ผลของการทำมิวเทชันกรดอะมิโนชนิดแอโรมาติกที่ผิวต่อรูปแบบการทำงานของ CHI60 จาก *Serratia* sp. TU09

นางสาวกมลทิพย์ ขัตติยะวงศ์

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EFFECTS OF MUTATION OF SURFACE AROMATIC RESIDUES ON THE MODE OF ACTION OF CHI60 FROM *Serratia* sp. TU09

Miss Kamontip Kuttiyawong

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By	Miss Kamontip Kuttiyawong
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Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Faculty of Science

(Professor Piamsak Menasveta, Ph.D.)

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(Associate Professor Chanpen Wiwat, Ph.D.)

กมลทิพย์ ขัตติยะวงศ์: **ผลของการทำมิวเทชันกรดอะมิโนชนิดแอโรมาติกที่ผิวต่อรูปแบบการทำงานของ CHI60 จาก *Serratia* sp. TU09** (Effects of mutation of surface aromatic residues on the mode of action of CHI60 from *Serratia* sp. TU09)
อ.ที่ปรึกษา: อ.ดร.รัฐ พิชญางกูร, หน้า 109. ISBN 974-14-2659-3.

ไคทิเนส 60 จาก *Serratia* sp. TU09 มีความเหมือนกับ ChiA จาก *Serratia marcescens* ซึ่ง CHI60 ประกอบด้วย 2 functional domains คือ *N*-terminal domain และ $(\alpha+\beta)_8$barrel catalytic domain ได้ทำการกลายพันธุ์กรดอะมิโน Tryptophan track บนผิวของ *N*-terminal domain และ catalytic domain ของ CHI60 (Trp33, Trp69, Trp245 และ Try418) และศึกษาแรงกระทำของกรดอะมิโนเหล่านี้ต่อไคทินชนิดต่างๆ คือ ไคทินที่ละลายน้ำ, ไคทินแบบอะมอร์ฟัส และไคทินเส้นใย เพื่อพิสูจน์ความเป็นไปได้ของความสำคัญของกรดอะมิโนเหล่านั้นต่อรูปแบบของการเร่งปฏิกิริยา, exo/endo และกลไกการทำงานของ Tryptophan track ที่ผิวของเอนไซม์ต่อการจับและการนำสารตั้งต้นไคทินเข้าสู่ catalytic site ในระหว่างการย่อยแบบ processive โดยใช้เทคนิค site-directed mutagenesis ในการเปลี่ยนกรดอะมิโน Tryptophan เป็น Phe, Tyr หรือ Ala

CHI60 มีรูปแบบในการย่อยไคทินได้ทั้งแบบ exo และ endo ซึ่งจะขึ้นอยู่กับชนิดของไคทินที่ใช้ โดยพบว่า CHI60 ใช้ endo mode ในการย่อยไคทินที่ละลายน้ำ, ใช้ทั้งสองรูปแบบในการย่อยไคทินแบบอะมอร์ฟัส และใช้ exo mode (processively) ในการย่อยไคทินเส้นใย

แรงกระทำระหว่างเอนไซม์และสารตั้งต้นไคทินเป็นแบบ hydrophobic ที่เกิดขึ้นระหว่างน้ำตาลกับเรสซิดิวส์ของกรดอะมิโน Tryptophan สามารถควบคุมรูปแบบของ CHI60 ในการย่อยไคทินได้ โดยการลดการจับอย่างจำเพาะของโมเลกุลเอนไซม์กับสารตั้งต้นไคทิน โดยการเปลี่ยนแปลงค่า ionic strength ของสิ่งแวดล้อม และการสร้างอนุพันธ์ของ CHI60 ที่กลายพันธุ์กรดอะมิโน Tryptophan ในบริเวณ Tryptophan track เป็น Phe หรือ Tyr จากผลการทดลองของ ionic strength ต่อการกลายพันธุ์กรดอะมิโน Tryptophan ใน Tryptophan track บริเวณ *N*-terminal domain และ catalytic domain ของ CHI60 แสดงให้เห็นว่า Trp33 บริเวณ *N*-terminal domain และ Trp275 บริเวณ catalytic domain มีบทบาทสำคัญต่อ processivity ของเอนไซม์ และเพื่อยืนยันรูปแบบของการย่อยไคทินของ CHI60 และตัวกลายพันธุ์ โดยตรวจสอบขนาดของผลิตภัณฑ์ที่ได้ด้วย TLC พบว่า CHI60 มีรูปแบบการย่อยไคทินแบบอะมอร์ฟัสเป็นแบบ endo และเมื่อศึกษาผลของอุณหภูมิที่ผลต่อการย่อยไคทินของ CHI60 พบว่าที่อุณหภูมิ 50°C CHI60 จะมีรูปแบบการย่อยแบบ endo มากกว่าที่อุณหภูมิ 37°C อย่างไรก็ตามที่อุณหภูมิ 50°C การทำงาน ของ CHI60 แบบ endo ลดลง ขณะที่ แบบ exo ของเอนไซม์เพิ่มขึ้น เมื่อทำการเพิ่ม ionic strength เมื่อวิเคราะห์ปริมาณผลิตภัณฑ์ที่ได้จากการย่อยไคทินด้วย CHI60 แสดงให้เห็นว่า CHI60 ทำงานแบบexo เพิ่มขึ้น และ มี processivity ในการทำงาน

นอกจากนี้พบว่า สัดส่วนของการทำงานที่อุณหภูมิ 37°C ต่อที่อุณหภูมิ 50°C ของตัวกลายพันธุ์เมื่อเปรียบเทียบกับ CHI60 มีความแตกต่างกัน ผลการทดลองแสดงว่า Trp245 น่าจะเป็นตัวนำไคทินแบบไม่ละลายน้ำเข้าสู่ catalytic cleft ส่วน W275F และ Y418A เป็นตัวช่วยเพิ่มจลนพลศาสตร์ของการย่อยไคทินที่ละลายน้ำที่อุณหภูมิสูง ซึ่งน่าจะเป็นผลมาจากการเพิ่มการปล่อยของผลิตภัณฑ์ในบริเวณ catalytic cleft และพบว่า W33F, W69F และ W245Y ซึ่งมีแรงกระทำแบบ hydrophobic ระหว่างเอนไซม์กับสารตั้งต้นไคทินลดลง นั้นก็แสดงให้เห็นว่า ตัวกลายพันธุ์เหล่านี้อาจสูญเสีย processivity จากการจับที่อ่อนลงเมื่ออุณหภูมิสูงขึ้น

ภาควิชา ชีวเคมี ลายมือชื่อนิสิต กมลทิพย์ ขัตติยะวงศ์

สาขาวิชา ชีวเคมี ลายมือชื่ออาจารย์ที่ปรึกษา

ปีการศึกษา 2549

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KAMONTIP KUTTIYAWONG: EFFECTS OF MUTATION OF SURFACE AROMATIC RESIDUES ON THE MODE OF ACTION OF CHI60 FROM *Serratia* sp.TU09 THESIS ADVISOR: RATH PICHYANGKURA, Ph.D., pp.109 ISBN 974-14-2659-3.

Chitinase 60 (CHI60) from *Serratia* sp.TU09 was cloned, expressed and characterized. The amino acid sequence of CHI60 was identical to ChiA from *Serratia marcescens*. CHI60 has two functional domains, the *N*-terminal domain and the $(\alpha/\beta)_8$ barrel catalytic domain. To study the mechanism and mode of action of CHI60, we mutated the exposed Trp residues in the tryptophan track in the *N*-terminus and the catalytic domain of CHI60 (Trp33, 69, 245, 275 and Tyr418). The interaction these of residues with different types of substrate, soluble, amorphous and crystalline chitin was studied, to investigate the possible role of these residues on the mode of catalysis, exo/endo mode, and mechanism by which the exposed tryptophan track functions on binding and guiding the substrate during processive hydrolysis. Site-directed mutagenesis of Trp residues to Phe, Tyr or Ala was carried out.

We found that CHI60 possesses both exo and endo mode of hydrolysis depending on type of substrate it hydrolyzes. CHI60 uses endo mode when it hydrolyzes soluble chitin involving only the catalytic domain, uses both endo and exo mode when it hydrolyzes amorphous chitin and uses exo mode when it processively hydrolyzes β-crystalline chitin.

We can influence the mode of hydrolysis by CHI60 between the enzyme and substrate by modulating the binding affinity of CHI60 to its substrate. This was accomplished by changing ionic strength of the environment, and/or generating derivatives of CHI60 with mutations on Trp residues in the Tryptophan track, where Trp was changed to Phe or Tyr. This is based on the effect that interaction between the sugar ring and Trp residues is a hydrophobic interaction.

We have successfully mutated Trp residues in the exposed tryptophan track at the *N*-terminus and the catalytic domain of CHI60. The effect of ionic strength on hydrolyzing activity of mutants suggested that Trp33 in the *N*-terminus and Trp275 in the catalytic domain may play an important role in the processivity of the enzyme. To further confirm the mode of hydrolysis of CHI60 and mutants, we studied the product size distribution by TLC when an amorphous substrate was used. On amorphous substrate CHI60 would be forced to hydrolyze the substrate via the endo mode, which would give multiple size products. We found that at 50 °C, CHI60 used endo mode more than at 37 °C. However at 50 °C, the endo mode of CHI60 was reduced while the exo mode of CHI60 was enhanced by increasing in ionic strength. We used HPLC analysis for further quantitative of product. The results demonstrated that the exo mode of CHI60 was enhanced at elevated ionic strength and substrate was processively hydrolyzed.

In addition, the difference in the ratio of the activity of the enzyme at 37 °C versus 50 °C of mutant comparing to WT CHI60, implied that Trp245 may function to guide the insoluble chitin into the catalytic cleft, and W275F, W418A may help to increase the kinetic of the hydrolysis at high temperature on PNAC by facilitating the dissociation of the product from catalytic cleft. On β-chitin, single mutants, W33F, W69F and W245Y, with reduced hydrophobic interaction between substrate-enzyme, lose their processivity at higher temperature probably due to weaker enzyme-substrate binding.

