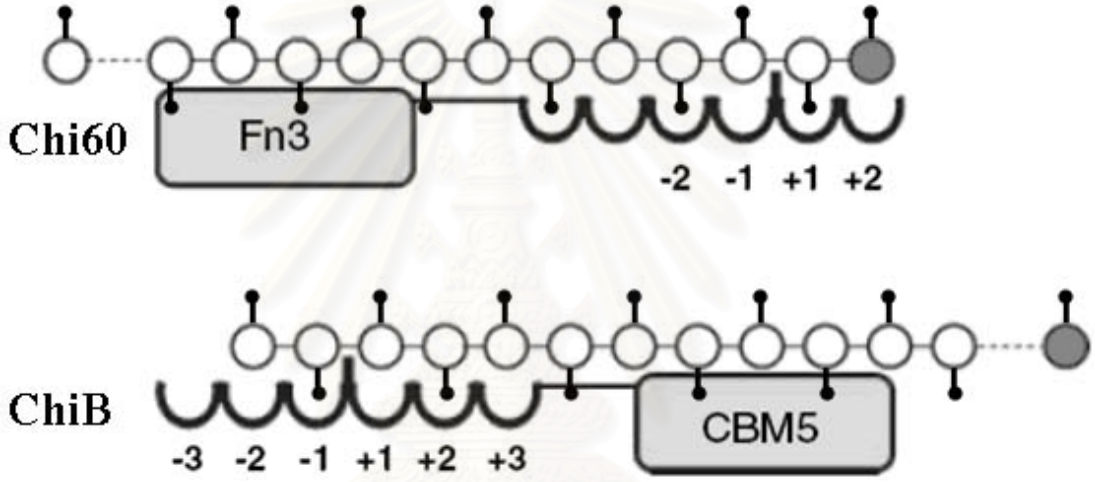

Figure 4.1 Schematic drawing of subsites, chitin binding domains and proposed orientation of polymer substrates in Chi60 and ChiB. Fn3, Fibronectin type 3 domain; CBM5, chitin binding module. Dotted lines indicate that the polymer substrates are much longer than shown in the figure. Reducing sugars are shown in grey. A correctly positioned *N*-acetyl group (symbolized by small black balls on sticks) in the -1 subsite is essential for catalysis (which is ‘‘substrate-assisted’’) to occur.

Table 4.1 The proposed mode of catalysis of chitinases.

Enzyme	β-chitin		Colloidal chitin	
	Exo-type	Endo-type	Exo-type	Endo-type
ChiA1	/	-	/	-
ChiA2	/	-	-	/
ChiA3	/	-	-	/
ChiA1ΔChBD	/	-	-	/
ChiA1ΔFnIIIDΔChBD	/	-	-	/
ChiA1-60N	/	-	/	-
ChiA1-60NΔFnIIIDΔChBD	/	-	-	/
Chi60-A1FnIIID&ChBD	/	-	/	/
Chi60	/	-	/	/
ChiB	/	-	/	/
RecSK-1	/	/	/	/

observed in Chi60. This phenomenon may be caused by the low fidelity of the catalytic domain of ChiA1 that yields a mixture of both products and the difference in the mode of catalysis of the different deletion derivatives of the enzyme present in each of enzyme preparation. According to model of crystalline chitin hydolysis, after the first hydrolysis at the second linkage from the reducing end, the chitin chain in the substrate binding cleft slides toward plus subsites and fills +1 and +2 subsites. Two NAG units is the minimum length of sliding to allow the next hydrolysis. Sliding by two NAG units is determined by the structure of the chitin chain itseft, rather than the protein structure around the catalytic site. This is the reason that hydrolysis product from β-chitin remained dimer.

The difference in the mode, exo and endo type, of catalysis of the different deletion derivatives of the enzyme, can be reflected by the increase in monomer/dimer ratio. The deletion mutants would bind less efficiently to the substrate resulting in enzymes which favors an endo type mode of hydrolysis over the processivity, exo type mode, of hydrolysis. This would result in odd number oligosacchairides which would later be hydrolyzed yielding NAG, thus rasing the ratio of monomer/dimer ratio of the hydrolytic product.

Wild-type ChiA1 and modified chitinases were first studied for their substrate specificity. The enzymes were assayed on soluble substrate, partially *N*-acetylated chitin (PNAC). The amount of enzyme which yields equal activity on PNAC was used to hydrolyze crystalline substrate, β-chitin, and amorphous substrate, colloidal chitin (Table 3.5 and Table 3.6). This is to ensure equal quantity of active catalytic domain present in each reaction. The differences in the activity observed would be a result from the change in substrate specificity of the ChiA1 derivatives. On β chitin we observed that the activity of the deletion mutants ChiA1ΔChBD and ChiA1ΔIIIΔChBD reduces as more of the *C*-terminal was deleted, Table 3.5. Interestingly, when colloidal chitin was use as substrate we observed the highest activity with ChiA1ΔChBD followed by ChiA1ΔFnIIIDΔChBD and wild-type ChiA1, Table 3.6.

When amorphous substrate was used the enzyme must preferentially hydrolyze via the endo-type mode, binding on the substrate, hydrolyzing, and releasing from the initial site to move to the next hydrolytic site. Therefore, an

enzyme which binds less tightly on the substrate would result in higher activity on amorphous substrates. This would explain the higher activity of ChiA1ΔChBD and ChiA1ΔFnIIIDΔChBD than wild-type ChiA1, observed. ChiA1-60N and ChiA1-60NΔFnDIIIΔChBD would result in the lower activity than wild-type ChiA1. Chi60-A1FnIIID&ChBD would not change activity when compare with Chi60. On the other hand, on crystalline substrates, the enzyme works processively, using exo-type activity, therefore the ability of the enzyme to hold on to the substrate is crucial for its high hydrolytic activity (Figure 4.2). When the chitin binding domain and fibronectin type III like domain, which was demonstrated earlier to assist in hydrolysis (Watanabe *et al*., 2003), was deleted, a reduction in activity of ChiA1 derivative, ChiA1ΔChBD and ChiA1ΔFnIIIDΔChBD, ChiA1-60NΔFnIIIDΔChBD was observed.