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Student's Signature Kamontip Kuttiyawong
Advisor's Signature

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LIST OF ABBREVIATIONS

A	Absorbance
bp	Base pair
BSA	Bovine serum albumin
°C	Degree Celsius
CCMM	Colloidal chitin minimum medium
DNA	Deoxyribonucleic acid
g	Gram
GlcNAc, NAG	*N*-acetyl-D-glucosamine
hr	Hour
kb	Kilo base
kDa	Kilo Dalton
L	Liter
M	Molar
mL	Milliliter
mg	Milligram
ng	Nanogram
µg	Microgram
µL	Microliter
min	Minute
rpm	Revolution per minute
w/w	weight by weight
Trp, W	Tryptophan
Phe, F	Phenylalanine
Tyr, Y	Tyrosine
Ala, A	Alanine
PNAC	Partially *N*-acetylated chitin

CHAPTER I

INTRODUCTION

Chitin

Chitin is the second most abundant biopolymer, after cellulose, which is a strong indication of its importance in nature. It is synthesized by different organisms with different chitin contents, which are given in Table 1.1 (Knoor, 1984). It is used as a major structural component of fungal cell walls and the exoskeletons of invertebrates including insects, arachnids, crustaceans and extracellular polymer of some bacteria (Muzzarelli, 1977). It is also found in common foods such as grains, yeast and mushrooms. In nature, chitin has been estimated annual production between 10^{10} and 10^{11} tones. The highest amount of chitin with respect to total dry weight is found in crustaceans. Thus crustacean shells were used as main source of chitin by most chemical industries.

Structure of chitin

Chitin is an insoluble linear β-1,4-linked homopolymer of *N*-acetyl-D-glucosamine (GlcNAc). It is chemically similar to cellulose, a polymer of β-1, 4-linked glucose and its derivatives. Chitosan is a copolymer of β-1, 4- linked glucosamine and *N*-acetylglucosamine (Figure 1.1). The successive sugar units in the chitin/chitosan chain are rotated 180° relative to each other. Thus, the functional and structural unit in these polymers is a disaccharide. The physical structure of chitin at the molecular level has been the subject of continuous study since 1920s, and the primary methods of analysis have been X-ray crystallography and infrared spectroscopy. Comparison of the X-ray data for chitins from different sources has revealed the existence in nature of more than one polymorphic form. Generally, the individual chain assumes an essentially linear structure, which undergoes one full twist, every 10.1-10.5 Å along the chain axis. Because each glycosidic unit in the chain is chiral, and all units are connected by an oxygen atom that C-1 of one glycosidic unit to C-4 of a next unit, a distinct left and right direction can be assigned to each polymer chain. Chitin in nature is a polymer of *N*-acetyl glucosamine, which

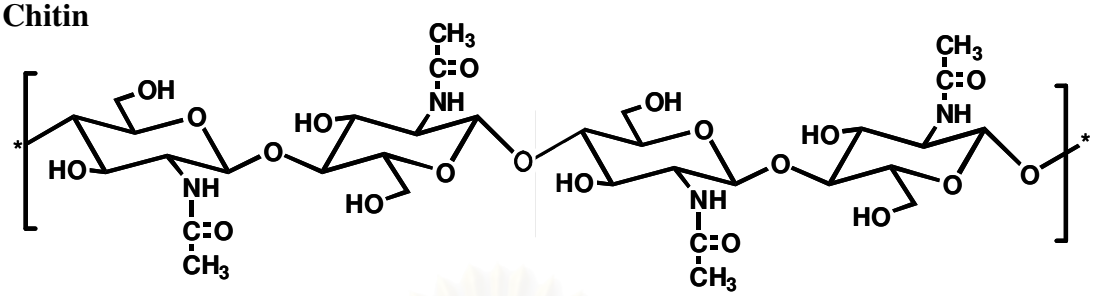
Table 1.1 Chitin content of selected crustacean, insect, molluscan organs and fungi

Type	Chitin content (%)	Type	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 (truefly)	54.8[c]
Crangon (shrimp)	69.1[c]	Pieris (sulfer 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]		
Lepas (barnacles)	58.3[c]	**Fungi**	
Molluscan organs		*Aspergillus niger*	42.0[d]
Clamshell	6.1	*Penicillium notatum*	18.5[d]
Oyster shell	3.6	*Penicillium chrysogenum*	20.1[d]
Squid, skeleton pen	41.0	*Saccharomyces cereviseae*	2.9[d]
Krill, deproteinized shell	40.2	*Mucor rouxii*	44.5
		Lactarius vaiiereus (mushroom)	19.0

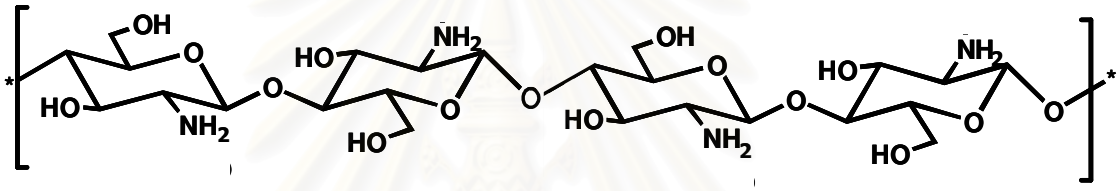
a. Wet body weight b. Dry body weight or total dry weight of cuticle

c. Organic weight of cuticle d. Dry weight of cell wall

Chitin



Chitosan



Cellulose

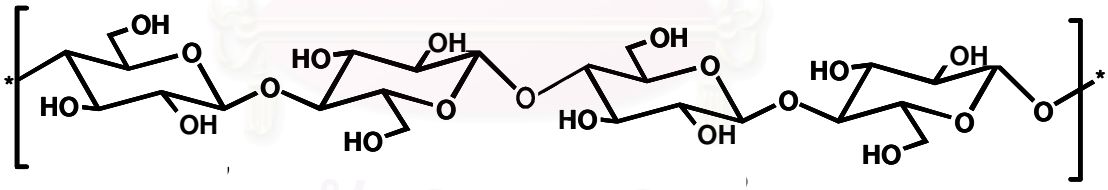


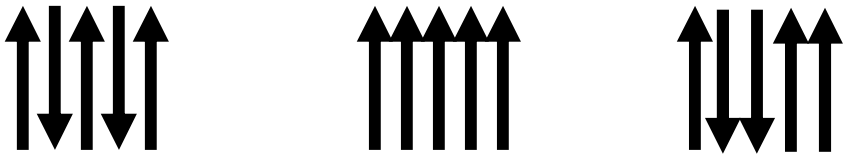
Figure 1.1 The structure of chitin, chitosan and cellulose

are arranged in 3 crystalline structural forms, an antiparallel (α-chitin), parallel (β-chitin) and mixture between antiparallel and parallel form (γ-chitin) depending on the origin (Figure 1.2A). Most chitin, including those from crustaceans, insects and fungi are in the so-called α-form, in which the unit cell is orthorhombic and the individual chains are arranged in antiparallel manner. The hydrophobic forces between the surfaces of the glucose rings further stabilize the stacking of the chains. Furthermore the arrangement of the CH_2OH side chains, dictating the type of intermolecular bonding along the chitin chain and resulting in the conformation of chitin.

In α-chitin, half of the -CH_2OH groups are bonded to carbonyl with in the same stack of chains, and half are bonded to the –CH_2OH groups on an adjacent stack. The existence of this intersheet bonding is probably responsible for the stability of the α-chitin structure, specifically its inability to swell in water. However, a rare second form known as β-chitin has been identified in four sources, the spines of the polychaete *Aphrodite*, the pen of the squid *Loligo*, the tubes of *Pogonophora* and the spines of certain marine diatoms. This chitin conformation has a monoclinic unit cell with the polymer chain arranged in a same sense, different from the α-chitin; the –CH_2OH groups are all hydrogen bonded to carbonyl group in the same stack of chain. Hence there is no hydrogen bonding between the stacks of the chains. A third form, γ-chitin has been reported from the stomach lining of *Loligo* only. It has a three-chain unit cell in which two up chains are followed by the down chain. However, this form has not been subjected to detailed analysis as in α and β forms, and it has yet to be established that this is truly a third structure, and not a distorted version of one of the others. The diagrammatic structure of all conformations of chitins are shown in Figure 1.2 B (Blackwell, 1969).

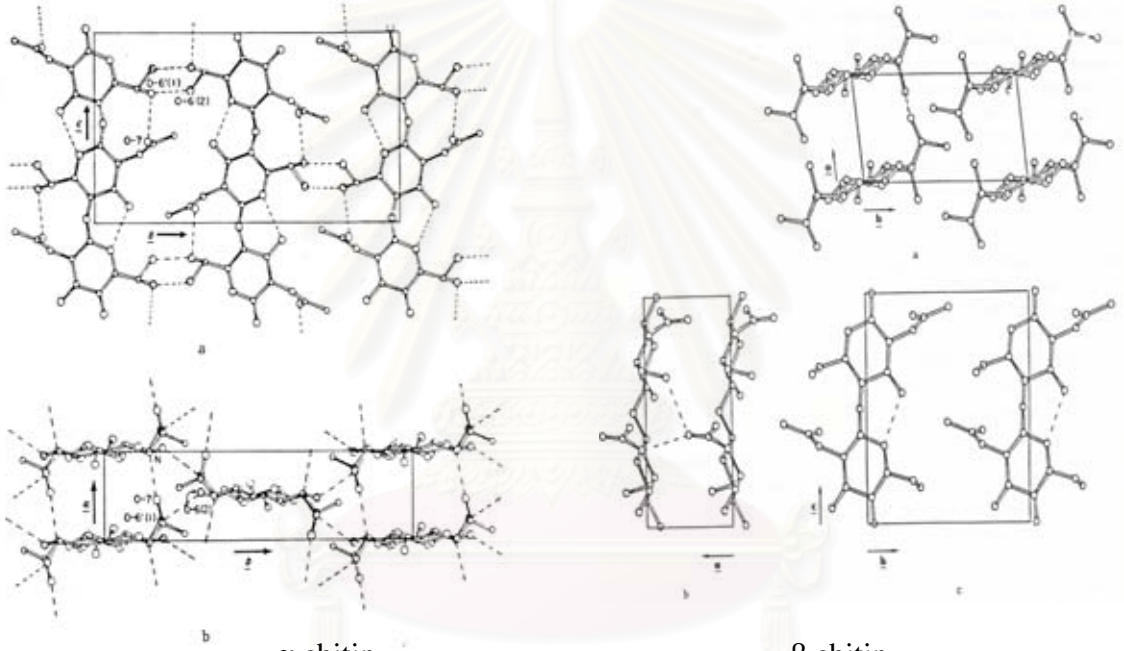
In recent years significant research has been directed toward the use of chitin and chitin derivatives in many fields such as effluent water treatment and drug delivery (Brine, 1984). For industrial and applied fields, chitin and its deacetylated form, chitosan, have been used to produce high value products such as cosmetics, drug carrier, food additives, semi-permeable membranes and pharmaceutical products (Table1.2) (Shahidi *et al.*, 1999; Riccardo and Martin, 1997 and Goosen, 1997). Chitin and chitosan (deacylated chitin) are used in such areas as bioremediation of heavy metal contamination (Cardenas *et al.*, 2001), paper and textile production (Shigemasa and Minami, 1996), as additives in animal feed (Austin *et al.,* 1981), and

A.



α-chitin β-chitin γ-chitin

B.



α-chitin β-chitin

Figure 1.2 Structure of α-chitin, β-chitin and γ-chitin

Structure of α-chitin, β-chitin and γ-chitin (A) and hydrogen bond between C=O-NH group of chitin chain from .α-chitin and β-chitin (B). The direction of poly *N*-acetyl-D-glucosamine chain is marked with an arrow.

Table 1.2 Current practical uses of chitin, chitosan and their derivatives

Area of Application	Example
Agriculture	- Plant seed coating
	- Fertilizer
Food Industry	- Color stabilization
	- Emulsifying agent
	- Clarification and deacidification of fruits and beverages
	- Dietary fiber
	- Fiber-optic sensor for determination ethanol beverages and organic acid
Medical	- Dressing materials for the burns and skin lesions of humans and animals
	- Wound - dressings
	- Artificial limbs
	- Carrier-drug conjugates
Wastewater Treatment	- Recovery of metal ions, pesticides and phenols
	- Removal of dye
Cosmetics	- Moisturizer
	- Face, Hand and Body creams
Paper	- Surface Treatment
	- Photographic Paper
Biotechnology	- Enzyme immobilization
	- Chromatography
	- Ultrafiltration membranes

as a component of consumer products such as cosmetics (Tsigos *et al*., 2000). Uses for chitin-derived products in the medical field include drug and vaccine delivery systems (Felt *et al.*,1998) wound and burn treatments (Muzzarelli *et al.*,1999 and Singla and Chawla, 2001), blood cholesterol lowering agents (Ylitalo *et al.*, 2002), anti-clotting agents (Drozd *et al*., 2001), antibacterial compounds (Cuero, 1999 and Helander *et al.*, 2001), and enhanced dissolution of some drugs such as ibuprofen (Bodek, 2002 and Ilango *et al.*, 1999). Chitinases have been shown to act as biocontrol and anti-fungal agents (De Boer *et al.*, 2001; Fung *et al*., 2002 and Liu *et al.*, 2002), as well as having anti-biofouling applications (Minhalma and Pinho, 2001). Chitinases can be used to create chitooligosaccharides of specific lengths, which have a variety of roles in the industrial and research applications mentioned above. Chitin-binding modules have been incorporated into protein expression vectors as fusion tags to facilitate the purification of recombinant proteins (Chong *et al*., 1997).

Chitinase

Chitinase is a glycosyl hydrolase that catalyzes the hydrolytic degradation of chitin. Chitinases are found in a wide variety of organisms that possess chitin as well as bacteria, fungi, nematodes, plants, insects, fish and human. The physiological functions of chitinases depend on their source (Table 1.3). Chitinases are broadly classified by their type of reaction that the enzymes catalyze and their substrate specificity, which is recommended by IUBMB (IUBMB; Nomenclature Committee of International Union of Biochemistry and Molecular Biology). However, the IUBMB classification is not helpful for understand evolutionary relationship among these enzymes. In 1991, Henrissat and his co-workers purposed a systematic comparison of the primary sequences of glycosyl hydrolases and grouped these enzynes into 35 families and presently updated to 106 families (http://afmb.cnrs-mrs.fr/CAZY/).

Classification of chitinase according to their mode of action

Chitinases are classified as endochitinases and exochitinases. The endochitinase activty is defined as the random cleavage at the internal points in the chitin chain. The exochitinase activity is defined as the processive hydrolysis staring at the non-reducing ends of chitin with the release continuously of $(GlcNAc)_2$ or

Table 1.3 Role of chitinases in different phyla

Organism	Role of chitinases	References
Bacteria	Mineralization of chitin, also in nutrition and Parasitism.	Flach *et al*., (1992); Connell *et al*., (1998).
Fungi	Physiological role in cell division, differentiation and nutritional role related to mycoparasitic activity (e.g. in *Trichoderma sp*.).	Kuranda *et al*. ,(1991); Gooday *et al*., (1992); Mellor *et al*. ,(1994); Alam *et al*.,(1995).
Plants	Defence against fungal and bacterial pathogens by degradation of their cell walls. Specific isoforms may play a role in embryo development, pollination and sexual reproduction.	Schlumbaum *et al*.,(1986); De Jong *et al*., (1992); Leung(1992); Kim and Chung (2002).
Insects	Developmental process of cuticle degradation at different larval stages.	Kramer and Fukamiso,(1985); Merzendorfer and Zimoch (2003).
Protozoa	Malarial parasites produce sufficient quantities of chitinase to penetrate the chitin containing peritrophic matrix of the mosquito midgut.	Huber *et al*., (1991); Langer *et al*. (2002).
Human	Chitotriosidase activity helps in defence against nematodal infections. Moreover, its enzymatic activity is markedly elevated in serum of patients suffering from lysosomal lipid storage disorders, sarcoidosis and thalassemia.	Escott *et al*. ,(1996); Choi *et al*. ,(2001); Aguilera *et al*,(2003); Gianfrancesco and Musumeci (2004).
Animal	The high chitinase level in goat and bovine blood (serum) might be a function of slow renal secretion which keeps the enzyme level comparatively low in case of abnormal lysozyme production (monocyticmyelomonocytic leukemias and renal diseases).	Lundblad *et al*. (1974).
Yeast	α–subunit of toxin secreted by *Kluyveromyces lactis* has chitinase activity which is most likely required for the _ subunit to gain entry to the sensitive cell. Chitinases has an essential role in cell separation during budding of the chitinous yeast *Saccharomyces cerevisiae*. *Saccharomyces cerevisiae* chitinase also used as antifungal.	Butler *et al*. ,(1991); Kuranda and Robbins, (1991); Carstens *et al*. ,(2003); David (2004)

GlcNAc. For glycosyl hydrolase (EC3.2.1.X), the first three digits indicate the type of reaction, substrate and sometimes reflects molecular mechanism as shown in table 1.4.

Classification of chitinase based on their amino acid sequences

Henrissat and his co-workers grouped chitinases and *N*-acetylhexosaminidases into three families 18, 19 and 20. Generally, chitinases are found in family 18 and 19. Family 18 contains several conserved amino acids, as shown in Figure 1.3A., which the enzymes of *Bacillus circulans, Serratia marcescens, Pyrococcus kadakaraensis and Trichoderma harzianum*. These sequences are found in the active site and include a glutamic acid (E) residue (residue in white letter) which played a crucial role in catalytic mechanism. Almost all of plant chitinases are grouped in family 19. The conserved amino acids from these family are represented by barley chitinase (*Hordeum valgare*), potato chitinase (*Solanum tuberosum*), pea chitinase (*Pisum sativum*) and *Arabidopsis thaliana*, as shown in Figure 1.3B. Two glutamic residues (residue in white letter) are important in the mechanism of the action of Family 19 (Henrissat *et al*., 1991).

Three dimensional structures of chitinases

Enzymes exhibit their function when the proteins fold properly to form appropriate three dimensional structures. Thus, information of the three-dimensional structure is essential for studying catalytic mechanism of the enzymes. X-ray crystallography and NMR spectroscopy are now important strategies for obtaining the three- dimensional structure of macromolecules. Since most of the chitinolytic enzymes have molecular weights of 15-80 kDa, X-ray crystallography is the main strategy for obtaining three-dimensional structures. Among the chitinolytic enzymes other than avian lysozymes, the crystal structure of 26 kDa chitinase from barley seeds, *Horduem vulgare* L., which is classified into family 19, was first solved by Robertus and his colleagues at the University of Texas (Hart *et al*., 1993). After that, the structures of a 60 kDa chitinase A from *Serratia marcescens* and a 29 kDa chitinase from rubber tree, *Hevea brasiliensis*, (hevamine) were solved and reported in the same journal (Perrakis *et al*., 1994 and Terwisscha *et al*., 1994). Both are family 18 enzymes. Comparison between the structures of family 18 and family 19 enzymes revealed a clear difference in their structures. The figure 1.4A and 1.4B

Table 1.4 Nomenclature of chitinolytic enzymes

Mode of action	Chitinolytic enzymes	EC No.
Random hydrolysis of the chain	Chitinase, 1,4-β-*N*-acetylglucosaminidase	EC3.2.1.14
Hydrolysis of terminal non-reducing sugar	[a,b] Early classification; Chitobiase β-D-acetylglucosaminidase	EC3.2.1.29 EC3.2.1.30
Successive removal of sugar unit from the non-reducing end	[c] Present classification; β-*N*-acetylhexosaminidase	EC3.2.1.52

[a]Recommendations of the Nomenclature Committee of the IUBMB, 1961 Chitobiase (chitobiase acetylaminodeoxyglucohydrolase (EC3.3.1.29) β-*N*-acetylglucosaminidase (β-2-acetylamino-2-deoxy-D-glucoside acetylamiondeoxyglucohydrolase)(EC3.2.1.14)

[b]Recommendations of the Nomenclature Committee of the IUBMB, 1978 EC3.2.1.29 deleted and the enzyme was included with β-*N*-acetylglucosaminidase

[c]Recommendations of the Nomenclature Committee of the IUBMB, 1992 Entry EC3.2.1.30 deleted and the enzyme β-*N*-acetylglucosaminidase with was included β-*N*-acetylhexosaminidase (EC3.2.1.52)

A.

```
B.circulans          IISVGGWTYSNRFFDGVDIDWETPVS
P.kodakaraensis      LISVGGWTLSKYFFDGVDLDWEYPVS
S.marcescens         LPSIGGWTLSDPFFDGVDIDWEFPGG
T.harzanum           ILSIGGWTWSTNFFDGVDIDWEYPAD
                     : *:**** *  ******:**E * .
```

B.

```
A.thaliana   KREIAAFLGQTSHETTGGWPTAPADGPYAWGYCFLREQNP-SDYCQASSEFPCASG
H.valgare    KREVAAFLAQTSHETTGGWATAPADGAFAWGYCFKQERGASSDYCTPSAQWPCAPG
P.sativum    KREVAAFFGQTSHETTGGWATAPADGPYSWGYCFKQEQNPASDYCEPSATWPCASG
S.tuberosum  KREIAAFFAQTSHETTGGWASAPADGPYAWGYCFLRERGNPGDYCPPSSQWPCAPG
             ***:***:.****E*****.:*****.::***** :E:.  .*** .*: :***.*
```


Figure 1.3 The local alignment of several chitinases from glycosyl hydrolase family 18 (A.) and glycosyl hydrolase family 19 (B.)

show the crystal structures of jackbean chitinase (family 19) and *Serratia marcescens* chitinase B (family 18). Jackbean chitinase is composed of two lobes, each of which is rich in α-helical structure. From a docking calculation of the chitinase and $(GlcNAc)_6$, the substrate binding cleft is estimated to lie between the two lobes (Hart *et al*., 1995). The hypothetical binding cleft is composed of two α-helices and three-stranded β- sheets. As described below, the cleft structure is conserved in other chitinolytic enzymes. On the other hand, the structure of family 18 chitinase from *S. marcescens* (chitinase A) is completely different from that of family 19 chitinase from barley seeds. The family 18 chitinase has a typical $(\beta/\alpha)_8$ barrel structure composed of eight α-helices and an eight stranded β-sheet. In addition to the main barrel domain, it has an *N*-terminal β -strand-rich domain and a small (β+α) domain. The crystal structures of other family 18 chitinases, e.g., chitinase B from *Serratia marcescens* (Perrakis *et al*. 1994) (Figure 1.4), hevamine from *Hevea brasiliensis* (Terwisscha van Scheltirga *et al.*, 1994), endo-β-*N*-acetyl-glucosaminidase F_1 from *Flavobacterium meningosepticum* (Roey *et al*., 1994), and endo-β- N-acetyl-glucosaminidase H from *Streptomyces plicatus* (Rao *et al.*, 1995), exhibit similar barrel structure. Apparently, in family 18 chitinases, the sequence homology results in the similarity in three-dimensional structure.