The *C*-terminal domain of chitinase A1 from *B*. *licheniformis* SK-1, which is connected to the *N*-terminal catalytic domain by fibronectin type III like domain, has been shown to be a chitin-binding domain. The chitinase A1 was similar to that of chitinase A1 from *B*. *circulans* WL-12, but it was only one fibronectin type III like domain (Watanabe *et al*., 1994).

The loss of this chitin binding domain results in a significant increase in colloidal chitin hydrolyzing activity. Similar results were also reported in yeast. Deletion of the chitin-binding domain of yeast chitinase increased the rate of chitin hydrolysis (Kuranda and Robins, 1991). The loss of the chitin binding domain of tobacco class I chitinase did not significantly affect the specific activity of the enzyme toward colloidal chitin, but it decreased antifungal activity to one-third when compared with that of intact chitinase (Iesli *et al*., 1993). Thus, it would seem that chitin-binding domains are not always important for enhancing catalytic activity, but they are required for conferring unique biological properties to the chitinases in various organisms.

The most interesting aspect of chitinase Al may be the presence of the one type III like domain located between the chitin-binding domain and the catalytic domain. The fibronectin type III like domain may participate in chitin binding activity; the deleting chitin binding domain did not affect chitin binding activity. The results obtained in this study indicate that type III like domain is important for chitin

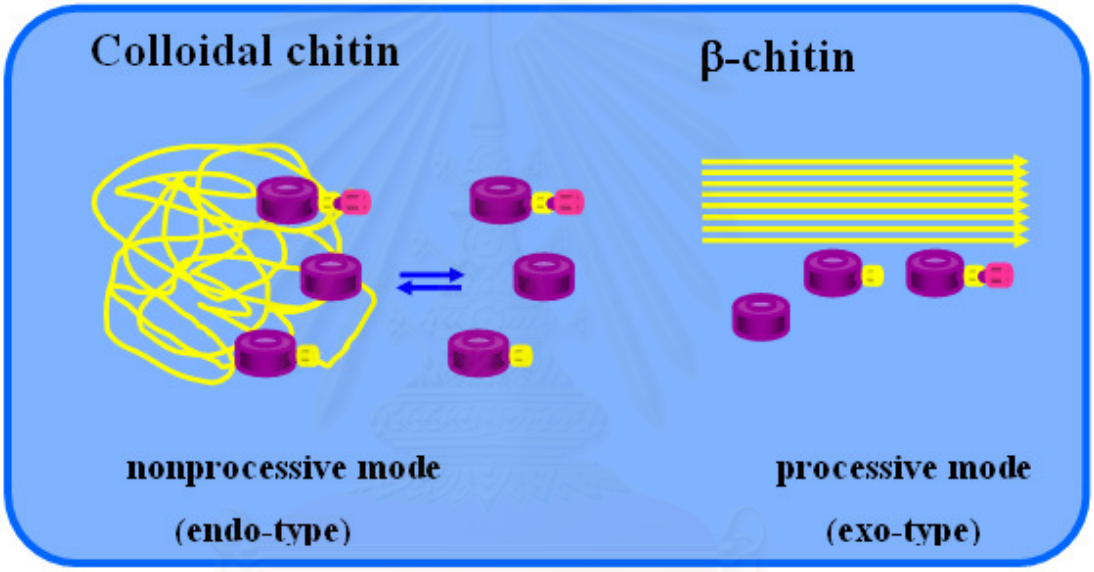

Figure 4.2 The model of mode catalysis of chitinase A1 from *B. licheniformis* SK-1.

binding and the hydrolysis of chitin by the enzyme. The type III like domain may have a structural role, for example, in maintaining an optimal distance and orientation between the catalytic domain and the chitin-binding domain. Bork and Doolittle, 1993 recently reported a systematic search and sequence analysis of eukaryotic and prokaryotic type III sequences, from which they proposed that bacterial type III like domain was initially acquired from animal sources. In prokaryotes, typeIII like domain has been found in other chitinases besides chitinase A1, as well as in a cellulase, a α-amylase, a bifunctional of-amylase-pullulanase, and PHB depolymerase. Whether this type III like domain has acquired from animal sources is not clear, but the fact that type III like domain is spread and maintained in the prokaryotic enzymes indicates a functional importance for this sequence.

Many chitinases are composed of a catalytic domain joined to one of more chitin-binding domains, as in the case of various insoluble polysaccharide hydrolases including cellulases. This kind of substrate-binding domain is functional not only for accumulating catalytic sites on the surface of substrates but also for disrupting hydrogen bonds in the crystalline region of substrates and thereby facilitating subsequent hydrolysis by the catalytic domains (Tomme *et al*., 1995).

A single chitin binding domain (ChBD) is present in Chi60, ChiB and ChiA1, and these domains are located on opposite side of the active site. It has been suggested tha the ChBDs contribute to exo-binding, at the reducing and non-reducing ends (Van Aalten *et al*., 2000; Hult *et al*., 2005) and that they may determine the directions of processivity (Hult *et al*., 2005).