Catalytic mechanisms of chitinases

Chitinases act by hydrolytically cleaving the β(1,4)-glycosidic linkages between GlcNAc residues. In general, this hydrolysis can occur in one of the two ways, either with retention of anomeric configuration in the product or with inversion. The catalytic mechanism of glycosyl hydrolases has been discussed on the basis of the mechanism of hen egg white lysozyme. But now, structural data are available for various chitinolytic enzymes. Thus, the catalytic mechanism of the enzymes should be reexamined considering the three-dimensional structures. The most powerful information has been obtained from X-ray crystal structures of the enzymes complexed with their substrate analogues. These structures were solved by several investigators, and their catalytic mechanisms were discussed from the relative locations of catalytic residues to the bound substrate analogues. Before discussing the

A.

B.

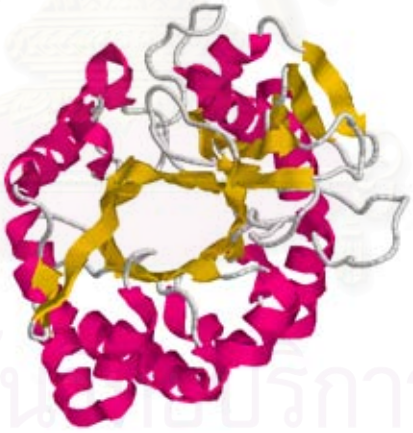


Figure 1.4 The ribbon drawing of a chitinase Family 18 and 19 The ribbon drawing of chitinase, (A) family 19 (jack bean chitinase; 1DXJ) and (B) family 18 (chitinase B from *Serratia marcescens*, 1E15) displayed by Rasmol 2.6.

mechanism of chitinolytic enzymes, however, the lysozyme mechanism should be reviewed for comparison.

Hen egg white lysozyme

Lysozyme catalysis takes place through the concerted action of two acidic residues, the general acid-base Glu35 and the nucleophile Asp52 as shows in Figure 1.5 (Scheme I) (Johnson *et al*., 1988 and Strynadka and James, 1991). At first, Glu35 acts as a general acid and protonates the glycosidic oxygen of the scissile bond, leading to bond cleavage and formation of a positively charged oxocarbonium intermediate at the sugar residue bound in subsite (-1). Hereafter, the subsite nomenclature proposed by Davies *et al*. (1997) is used in this article. Asp52, located on the opposite side of the scissile bond, has been proposed to either stabilize the oxocarbonium intermediate through electrostatic interactions or form a covalent intermediate through a bond to the C1 atom of the intermediate sugar residue at subsite (-1). After forming the intermediate state, the leaving oligosaccharide fragment diffuses out of the active site and is replaced by a nucleophilic water molecule. The water molecule then attacks the C1 atom of the reactive intermediate with the aid of the negatively charged Glu35 acting as a general base. The configuration at the anomeric carbon atom is retained (Dahlquist *et al*., 1969), because the water molecule attacks the intermediate from the β-side. This is commonly referred to as the double displacement mechanism of hydrolysis.

Family 19 Chitinases

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 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 1H-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 Figure 1.5, scheme II. 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 α-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 α-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 (shows in figure 1.5, Scheme III) 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 marcescens* 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 eliminated 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 a 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).

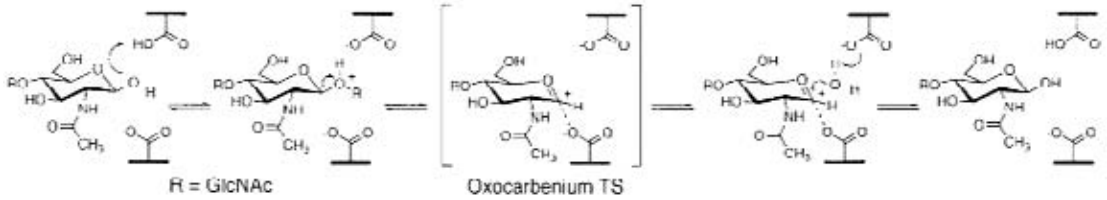
The lastest proposed catalytic mechanism in chitinase A from *Serratia marcescens* suggested that residues Asp313 and Try390 along with Glu315 play a central role in the catalysis (Yannis *et al*., 2001). Yannis and coworkersproposed that after the protonation of the substrate glycosidic bond, Asp313 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 intermediate, the acetamido group of -1 sugar comes close to O5 atom in a way that could allow a modified “substrate assisted” reaction, shows in figure 1.4, scheme IV.

Substrate binding mechanism

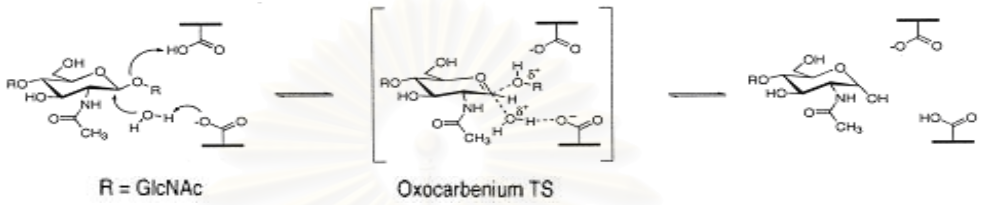
Hen Egg White Lysozyme

Hen egg white lysozyme has well-known binding subsites, so called A, B, C, D, E, and F, and the glycosidic bond cleavage takes place between sites D and E. According to the subsite nomenclature proposed by Davies *et al*., 1997, the binding subsites can be written as (-4)(-3)(-2)(-1)(+1)(+2). The subsite structure was estimated from model building of the lysozyme-$(GlcNAc)_6$ complex based on the crystal structure of the complex with $(GlcNAc)_3$, and can be confirmed from experimental time-courses of oligosaccharide degradation and product formation obtained by high performance

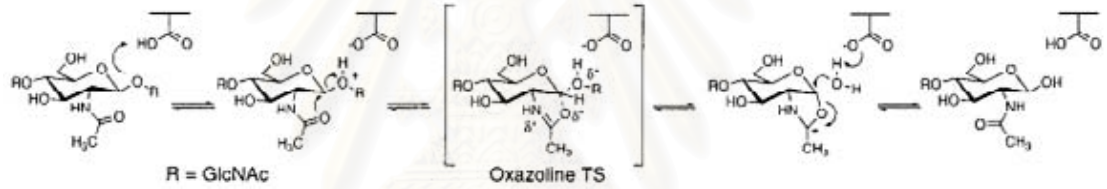
Scheme I



Scheme II



Scheme III



Scheme IV

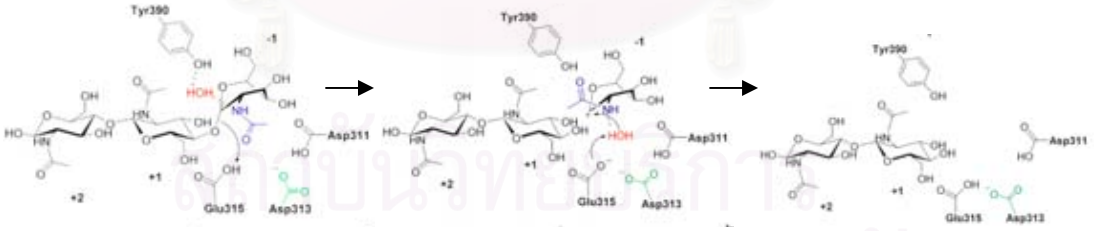


Figure 1.5 Chitinase mechanism Mechanisms of lysozyme (scheme I), family 19 chitinase(scheme II) and family 18 chitinase (scheme III, IV).

liquid chromatography (HPLC) (Masaki *et al*., 1981 and Fukamizo and Hayashi, 1982). As shown in Figure 1.6A, the amino acid residues participating in the substrate binding are Asn59, Trp62, Trp63, and Ala107 at subsite (-2), Trp62 at subsite (-3) (not shown in the figure), and Asp101 at subsites (-3) and (-4) (Imoto *et al*., 1972). At subsites (+1) and (+2), Arg114 and Phe34 are considered as contact sites for "right-sided" binding mode, and Arg45, Asn46, and Thr 47 for "left-sided" binding mode (not shown in the figure) (Pincus and Scheraga, 1979).