Enzymes acting on cellulose or chitin face the challenges of associating with the insoluble substrate, disrupting crystal packing, and guiding a single-polymer chain into the catalytic center. In addition to their catalytic domain, cellulases and chitintases often contain one or multiple so-called carbohydrate-binding modules (CBMs) (Baraston *et al*, 2004), which are beneficial for enzyme efficiency because they adhere to and sometimes disrupt the substrate (Din *et al*., 1994; Watanbe *et al*., 1994; Suzuki *et al*., 1999; Garrard *et al*., 2000; Lehtio *et al*., 2003; McCartney *et al*., 2004). Recently, it has been shown that chitin-degrading microorganisms produce a separate noncatalytic protein whose function is to disrupt the crystallinity of the

substrate, thus dramatically increasing the efficiency of the hydrolysis by chitinases (Vaaje-Lolstad *et al*., 2005)

Analysis of the degradation products of chitinases

Hydrolytic product of wild type ChiA1, Chi60, ChiB and its modified enzymes were examined using colloidal chitin and β-chitin as the substrate. Colloidal chitin was hydrolyzed by wild type ChiA1 and the modified enzyme producing dimer as a major product and monomer and trimer as a minor product as shown by results from TLC and HPLC.

Generally, enzymatic degradation of polysaccharides occurs from one of the chain ends (exo-mechanism) or from a random point along the polymer chain (endo-mechanism). Each of these two mechanisms can occur in combination with a processive mode of action, meaning that the substrate is not released after successful cleavage but slides through the active site for the next cleavage event. Processivity reduces the search space for enzymes and is thought to be especially important when degrading insoluble substrates. Enzymatic degradation of crystalline polysaccharides is difficult to study because the insoluble substrate is not amenable to straight forward biochemical analysis and soluble intermediate oligosaccharide products are degraded fast and, therefore, difficult to detect. Thus, usually the only detectable products during degradation of chitin or cellulose are mono-, di-, and trisaccharides, which are typical end products. Processivity often is assessed by comparing the production of soluble and nonsoluble reducing ends, but this approach cannot discriminate between all possible modes of action (exo-acting enzymes will yield high soluble/nonsoluble ratios regardless of processivity, and so will endo-acting enzymes with a high degree of processivity). Processivity also may be assessed roughly by studying the ratio between produced dimers and monomers.

It is very unlikely that monomers are produced directly from chitin. Instead, it is produced when trimer NAG_3 is degraded producing dimer NAG_2 and monomer NAG. Because of the 180° rotation between consecutive sugar units, processive action on chitin will yield dimers, while trimers can only be produced by an endo-chitinase in the first hydrolytic step. Thus, the molar amount of monomer seen after complete degradation of the substrate may be taken to represent the cumulative molar

amount of trimer formed during the degradation reaction. The molar amount of dimer directly produced from chitin (i.e. not from degradation of trimer) equals the observed molar amount of dimer minus the observed molar amount of monomer.

NAG/NAG_2 ratio can give information on substrate-binding mode and: or processivity. The high M/D (≅ 1) indicates the high processive binding, endo-type mode of catalysis, while low M/D (< 1) ratio indicates the high processivity exo-type mode of catalysis.

The NAG/NAG_2 and NAG_2/NAG_3 ratio are interesting because it can give information concerning the substrate-binding modes and/or processivity (Teeri et al., 1998; Medve et al., 1998). Therefore, processivity may be assessed by studying the NAG/NAG_2 and NAG_2/NAG_3 ratios in product mixtures. Degradation of chitin that resulted in high NAG/NAG_2 ratios and in low NAG_2/NAG_3 ratios indicate that the enzyme had a nonprocessive endo-mode, but low high NAG/NAG_2 and high NAG_2/NAG_3 ratios indicate that enzyme had a processive exo-mode (Horn *et al*., 2006a).

The NAG_2/NAG_3 ratios of the intact ChiA1 showed NAG_2/NAG_3 ratios higher than the truncated derivatives (Table 3.1). These result explained that the intact ChiA1 has higher processivity than the trancated proteins. Therefore, ChiA1 was exo-type mode, while ChiA2 and ChiA3 were endo-type mode for catalysis of amorphous substrate.

In addition, the NAG/NAG_2 and NAG_2/NAG_3 ratios of ChiA1, ChiA2 and ChiA3 were shown in Table 3.3. The results indicated that the ChiA1 had exo-type mode of hydrolysis when degrading colloidal chitin, following ChiA3 and ChiA2, respectively. However, the increased NAG/NAG_2 and reduced NAG_2/NAG_3 ratios of ChiA2 and ChiA3 may be caused by the deletion of fibronectin type III like domain and chitin binding domain which resulted in the lost of processivity.

The homologous recombinant

Homologous recombination using *E. coli* strain JC8769 was performed to generate recombinant chititinase. The recombinants were isolated and screened on colloidal chitin minimum medium and assayed NAG_4 hydrolysis. Four recombinants were screened from 110 colonies (RecSK1-RecSK-4). RecSK-1 to RecSK-4 were expressed and assayed activity on PNAC. RecSK-1 was stable and high chitinase

productivity. Therefor, RecSK-1 was further studied. To confirm RecSK-1, plamid was sequenced and analyzed. The DNA sequence was confirmed that RecSK-1 composed of truncated mutant Chi60 with deletion of residues 419-563. To further confirm the chitinase activity of RecSK-1 was checked. The RecSK-1 was expressed and characterized of some properties.

Chimeric chitinase has an optimum pH at pH 4.0 and optimum temperature at 60 °C on PNAC. The enzyme hydrolyzed PNAC the best, followed by BC and CC, respectively. Interestingly, the chimeric chitinase hydrolyzed pNP-NAG_2 and NAG_2 to produce monomer of NAG. In addition, the chimeric enzyme is the novel enzyme which hydrolyzes pNP-NAG_2 and NAG_2. However, the family 18 chitinase was not hydrolyzed pNP-NAG_2 and NAG_2 to produce monomer of NAG.

These possible machanisms are how chitin with odd number of residues is produced. Three possible mechanisms are considered. First, chimeric enzyme shows a mixed exo/endo mode of action, with the exo attack creating chitin with odd number of residues. This mechanism is unlikely because the observed number of endo attacks combined with the degree of processivity. Second, in the processive mode of action of chimeric enzyme can move along the polymer chain by an odd number of sugar units. This mode, however, require a rotation of the substrate by 180° along the chain axis for the glysosidic bond (Sikorski *et al*., 2005). Third, the chitin with an odd number of residues are created by rehydrolyis of longer even numbered oligomers produced by processive enzyme action contain cleavable sequences inaccessible to the enzymes during processive action because of the inability of the enzymes to move between productive complexed by an odd number of sugar units. Thus, the rebinding of even-numbered oligomer may be productive, and this will predominately lead to the formations of oligomers with an odd number of residues (Sikorski *et al*., 2006).

Production of *N*- *N′* diacetylchitobioase (NAG_2) and *N*-actylchitooligosaccharide

The enzymatic degradation of the closely related insolublepolysaccharides cellulose (1,4)-linked glucose and chitin (1,4)-linked *N*-acetylglucosamine is of large biological and economical importance. In recent review papers on the potential of biofuels, Ragauskas *et al*., 2006 and Farrell *et al*., 2006 emphasized the importance of improved cellulosic technologies and better cellulolytic enzymes for conversion of

biomass to easily fermentable compounds such as glucose. Large efforts to improve cellulosic technologies are underway, stimulated by, among others, the U.S. Department of Energy (www1.eere.energy.gov/biomass). Chitin is the most important nonplant structural biopolymer, occurring in, e.g., the exoskeletons of invertebrates, fungal cell walls, and the digestive tracts of insects. Chitin is available in large quantities as an underutilized waste product (e.g., shrimp shells). It is used for production of glucosamine and chitosan and also could be converted to bioactive oligomeric compounds or building blocks for bioactive glycoconjugates if efficient enzyme technology were available. Chitin turnover plays a role in many important processes, including transmission of the malaria parasite, infectivity of insect viruses, plant defense responses, and modulation of immune responses and asthmatic inflammation in humans. Because chitin does not occur in humans, chitin metabolism is an interesting target area for development of drugs and pesticides.

There are several ways to manipulate enzymic reactions towards synthesis and away from hydrolysis, e.g., the use of high salt concentrations or organic solvents. Usui *et al*., 1990 used or added ammonium sulfate successfully in formation of NAG_6 and NAG_7 from NAG_2 and synthesized pNP-NAG_5 from NAG_5 and NAG_2 in 60% methanol (Usui *et al*., 1988). Yoon, (2005) used solvent to formation of NAG_3, NAG_4 and NAG_5 from NAG_2 by transglycosylation of lysozyme in 50% organic solvent-buffer.

Kuttiyawong, 2006 found that the mode of Chi60 would be modulating by increasing the ionic strength. The double mutants of Chi60, such as W33F/W245F was not affect by ionic strength and the enzyme used only exo-type mode. So, in this experiment the W33F/W245F was selected to produce the NAG_2 in the optimum condition with increased the ionic strength with 2 M. The reaction was using dialysis tubing as a convenient separator (Matsuoka, et al., 2000). This method was successful to produce high yield of NAG_2.

Colloidal chitin was used to prepare oligosaccharide by partially purified ChiA1ΔChBD using dialysis tubing. The results showed a mixture of oligomers monomer to pentamer. In addition, when the ethanol was added in the reaction, the long oligosaccharides were increased. The results suggested that the enzyme produced long oligosaccharides by transglycosylation or increasing endo-type activity

of enzyme. It is known that the addition of organic solvent to the reaction mixture improves the efficiency of enzyme glycolylation (Murata *et al*., 2000). For example, enzymatic polymerization to systhesis cellulose has been achieved successfully in an acetate buffer-acetonitrile mixture (Kobayahi *et al*., 1991).

CHAPTER V

CONCLUSION

The chitinolytic machinery of *Bacillus licheniformis* SK-1 consist of processive exo enzyme on chitin, ChiA1, and a non processive endo enzyme, ChiA2 and ChiA3. Thus, when *B. licheniformis* applies its battery of chitinases to degrade chitin, the degradation this auto-degradation of ChiA1 is likely to supply the exo-and endo-type enzyme allowing the enzyme to work efficiently to various substrates.

ChiA1, Chi60 and ChiB are all both exo-type and endo-type mode depending on type of substrate. When Chi60 and ChiB hydrolyze amorphous substrate they use endo-type mode, while hydrolyze crystalline substrate they use exo-eype mode. In addition, intact ChiA1 is true exo-type on crystalline and amorphous substrate, while the truncated chitinases are endo-type on amorphous substrate. ChiA1 derivative with high endo type activity is a good candidate for oligosaccharide production.

This study suggested that the functional domain of ChiA1, fibronectin type III like domain and chitin binding domain, could change the substrate specificity and the mode of catalysis. Chitinase derivatives with high endo-type activity should be a good candidate for oligosaccharide production. Interestingly, the chimeric chitinase (RecSK-1) can hydrolyze pNP-NAG_2 and NAG_2 to produce monomer of NAG. In addition, the chimeric enzyme can be useful for the preparation of *N*-acetyl-D-glucosamine from chitin.

Chi60 mutant, Chi60W33F/W245F, was used to prepare *N-N′* diacetylchitobiose from β-chitin and was separated the product by dialysis. The HPLC yields of NAG and NAG_2 from the hydrolysis of β-chitin were 85 mg and 565 mg, respectively, representing 9% and 57% (w/w). In addition, the ChiA1ΔChBD was used to prepare oligosaccharide from colloidal chitin. In the condition used, chitotetraose can be prepared.

In this thesis demonstrated that the different hydrolysis mode of enzymatic hydrolysis can be used to prepare NAG or *N*-acetylchitooligosaccharide. In addition, exo-chitinase can be used for the production of *N*-acetylchitobiose while endo-chitinase can be used for the production *N*-acetylchitooligosaccharide.