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 Tyr 123 and Asn124 (Figure 1.6B). 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 1.6C 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

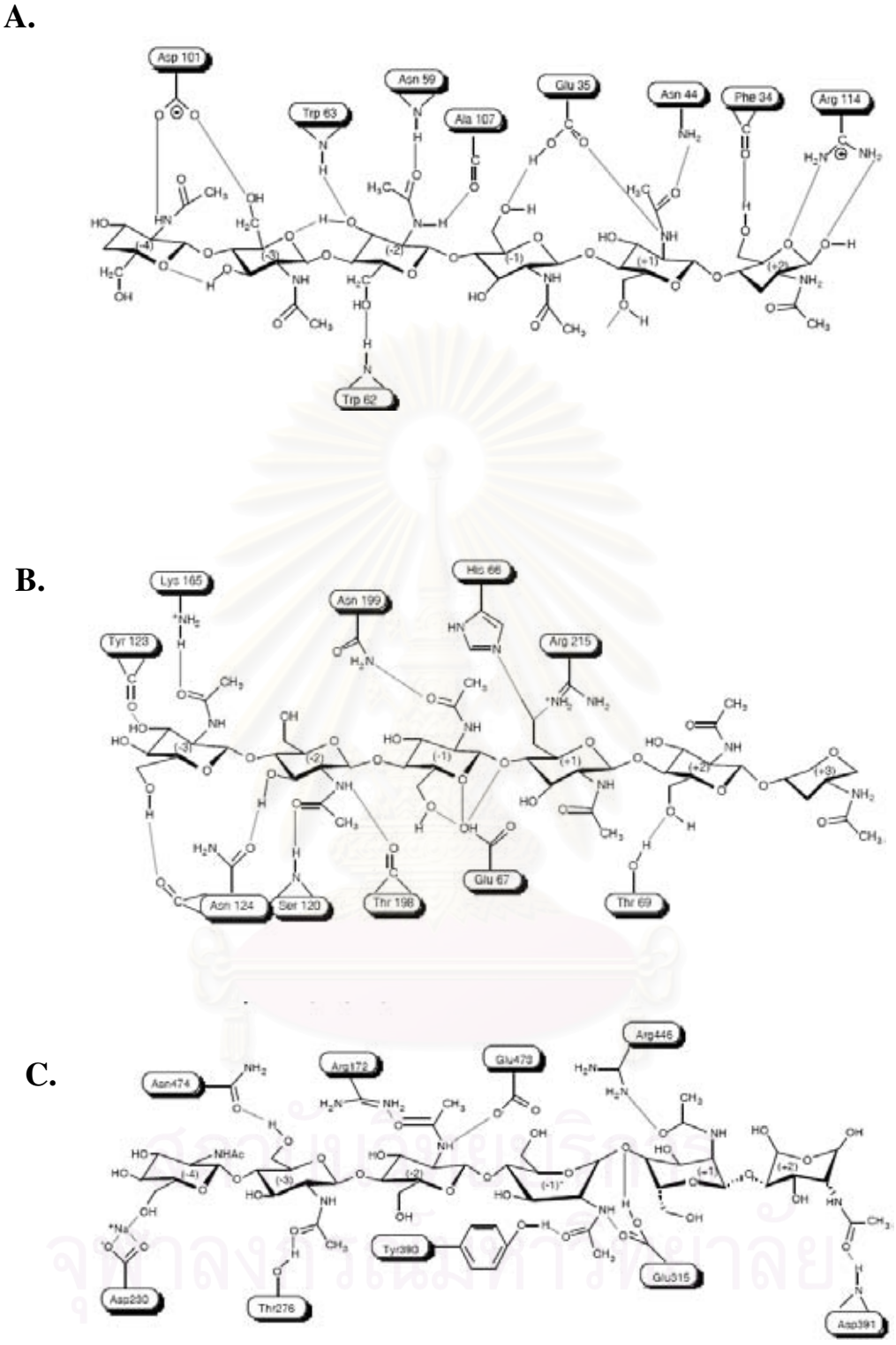


Figure 1.6 Interaction between hen egg white lysozyme and $(GlcNAc)_6$ (A), barley chitinase and $(GlcNAc)_6$ (B) and *S. marcescens* chitinase A and $(GlcNAc)_6$ (C). Hydrogen bonding interaction were from molecular dynamics silimulation (Brameld and Goddard, 1998).

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). 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 1.7B). 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 chitinase.

Biotechnological applications of chitinase

Practical applications of chitinase include its use in the preparation of protoplasts from fungi (Yabuki *et al*., 1984), as a protective agent against plant-pathogenic fungi (Sundheim *et al*., 1998) and in the production of chitio-oligosaccharides as biologically active substances (Usui *et al*., 1990).

Chitinases are reported to dissolve cell walls of various fungi, a property that has been used for the generation of fungal protoplasts (Anjani Kumari and Panda, 1992 and Ramaguera *et al.*, 1993). Chitinases from *Streptomyoes* was found to be active in the generation of protoplasts from *Asperigillus oryzae* and *Fusarium solani* (Skujins *et al*., 1965). An enzyme complex from *Bacillus circulans* WL-12 with high chitinase activity was effective in generating protoplasts from *Phaffia rhodozyme* (Johnson *et al*., 1979). A mixture of commercially available chitinase and cellulose was used to release viable protoplasts from *Caprinus macrorhizus* (Yanagi and Takebe, 1984). Chitinase from *Trichoderma harzianum* was the most efficient generator of protoplasts from different fungi (Kitomoto *et al.,* 1988 and Anjani Kumari and Panda, 1992).

Chitinase-producing organisms are used in agriculture as an effective biocontrol agent against a number of phytopathogenic fungi (Kapat *et al*., 1996 and Elad *et al*., 1982). Chitinase from *T. harzianum* was found to be active against broadest range of pathogens and soil-borne fungi (Irene *et al*., 1994).

Aeromonas caviae controlled infection *by Rhizoctonia solani* and *Fusarium oxysporum* in cotton and *Sclerotium rolfsii* in beans by producing chitinase (Inbar and Chat, 1991). Chitinase produced by *Serratia marcescens* was effective against pathogenic fungi *S. rolfsii* (Ordentlich *et al*., 1988) and larvae of *Galleria mellonela* (Lysenko, 1976). A culture filtrate of *Aphanocladium album* strongly inhibited growth of *Necteria haematcocca* in the pea (Kunz *et al*., 1992).

Chitinase-producing organisms are effectively used in the bioconversion process to treat shellfish waste and also to obtain value-added products from such wastes (Revah-Moiseev and Carroad, 1981; Tom and Carroad, 1981 and Carroad and Tom, 1978). Thus, chitinolytic enzymes have been purified and cloned from many microorganisms, and their enzymatic properties have been investigated.

Chitinase A in *Serratia marcescens*: a representative of family 18 chitinase

Chitinase A in *Serratia marcescens* is the representative of family 18 chitinase, and its chitinolytic machinery is one of the best-characterized chitinolytic machinery known data.

Protein related to chitin degradation

The chitinase-encoding genes have been identified so far from the bacterium and it was designated as *chiA*, encoded ChiA as a 563-residue precursor, then it was secreted from the cells to the culture medium with concomitant cleavage of an N-terminal signal peptide. The resulting enzyme has 540 residues and a calculated molecular mass of 58.5 kDa (Perrakis *et al*., 1994).

Chitinases export and localization

During secretion to the extracellular, ChiA is processed by cleaving of the N-terminal signal peptide. N-terminal signal peptides are signatures of *sec*-dependent proteins exported to the periplasm. When protein reaches the periplasm, its signal peptide is cleaved by a periplasmic signal peptidase (Nathan *et al*., 2003). But how ChiA is transported across the outer membrane after this cleavage still remains unknown.

Biochemical characteristics of chitinases

Pure ChiA shares several biochemical characteristics. A broad pH optimum for its is around pH 5.0-6.0; a temperature optimum is between 50-60 °C. The enzyme is fairly stable, with half-lives of more than 10 days at 37°C, pH 6.1. In addition, the enzyme not only cleave [$(GlcNAc)_2$] from poly *N*- acetyl-glucosamine, but also can produce monomer GlcNAc from long $(GlcNAc)_n$ substrate. ChiA has endo- and exo-chitinase activity while *Serratia marcescens* ChiB is a true exochitinase (Brurberg *et al*., 1996).

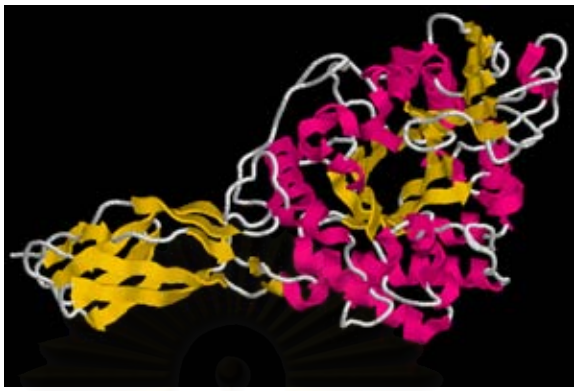
Structure of ChiA

The structure of ChiA is unique because of its completeness, which comprises three domains: an all beta-strand amino-terminal domain similar to fibronectin type III domain, a catalytic $(\beta/\alpha)_8$ barrel domain, and a small $(\alpha+\beta)$-fold domain, which is inserted in the catalytic $(\beta/\alpha)_8$ barrel domain (Uchiyama *et al.*, 2001) (figure 1.7A). This characteristic means that it has a tendency to undergo the proteolytic cleavage, and the flexibility between individual domains may assist the protein in directing the substrate chain into the cleft at the catalytic domain (Aronson *et al*., 2003). The amino-terminal (ChiN) domain is a putative chitin-binding domain. This domain is likely to interact with the substrate by elongating the substrate-binding cleft but may not be involved in the initial binding formation (Perrakis *et al*., 1997).

Comparison of structures and enzymatic activities

All family 18 chitinases hold a TIM barrel structure as a scaffold bearing the active site. And down into the TIM barrel core, there is a string of conserved aspartic acid residues forming the DxxDxDxE motif that is also observed in all family 18 members. Although ChiA and ChiB both have the catalytic domain and the sugar binding domain, there are some differences between each other. The sugar-binding domain in ChiB is located at the reducing side of the active site and in that the substrate binding cleft is blocked at the nonreducing side of subsite –3 by porch loop. This phenomenon provides a structural explanation for the exoactivity of ChiB and shows that the enzyme degrades its polymeric substrates from the nonreducing end.

A.



B.

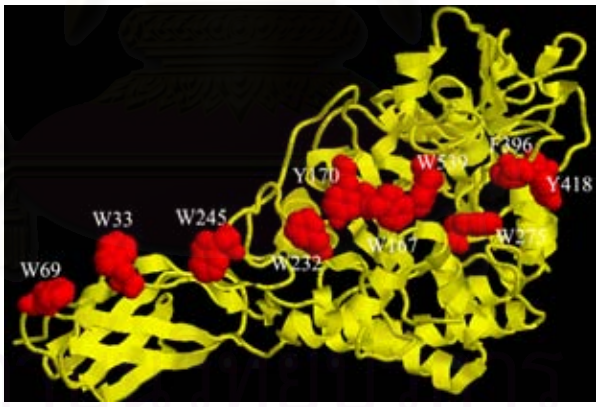


Figure 1.7 The structure of ChiA from *Serratia marcescens* ChiA (A) has three domains: N-terminal domain, catalytic TIM barrel domain, and (β+α) domain. The aromatic residues of ChiA from *Serratia marcescens* (B), which the aromatic residues, are labeled with white letter and are considered to participate in the sugar residue binding by hydrophobic interaction.

However, the fibronectin III-like ChiN domain of ChiA extends the substrate binding cleft at the nonreducing end, which suggests that ChiA carries out exo-degradation of the chitin chain from the reducing end. Interestingly, the porch loop and flexible loop (forming "roof" of the tunnel in ChiB) are absent in ChiA, thus make ChiA a less tunnel-like character. Therefore chitin chain can extend on both sides of the active site, which suggests that the enzyme could have some endo-activity (Van Aalten *et al*., 2000).

Catalytic mechanism of chitinase A in *S. marcescens*

All enzymes in family 18 have a retaining catalytic mechanism, but chitinases are slightly different in that the active sites of the enzymes lack the second acidic residue that is needed to stabilize the oxocarbonium intermediate formed in the classical double displacement mechanism. The catalytic mechanism of chitinases is a substrate-assisted mechanism in which initial protonation is followed by a nucleophilic attack of the *N*-acetyl group of the sugar in the –1 subsite (Van Aalten *et al*., 2001).

Residues related to substrate binding of ChiA

Substrate binding is controlled by a series of aromatic residues. Three aromatic residues linearly aligned on the surface of ChiA, Trp33 and Trp69 in the N-terminal domain and Trp245 in the catalytic domain are key residues for the binding activity of ChiA to insoluble single chitin chain (Uchiyama *et al*., 2001). On the other hand, Phe232, close to the catalytic cleft, is essential for the degradation of insoluble chitin but not related to chitin binding activity. The relative location of Phe232 to the substrate-binding cleft thus suggests that Phe232 carries out the important role in guiding and introducing the chitin chain into the catalytic groove (Uchiyama *et al*., 2001). When the chitin chain sliding through the catalytic cleft to the active site, the second linkages are formed from the reducing end and progressively cleave $(GlcNAc)_2$ units from chitin polymer. In this process, Tyr170 at subsite –5, Trp167 at subsite –3, Trp539 at subsite –1, Trp275 at subsite +1 and Tyr418 and Phe396 at subsite +2 in the catalytic cleft are indicated to play major roles in holding and sliding the chitin chain. Trp275 and Phe396 form opposite sides of the cleft, stacking against the hydrophobic faces of the GlcNAcs. In contrast, the polysaccharide chain in the groove lies flat and every other GlcNAc unit in this region has its hydrophobic face

aligned with an aromatic amino acid in the odd-numered subsite (-1,-3,-5), whereas sugars in the even-numbered subsites have their hydrophobic surfaces exposed (figure 1.7B) (Aronson *et al*., 2003).

From our previous work, we have successfully cloned and characterized chitinase 60 (CHI60) from *Serratia* sp.TU09, previously identified as *B. cepacia* TU09 (Kamontip, 2001). CHI60 was found to be identical to the previously characterized ChiA from *Serratia marcescens*. The amino acid sequence and the 3D structure of CHI60 exhibited 100% homology to Serratia marcescens ChiA. Due to characteristics of Chi60, which similar to ChiA. ChiA is a template for structure study and the studied of ChiA was used as a data for our hypothesis.

In this study, we have mutated the exposed tryptophan track in the N-terminal domain and the catalytic domain of *Serratia* sp.TU09 chitinase 60 (Trp-33, Trp-69, Trp-245, Trp-275 and Tyr-418), and studied the interaction of these residues on different types of substrates, soluble chitin, amorphous chitin and crystalline chitin. To investigate the possible importance of the Trp-69, Trp-33, Trp-245, Trp-275 and Tyr-418 in the mode of catalysis, exo- endo-type, and mechanism of how the exposed tryptophan track functions in binding and guiding the substrate during processive hydrolysis. The site-directed mutagenesis of the aromatic residues (Trp) to phenylalanine, tyrosine or alanine was carried out.

CHAPTER II

MATERIALS AND METHODS

2.1 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-Rad, U.S.A.; Submarine Agarose Gel Electrophoresis unit

Incubator: Model 1H-100, Gallenkamp, England

Incubator shaker: Model G-76, New Brunswick Scientific Co.,Inc., 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

2.2 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.

Ampicillin: 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, England

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 *Hin*d 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: Sigma, U.S.A.

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

2.3 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.

Calf Intestine Alkaline Dephosphorylase (CIAP): New England BioLabs, Inc., U.S.A.

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

Pfu DNA polymerase: Promega, U.S.A.

2.4 Bacterial Strains

Serratia sp.TU09, isolated from Bangkok, Thailand, South-East Asian soil.

Escherichia coli DH5α was the host strain used throughout the construction and production of plasmid carrying the wild-type *CHI60* gene and the *CHI60* gene with various mutations.

E. coli DH5α with genotype *F′*, ∅80δ lacZΔM15, Δ(lacZYA-argV169), endA1, recA1, hsdR17 ($r_K.m_{K+}$), deoR, thi-1, supE44, λ⁻gyrA96, relA1 (Dower, 1988) was purchased from GIBCOBRL, U.S.A.

2.5 Plasmids

Plasmid pBluescript SK⁻ (stratagene) was used as a cloning vector and carrying the wild-type *CHI60* gene from *Serratia* sp.TU09 (pKKCHI60) was described previously.

Plasmid pGEM® –T Easy (Promega) was used for cloning PCR fragments into *E. coli*.

2.6 Media Preparation

2.6.1 Luria-Bertani (LB) medium (Sambrook and Russell, 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 minutes.

2.6.2 Colloidal chitin minimum medium (CCMM)

Medium for cultivation and enzyme production from *Serratia* sp.TU09 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.

2.7 Cultivation of Bacteria

2.7.1 Starter inoculum

A colony of *Serratia* sp.TU09 was grown in 2 mL of LB medium at 30°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 hours.

2.7.2 Culture conditions

CHI60 and its mutants were produced in *Escherichia coli* DH5α cells carrying the plasmid pKKCHI60 or its derivatives. Starter culture condition of *E. coli* contained chitinase gene (*CHI60*) 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 in shaking water bath for chitinase production and chromosomal DNA extraction. Cells were collected by centrifugation at 5,000 g for 20 minutes at 4°C. For chitinase production, culture broth with crude chitinase enzyme was concentrated by aqua sorb and then dialyzed against 10 mM sodium phosphate buffer (pH 7.0). Concentrated crude chitinase enzyme was kept at 4°C for further characterization (Kamontip, 2001).

2.8 Substrate preparations

2.8.1 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 hours. Then, added 750 g crushed ice with vigorous shaking. In this step, squid pen chitin was dissolved in NaOH solution and the layer of ice covered around the flask was appeared. The mixture was stirred overnight at 4°C. Next, conc. HCL (12M) was added until the pH was 7. Added 2 volume of cold acetone to precipitate partially *N*-acetylated chitin (PNAC) and PNAC was collected by filtrated by Buchner funnel. PNAC was dialyzed with water to remove salt. Finally, PNAC was lyophilized.

2.8.2 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 6 hours on ice with magnetic stirrer. After stirred for a while at 37°C, the chitin hydrolysate was deprotonated by filtrated into 4,000 mL of chilled distilled water. The milk-like mixture was kept standing overnight at -20°C. The colloidal chitin was collected by centrifugation at 8,000 g for 15 minutes, then resuspended

with distilled water to wash the pellet. The pellet was washed until the pH is between 6-7.

The colloidal chitin was resuspended in steriled distilled water and kept at 4°C. Take 1 mL to determine wet weight and dry weight. The colloidal chitin solution can be kept at this condition for at least 2 years.

2.8.3 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 5% β-crystalline chitin solution can be kept at 4°C for at least a year.

2.9 Scanning electron microscopy (SEM)

A 4 mg/mL (dried weight) colloidal chitin and 50 mg/mL (dried weight) β–crystalline chitin were scanned by scanning electron microscope (JEOL: model JSM-5800LL) at 15 kV.

2.10 Identification of Bacterium producing chitinase

Bacteria strain TU09 was grown on colloidal chitin minimum medium plate then identify by biochemical method at The National Institute of Health, Department of Medical Science, Ministry of Public Health. The identity was confirmed by 16S rRNA sequence comparison. The amplification method of 16S rRNA is described below.

2.10.1 Polymerase Chain Reaction (PCR) amplification

Primer for generated 16S rRNA gene

A partial DNA sequence for the 16S rRNA gene was amplified by using 5'-AGA GTT TGA TCC TGG CTC AG -3' (pA) as forward primer and 5'-AAG GAG GTG ATC CAG CCG CA-3' (pH') as reverse primer (Ulrike *et al*., 1989). The conditions for 16S rRNA gene amplifications is described in Table 1. The amplified PCR product was cloned into pGEM T-easy and transformed to *E.coli* DH5α. The plasmid containing 16S rRNA gene was sequenced using the dideoxy-chain termination method with CEQ^{TM}8000 Genetic Analysis System. The sequence of 16S

Table 2.1 Cycling Parameters for the 16S rRNA gene amplifications

Segment	Cycles	Temperature	Time
1	1	95°C	1 minute
2	25-30	94°C 55°C 72°C	1 minute 2 minutes 3 minutes
3	1	72°C	5 minutes

After final extension, the PCR products were kept at 4 °C

rRNA gene was aligned with others obtained from GenBank using Basic Local Alignment Search Tool (BLAST)(Altschul *et. al.*, 1997).

2.11 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 were measured colorimetrically with ferric cyanide reagent by a modification of the Schales' procedure (Imoto and Yagishita, 1971) using GlcNAc as the standard.

2.11.1 Determination of chitinase activity by measuring reducing sugar

Chitinase activity was assayed by measuring reducing sugar (Imoto and Yagishita, 1971) produced from a mixture of 750 μL containing 1 mg/mL of colloidal chitin and the desired amount of enzyme in 0.1 M citrate buffer pH 5.0. The mixture was incubated at 37°C for 30 minutes. The reaction was stopped by adding 1.0 mL color reagent (made by dissolving 0.5 g of potassium ferric cyanide in 1 liter of 1.5 M Na_2CO_3) and heat to 100°C for 15 minutes. Small particles were removed from the mixture by centrifugation at 5,000 g for 10 minutes. The absorbance of the sample (A1) at 420 nm was measured by a spectrophotometer versus water. A blank value (A0) was obtained when boiled 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*-acetyl glucosamine equivalent) per minute.

2.12 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.

2.13 Recombinant DNA Techniques

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

2.14 Selection of amino acid residues for reduction of hydrophobic interaction

2.14.1 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 from *Serratia* sp.TU09 was submitted to the SWISS MODEL using protein of ChiA from *Serratia marcescens* complexed with the octamer substrate deposited in Protein Data bank (accession number 1EHN) as template. The resulting protein structure model can be visualized and analyzed using the integrated tool (Raswin program).

2.14.2 Selection of amino acid residues

3D-structure of CHI60 *Serratia* sp.TU09 was superimposed on the structure of inactivated *S. marcescens* ChiA complexed with $(GlcNAc)_8$ and observed the exposed aromatic residues interacting with the GlcNAc units. Then, the interested aromatic residues were studied. The residues were mutated to phenylalanine, tyrosine or alanine for decreased hydrophobic interaction between sugar and enzyme.