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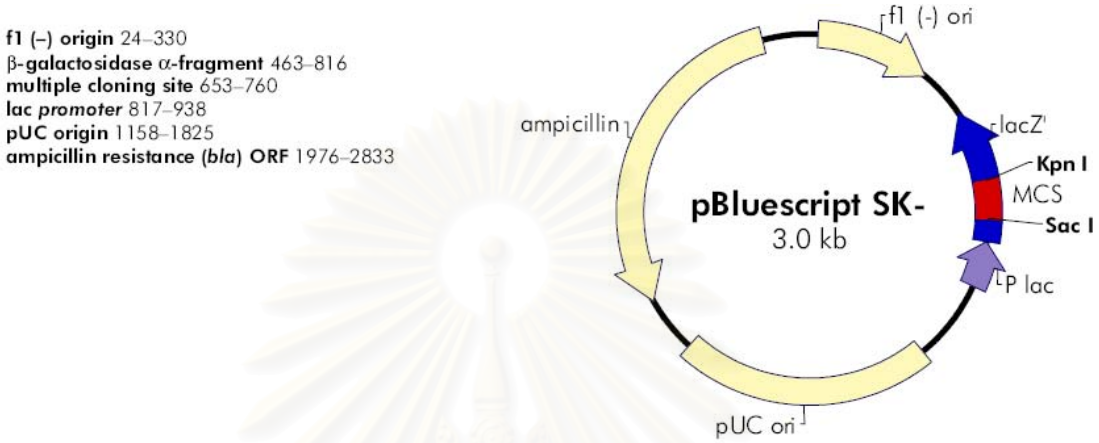
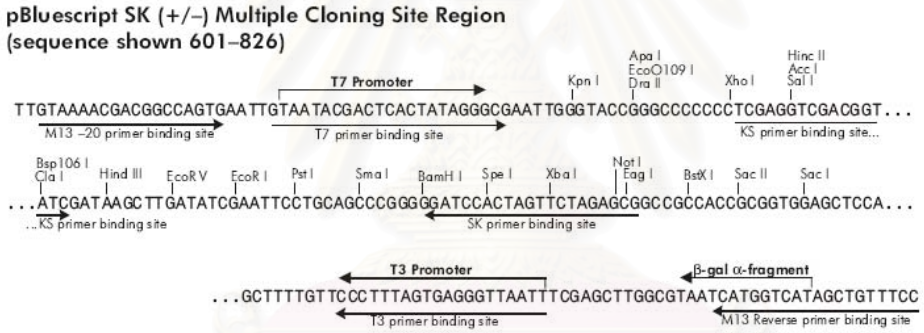
APPENDICES

APPENDIX A

Restriction map of pBluescript SK (-)





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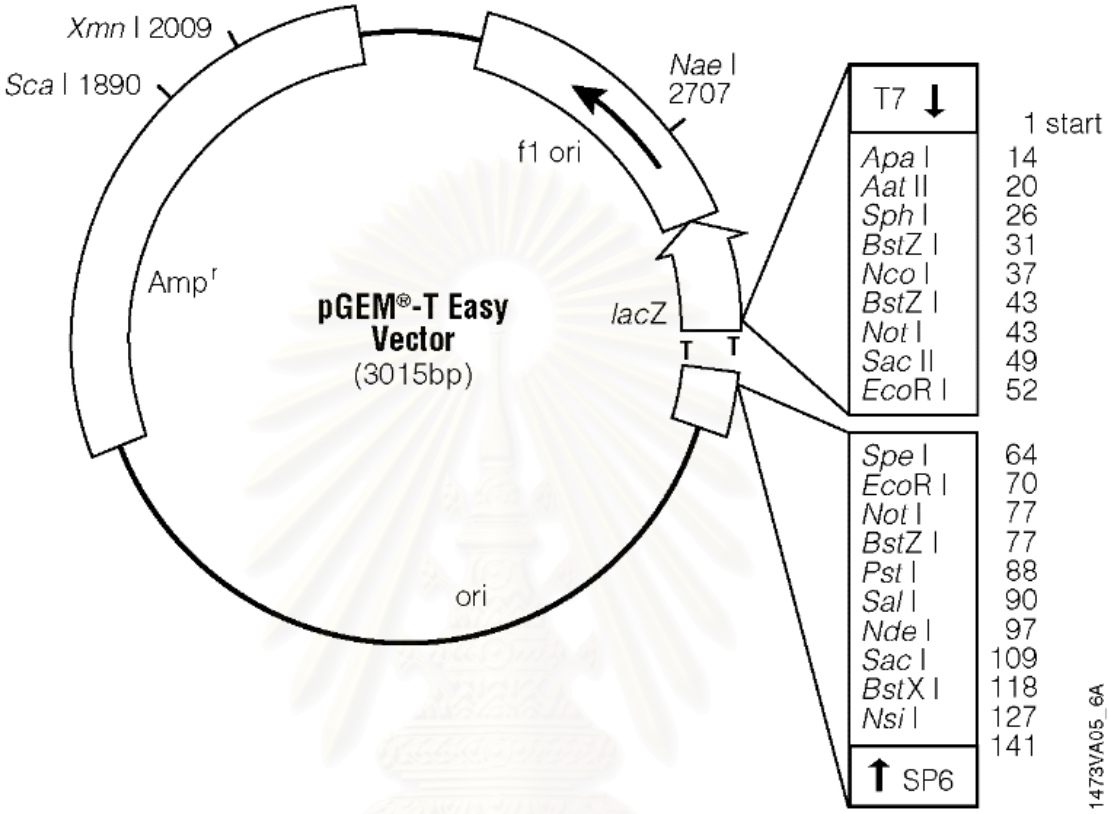