2.15 Site-directed Mutagenesis

Site-directed mutagenesis was carried out by polymerase chain reaction using a modified method from QuickChange site-directed mutagenesis kit (Stratagene) and pKKCHI60 as a template. The site-directed mutagenesis method is performed using *Pfu* DNA polymerase (Promaga) and a temperature cycler. *Pfu* DNA polymerase replicates both plasmid strands with high fidelity and without displacing the mutant oligonucleotide primers. The basic procedure utilizes a supercoiled double-stranded DNA (dsDNA) vector with an insert of interest and two synthetic oligonucleotide

primers containing the desired mutation (see Figure 1). The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during temperature cycling by *Pfu* DNA polymerase. Incorporation of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. Following temperature cycling, the product is treated with *Dpn* I. The *Dpn* I endonuclease (target sequence: 5´-Gm6ATC-3´) is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. DNA isolated from almost all *E. coli* strains is dam methylated and therefore susceptible to *Dpn* I digestion. The nicked vector DNA containing the desired mutations is then transformed into DH5α competent cells. The small amount of starting DNA template required to perform this method, the high fidelity of the *Pfu* DNA polymerase, and the low number of thermal cycles all contribute to the high mutation efficiency and decreased potential for generating random mutations during the reaction. The mutant clones were selected after sequencing the entire open reading frame to ensure that the desired mutation was only mutation in each mutated gene. 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. To create the gene encoding CHI60 with a double mutation (W33F/W69F/Y), the second mutation was introduced into the gene encoding W33F by using the primers for W69F/Y mutation, double mutation (W33F/W245F/Y), the second mutation was introduced into the gene encoding W33F by using the primers for W245F/Y mutation, a double mutation (W33Y/W245F/Y), the second mutation was introduced into the gene encoding W33Y by using the primers for W245F/Y mutation and double mutation (W69Y/W245F/Y), the second mutation was introduced into the gene encoding W69Y by using the primers for W245F/Y mutation.

2.15.1 Oligonucleotide primer

Mutagenic primers were synthesized by BSU. They were used in the mutagenesis of Phe33, Phe69, Phe245, Phe275 and Tyr418. These oligonucleotide used in the study are listed in Table 2. The conditions for site-directed mutagenesis amplifications is described in Table 3.

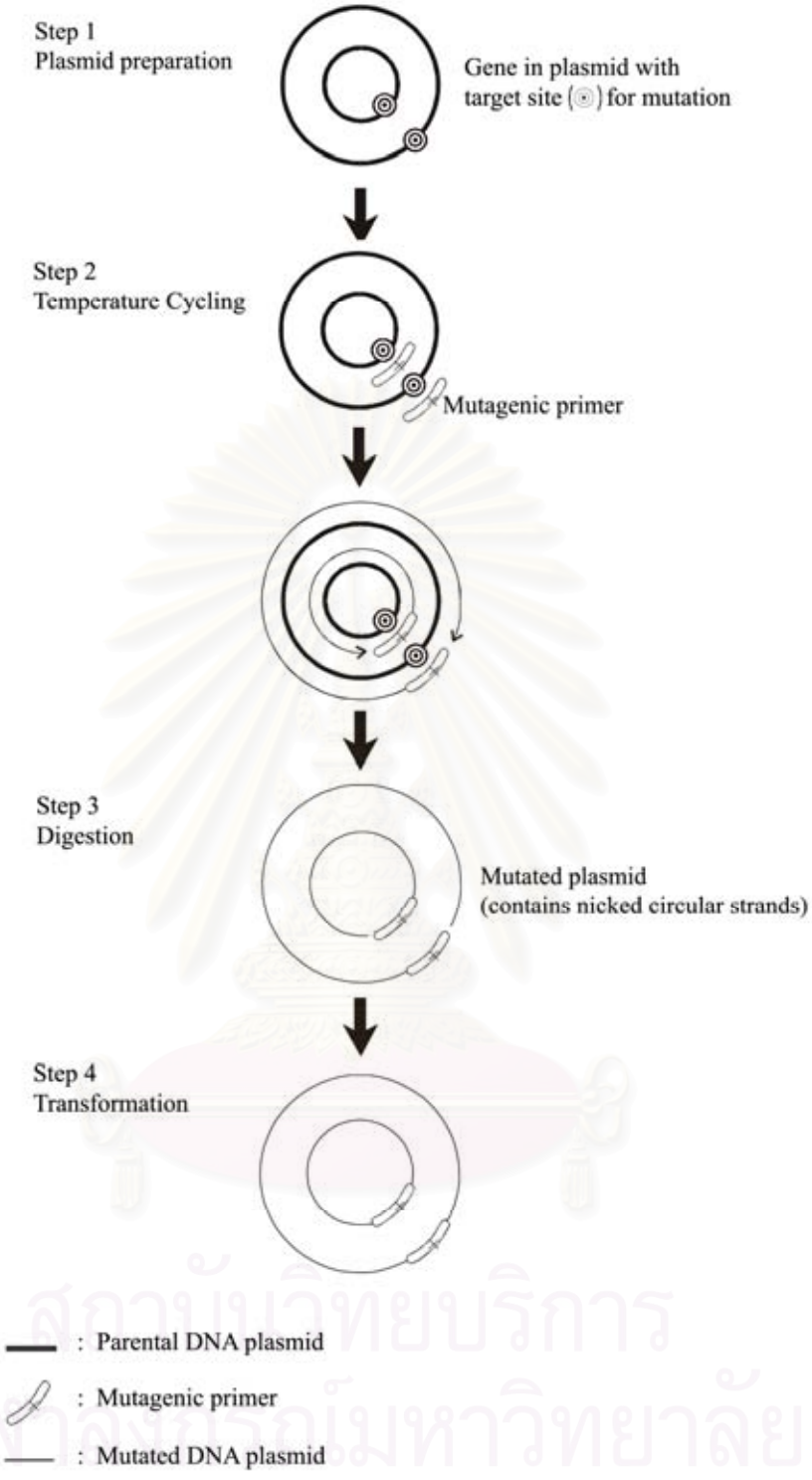


Figure 2.1 The overview of site-directed mutagenesis method

Table 2.2 Mutagenesis oligonucleotide primers Mutated bases are shown by underline type.

Primers	Sequences
Trp-33→Phe (W33F/Y)	5'-CGA CCA TCG CCT (T/A)CG GCA ATA CCA A-3' 5'- TAT TGC CG(T/A) AGG CGA TGG TCG GCT T -3'
Trp-69→ Phe (W69F/Y)	5'- GGA ATT TAT (A/T)CA ATG GCG AC -3' 5'- CGC CAT TG(A/T) ATA AAT TCC AG -3'
Phe-245→ Phe (W245F/Y)	5'- CGT TAC CGC CT(T/A) CGA TGA CC -3' 5'- GGT CAT CG(A/T) AGG CGG TAA CG -3'
Trp-275→ Ala (W275A)	5'- GAT CGG CGG CGC GAC GCT GT -3' 5'- GAC AGC GTC GCG CCG CCG AT -3'
Trp-275→ Phe (W275F)	5'- GAT CGG CGG CTT TAC GCT GT -3' 5'- GAC AGC GTA AAG CCG CCG AT -3'
Tyr-418→ Ala (Y418A)	5'- CCG GAC ACC GCT GCC ACC AC -3' 5'- CCG TGG TGG CAG CGG TGT CC-3'

Table 2.3 Cycling Parameters for the site-directed mutagenesis

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	16	95°C 55-60°C 72°C	30 seconds 1 minute 12 (2 minutes/kb of plasmid length)
3	1	72°C	5 minutes

2.16 Purification of CHI60 and Its Mutants

For chitinase purification, crude enzyme was concentrated by ultrafiltration (cut off 30,000 Da), CHI60 and its mutants were purified by DEAE-cellulose column, and chitin affinity adsorption, respectively. Crude chitinase was applied to a DEAE-cellulose column previously equilibrated with 20 mM Tris-HCl buffer pH 7.0. Then, unbound proteins from DEAE-cellulose column was incubated with colloidal chitin in 0.5 M Tris-HCl buffer pH 7.0 at 4°C for 3 hours. After that, the unbound protein was washed by 0.5 M Tris-HCl buffer pH 7.0 for two times and the chitinase-colloidal chitin complex were collected by centrifugation at 8,000 g for 20 minutes at 4°C. The purified chitinase was eluted with 50 mM Tris-HCl buffer pH 7.0 and incubating the chitinase-colloidal chitin complex at 37°C for 2 hours. The purified chitinases were dialyzed by ultrafiltration (cut off 30,000 Da) with 50 m M Tris-HCl buffer pH 7.0 to remove the hydrolyzing product. The purified chitinases were kept at 4°C for further characterization.

2.17 Characterization of CHI60 and its derivative

2.17.1 Effect of temperature on the hydrolyzing activity of CHI60 and its derivative on various substrates

The effect of temperature on the hydrolyzing activity of CHI60 and its derivative was determined on partially *N*-acetylated chitin, colloidal chitin and β-crystalline chitin. The reaction mixture (total 750 µl) contained purified chitinase and 0.5 mg/mL of PNAC and each substrate (1mg/mL of colloidal chitin, or 10 mg/mL of β-crystalline chitin) in 0.1 M sodium citrate buffer, pH 5.0. The mixture was incubated at 37°C or 50°C.

2.17.2 Effect of ionic strength on the hydrolyzing activity of CHI60 and its derivative

The effect of ionic strength on enzyme activity was determined by incubating the reaction mixtures at different sodium chloride (NaCl) concentration ranging from 0-2 M, and assaying the enzyme by measuring the concentration of reducing sugar. In this study, the amount of enzyme which yields equal activity on soluble substrate (PNAC) was used to hydrolyzed amorphous substrate (colloidal chitin) and crystalline

substrate (β-crystalline chitin). The reaction mixture (total 750 µl) contained 250 mU, 1,200 mU and 2,400 mU of purified chitinase for PNAC, colloidal chitin and β-crystalline chitin, respectively and 0.5 mg/mL of PNAC, 1 mg/mL of colloidal chitin, and 10 mg/mL of β-crystalline chitin of each substrate in 20 mM sodium citrate buffer, pH 5.0. The mixture was incubated at 37°C.

2.17.3 Estimation of molecular weight

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 (Trudel and Asselin, 1989) using a 10% or 12.5% acrylamide gel containing 0.001% (w/v) glycol chitin. Sample solution of enzyme were denatured by heating at 100°C for 5 minutes 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. The electrophoresis was performed at constant current of 20 mA per slab, at room temperature on a Mimi-Gel electrophoresis unit from cathode towards anode. Prestained proteins marker, broad rang 6-175 kDa, was used. After electrophoresis, proteins were stained with 0.25 % Coomassie Brilliant Blue R-250 at room temperature for one hour then destaining with a mixture of 10 % (v/v) acetic acid and 25 % (v/v) methanol.