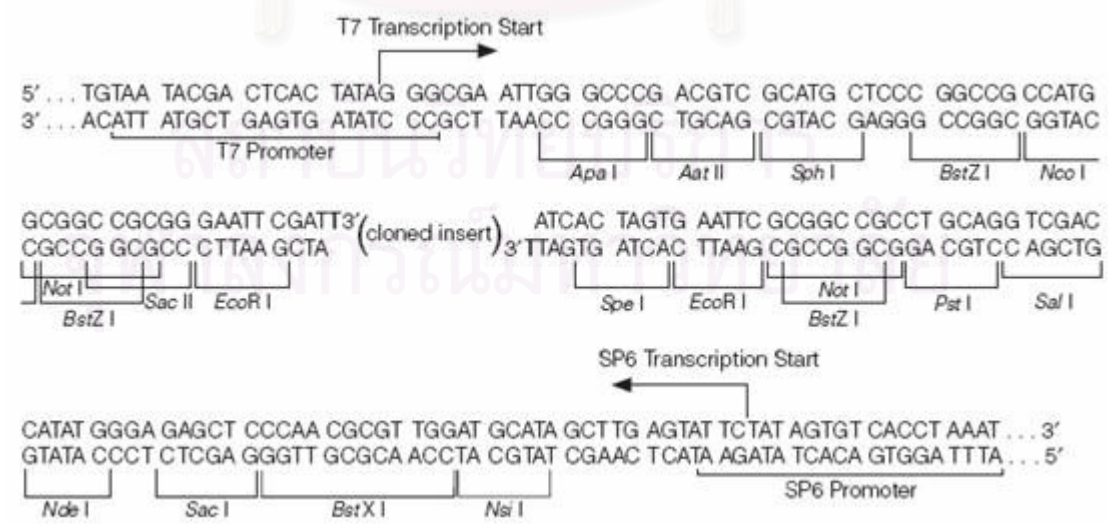

Restriction map of pET19b (+)

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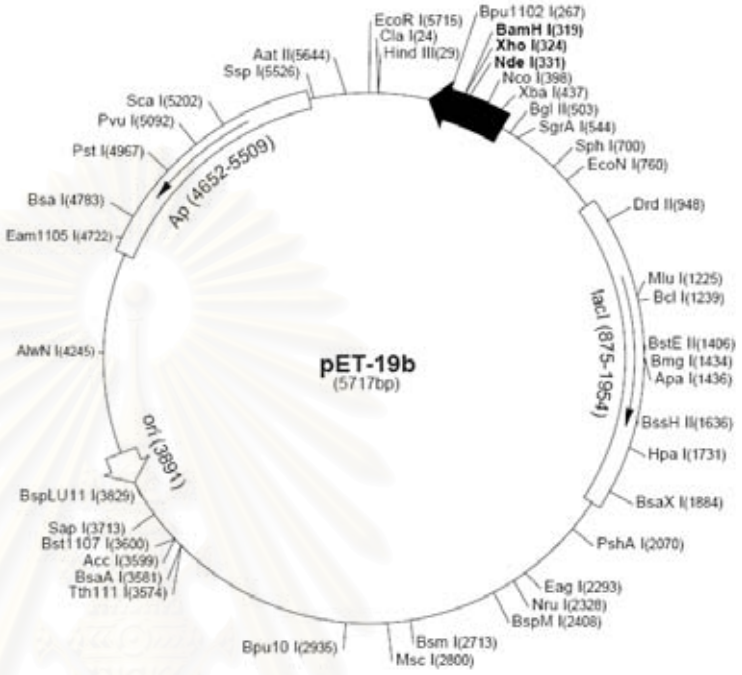

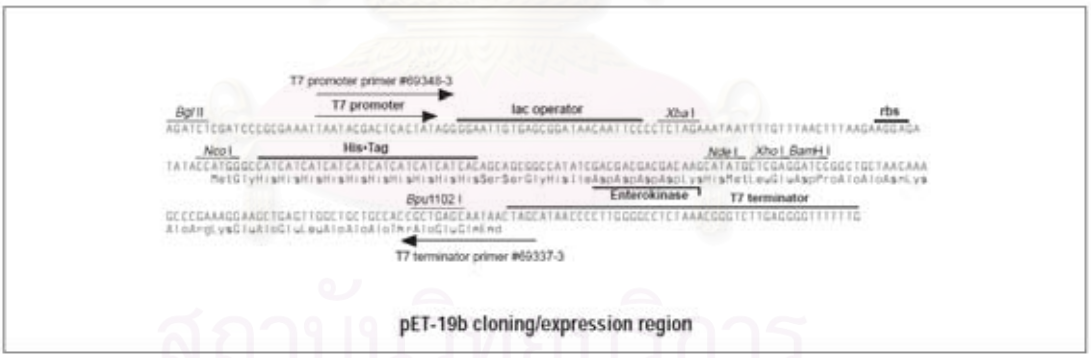

APPENDIX B

Substrate preparations

Partially *N*-acetylated chitin (PNAC)

Partially *N*-acetylated chitin (PNAC) was prepared from squid pen chitin. A 10 g flake squid pen chitin in 250 mL 40% (w/w) NaOH was vacuumed for 4 hr. Then, the 750 g of crushed ice was added and vigorously shaked. In this step, the layer of ice was appeared and covered around the flask. The mixture was stirred for 4-6 hr at 4°C. Next, the solution was adjusted pH until pH 7.0 with conc. HCl. Two volume of cold acetone was added to precipitate PNAC. PNAC was collected by filtration and dialyzed with against water to remove salt. Finally, PNAC was lyophilized.

Colloidal chitin

Colloidal chitin was prepared from flake shrimp shell chitin by the methods described by Jeuniaux, (1966) and Yamada and Imoto, (1981) with some modification. A 40 g of shrimp shell chitin was hydrolyzed by adding 400 mL of conc. HCl (12M) and stirred for 4-6 hr on ice with magnetic stirrer. The solution was stirred for a while at 37°C. The hydrolyzed chitin was filtrated in 4,000 mL of chill distilled water. The milk-like mixture was kept for overnight at 4°C. The colloidal chitin was collected by centrifugation at 8,000g for 15 min and then resuspended with distilled water to wash the pellet. The pellet was washed until the pH 6-7.

The colloidal chitin was resuspended in distilled water and kept at 4°C. The solution was determined the percent dry weight. The colloidal chitin was added 0.05% NaN_3 and kept at 4°C for at least 2 years.

β-crystalline chitin

β-crystalline chitin was prepared from squid pen chitin. A 5 g flake squid pen chitin was grinded in a high speed blender at 10,000 rpm with water until squid pen chitin swell in water. The solution was added 0.05% NaN_3 and kept at 4°C for at least a year.

APPENDIX C

Standard curve of *N*-acetyl-D-glucosamine for chitinolytic activity assay by colorimetric method.

Standard curve for *N*-acetyl-D-glucosamine (GlcNAc) was made by determining the absorbance value at 420 nm of standard *N*-acetyl-D-glucosamine according to the method of Schale.

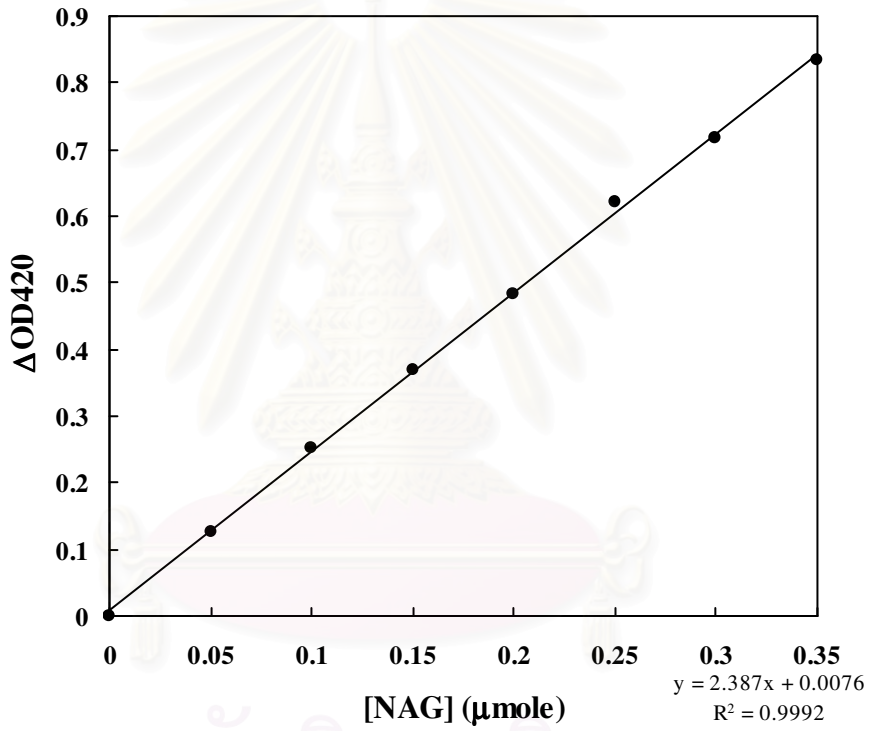

Figure C1. Correlation between final concentration of standard *N*-acetyl-D-glucosamine and optical density (absorbance) at 420 nm.

Standard curve of *N*- *N′* diacetylchitobiose for reducing sugar assay by colorimetric method.

Standard curve for *N*- *N′* diacetylchitobiose were made by determining the absorbance value at 420 nm of standard *N*-acetyl-D-glucosamine according to the method of Schale.

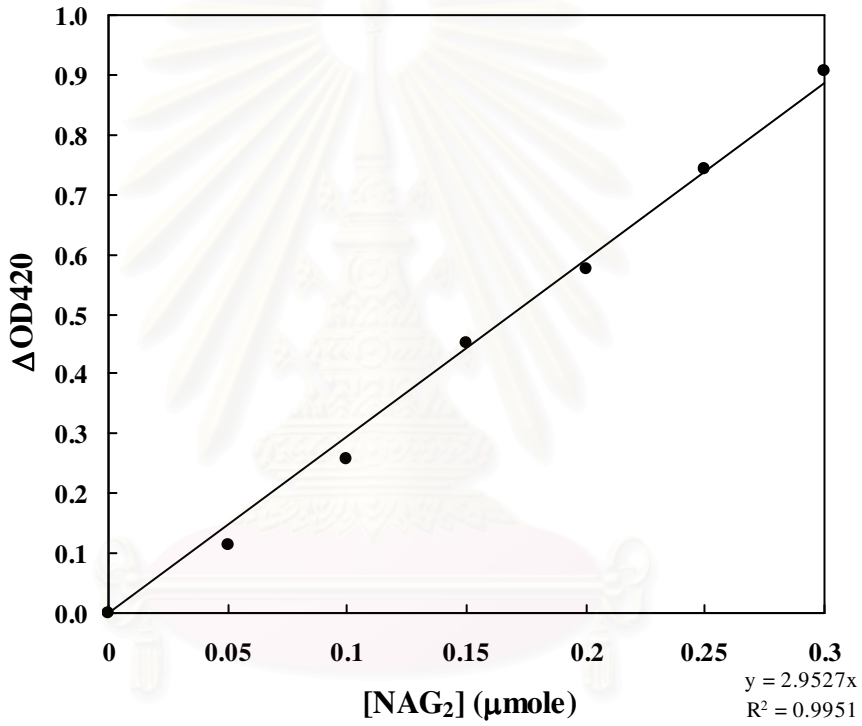

Figure C2. Correlation between final concentration of standard *N*-*N′*-diacetylchitobiose and optical density (absorbance) at 420 nm.

Standard curve of p-nitrophenol for chitobiose activity assay by colorimetric method.

Standard curve for p-nitrophenol was made by determining the absorbance value at 420 nm of standard p-nitrophenol according to the colorimetric method.

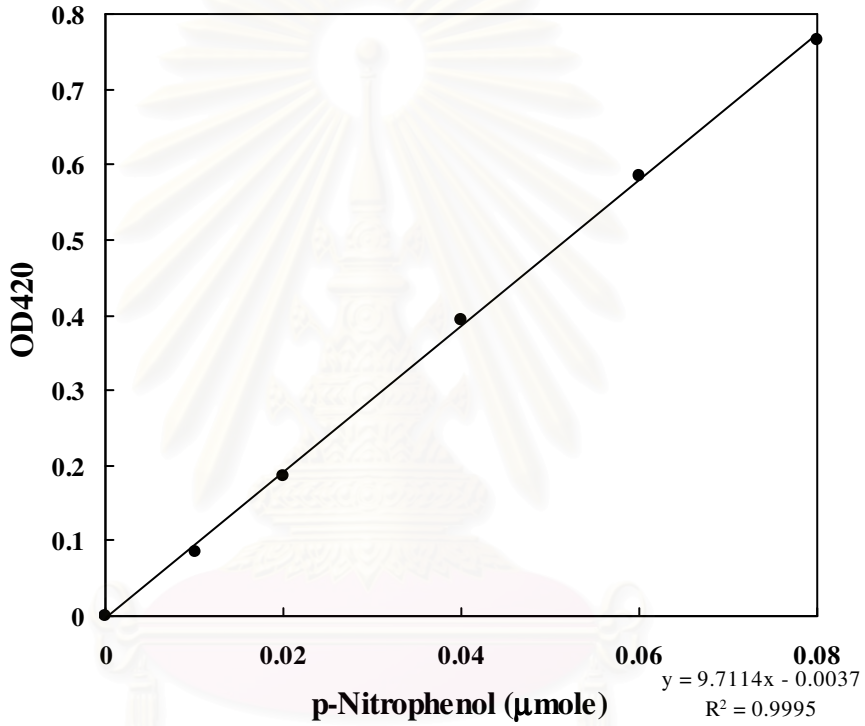

Figure C3. Correlation between final concentration of standard p-nitrophenol and optical density (absorbance) at 420 nm.

APPENDIX D

Standard curve of protein concentration by Bradford's colorimetric method.

Standard curve for bovine serum albumin (BSA) was made by determining the absorbance value at 595 nm of BSA according to the method of Bradford.

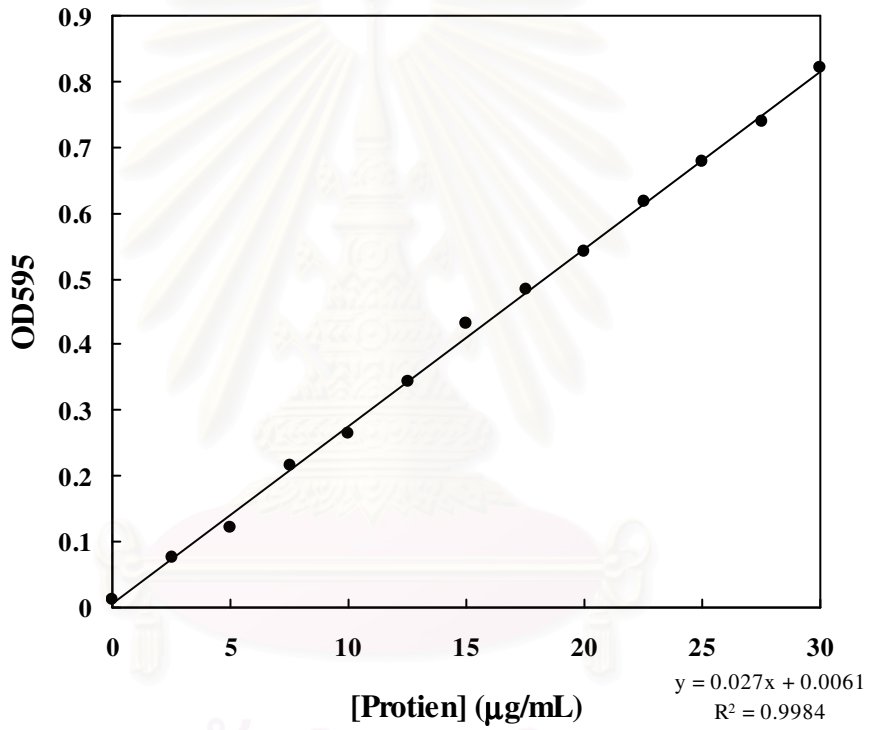

Figure D. Relationship between standard protein (BSA) concentration and optical density (absorbance) at 595 nm.

APPENDIX E

Sumitted sequence

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ACCESSION   EF011101
VERSION     EF011101.1  GI:116109048
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SOURCE      Bacillus licheniformis
  ORGANISM  Bacillus licheniformis
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REFERENCE   1  (bases 1 to 4272)
  AUTHORS   Kudan,S. and Pichyangkura,R.
  TITLE     Cloning and characterization of thermostable chitinase from
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  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 4272)
  AUTHORS   Kudan,S. and Pichyangkura,R.
  TITLE     Direct Submission
  JOURNAL   Submitted (09-SEP-2006) Biotechnology Program, Faculty of Science,
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     3601 cggcagtaaa ggagaatacg gtatcggcaa taccttaacg agaacgatgt atcataaatt
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     4261 tgtacggcaa ga
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BIOGRAPHY

Mr. Sanya Kudan was born on June 4th 1974 in Sukhothai. After He finished Mattayom VI in 1991 from Mattayom Wat Nongkhaem School, he was enrolled in the Biotechnology, Ramkhamhaeng University and graduated with a B.S. in 1997. He graduated M.Sc. in Biochemistry at Chulalongkorn University in 2001. He has working at Department of Biotechnology, Faculty of Science, Ramkhamhaeng University. Then, continued studying for Ph.D. in Biotechnology program at Chulalongkorn University in 2002.