The protein band containing chitinase activity after SDS-PAGE was detected by incubating gel overnight at 37°C with reciprocal shaking in 100 mM sodium citrate buffer (pH 5.0) 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.

2.17.4 Analysis of the degradation products of Chitinase by HPLC

The 25 mU/mL purified chitinases (the amount of enzyme which yields equal activity on colloidal chitin) were used to digest 12 mg/mL colloidal chitin. The 2.5 mU/mL purified chitinases (the amount of enzyme which yields equal activity on β-crystalline chitin) were used to digest 60 mg/mL of β-crystalline chitin. The mixture was incubated in 20 mM sodium citrate buffer, pH 5.0 and different NaCl concentration ranging from 0-2 M, at 37°C, 1 hour. The product at the end of 1 hour

was boiled for 15 minutes and centrifuge at 8,000 g in a bench-top centrifuge for 10 minutes. The reaction mixtures were applied to activated charcoal column for salt elimination. The method is described below. Then 300 μL of the degradation product was withdrawn and mixed with 700 μL of acetonitrile, and filtered through a 0.45 micron filter. The product was then analyzed by HPLC. The used condition was; Shodex Asahipak NH3P column, mobile phase 300 mL water: 700 mL acetonitrile, flow rate 1.0 mL/min.

2.17.5 Analysis of the degradation products of Chitinase by Thin layer chromatography (TLC)

The reaction products of *N*-acetyl-chitooligosaccharides were analyzed by silica-thin layer chromatography (TLC). Aliquotes (10 μl) of the reactions mixtures were chromatographed on silica gel plate with isopropanol-ethanol-water (5: 2: 1[v/v/v]) and TLC plates were run twice. The product were detected by dipping the plate in aniline-diphenylamine reagent (4 mL of aniline, 4 g of diphenylamine, 200 mL of acetone and 20 mL of 85% phosphoric acid) and baking it at 130 °C for 3 min (Tanaka *et al*., 1999).

2.17.6 Salt elimination from degradation products

Activated charcoal column was used for salt elimination. The reaction mixture was applied to a activated charcoal column. Then, the unbound was washed by water for 3 times (salt was eliminated from this step). The degradation product was eluted by 60% ethanol. After that the mixture was dried at 60°C and the degradation products was dissolved in water and kept at room temperature for analysis.

CHAPTER III

RESULTS

Identification of bacterial strain TU09 producing chitinase

Biochemical identification

Bacterial strain TU09 was first identified at The Thailand Institute of Scientific and Technological Research (TISTR), Ministry of Science and Technology, as *Burkholderia cepacia* (Krusong, 1999). Since the cloned CHI60 gene was identical to *Serratia marcescens* ChiA. Therefore, this bacterium was reidentified by The National Institute of Health, Department of Medical Science, Ministry of Public Health. The results of biochemical characteristics are listed in Table 3.1. This strain was a gram negative rod shape bacteria and a γ- hemolysis, which grew both aerobically and anaerobiclly on LB agar. The colonies on nutrient agar were convex circular. This bacterium was positive for Voges-Proskauer (VP) reaction and produce DNase. It was fermentative and produced acid from most sugars, except raffinose. From these results, bacteria strain TU09 was identified as *Serratia liquefaciens*.

Characterization of a gene coding for 16S ribosomal RNA

The partial 16S ribosomal RNA gene of strain TU09 was *in vitro* amplified via the PCR. The amplified PCR product of the 16S rRNA of *Serratia* sp.TU09 was ligated to pGEM T-easy and transformed to *E. coli* DH5α. The plasmid containing a 1.5 kb of 16S rRNA (p16STU09) was digested with *Eco*RIto confirm the presence of the insert, as shown in Figure 3.1. The result showed that p16STU09 was digested by *Eco*RI at 3 sites, two sites in pGEM T-easy and another site in the 16S rRNA gene. The inserted fragment of p16STU09 was sequenced using the dideoxy-chain termination method with CEQ^{TM}8000 Genetic Analysis system. The result showed 16S rRNA gene of bacteria strain TU09 was 1537 bp in length (Figure 3.2). Sequence alignment using BLAST program demonstrated that 16S rRNA gene of strain TU09 has 99% homology with of *Serratia marcescens* (AB061685), 1532 bp in length (is shown in Figure 3.3). Strain TU09 was biochemically identified twice, which

Table 3.1 Biochemical characteristics of bacterial strain TU09

Characteristics	**Reaction**	**Reaction (Krusong, 1999)**
Gram reaction	-ve	-ve
Fermentative production of acid from:		
-Glucose/Gas	+/-	+
-Lactose	+	
-Maltose	+	+
-D-Xylose	+	
-Rhamnose	+	
-Sucrose	+	
-Adonitol	+	
-Arabinose	+	-
-Inositol	+	
-Sorbitol	+	
-Raffinose	-	
-Salicine	+	
-PDA	-	
-Lysine	+	
-Arginine	-	
-Ornithine	+	
Test:		
-TSI	A/A	
-Molility	+	
-Indole	-	-
-Citrate	-	+
-Urease	-	-
-Esculin	+	+
-Malonate	-	
-VP	+	
-DNase	+	

Remark: -ve = Gram negative bacteria

\+ = Positive reaction

\- = Negative reaction

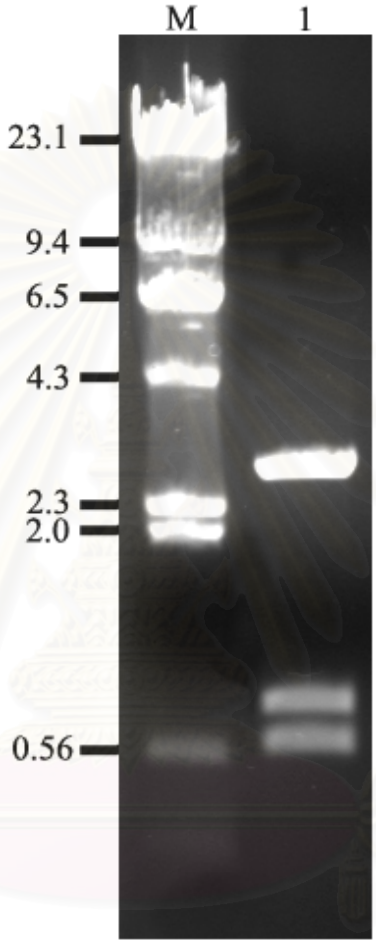


Figure 3.1 PCR product of 16S rRNA of bacteria strain TU09

Lane M: λ/*Hin*dIII Marker

Lane 1: p16STU09/*Eco*RI

```
Sequence 1537 BP; 383 A; 350 C; 487 G; 317 T; 0 other;
AGAGTTTGAT CCTGGCTCAG ATTGAACGCT GGCGGCAGGC TTAACACATG CAAGTCGAGC        60
GGTAGCACGG GGGAGCTTGC TCCCTGGGTG ACGAGCGGCG GACGGGTGAG TAATGTCTGG       120
GAAACTGCCT GATGGAGGGG GATAACTACT GGAAACGGTA GCTAATACCG CATAACGTCG       180
CAAGACCAAA GAGGGGGACC TTCGGGCCTC TTGCCATCAG ATGTGCCCAG ATGGGATTAG       240
CTAGTAGGTG GGGTAATGGC TCACCTAGGC GACGATCCCT AGCTGGTCTG AGAGGATGAC       300
CAGCCACACT GGAACTGAGA CACGGTCCAG ACTCCTACGG GAGGCAGCAG TGGGGAATAT       360
TGCACAATGG GCGCAAGCCT GATGCAGCCA TGCCGCGTGT GTGAAGAAGG CCTTCGGGTT       420
GTAAAGCACT TTCAGCGAGG AGGAAGGTGG TGAACTTAAT ACGTTCATCA ATTGACGTTA       480
CTCGCAGAAG AAGCACCGGC TAACTCCGTG CCAGCAGCCG CGGTAATACG GAGGGTGCAA       540
GCGTTAATCG GAATTACTGG GCGTAAAGCG CACGCAGGCG GTTTGTTAAG TCAGATGTGA       600
AATCCCCGGG CTCAACCTGG GAACTGCATG TTGAAACTGG CAAGCTAGAG TCTCGTAGAG       660
GGGGGTAGAA TTCCAGGTGT AGCGGTGAAA TGCGTAGAGA TCTGGAGGAA TACCGGTGGC       720
GAAGGCGGCC CCCTGGACGA AGACTGACGC TCAGGTGCGA AAGCGTGGGG AGCAAACAGG       780
ATTAGATACC CTGGTAGTCC ACGCTGTAAA CGATGTCGAT TTGGAGGTTG TGCCCTTGAG       840
GCGTGGCTTC CGGAGCTAAC GCGTTAAATC GACCGCCTGG GGAGTACGGC CGCAAGGTTA       900
AAACTCAAAT GAATTGACGG GGGCCCGCAC AAGCGGTGGA GCATGTGGTT TAATTCGATG       960
CTACGCGAAG AACCTTACCT ACTCTTGACA TCCAGAGAAC TTAGCAGAGA TGCTTTGGTG      1020
CCTTCGGGAA CTCTGAGACA GGTGCTGCAT GGCTGTCGTC AGCTCGTGTT GTGAAATGTT      1080
GGGTTAAGTC CCGCAACGAG CGCAACCCTT ATCTTTTGTT GCCAGCGGTT CGGCCGGGAA      1140
CTCAAAGGAG ACTGCCAGTG ATAAACTGGA GGAAGGTGGG GATGACGTCC AAGTCATCCA      1200
TGGCCCTTAC GAGTAGGGCT ACACACGTGC TACAATGGCG TATACAAAGA GAAGCGACCT      1260
CGCGAGAGCA AGCGGACCTC ATAAAGTACG TCGTAGTCCG GATTGGAGTC TGCAACTCGA      1320
CTCCATGAAG TCGGAATCGC TAGTAATCGT AGATCAGAAT GCTACGGTGA ATACGTTCCC      1380
GGGCCTTGTA CACACCGCCC GTCACACCAT GGGAGTGGGT TGCAAAAGAA GTAGGTAGCT      1440
TAACCTTCGG GAGGGCGCTT ACCACTTTGT GATTCATGAC TGGGGTGAAG TCGTAACAAG      1500
GTAACCGTAG GGGAACCTGC GGCTGGATCA CCTCCTT                               1537
```

pA 5 ' AGAGTTTGATCCTGGCTCAG 3 '

pD 5 ' CAGCAGCCGCGGTAATAC 3 '

pF 5 ' CATGGCTGTCGTCAGCTGGT 3 '

pH 5 ' TGCGGCTGGATCACCTCCTT 3 '

Figure 3.2 Nucleotide sequence of 16S rRNA of *Serratia* sp.TU09

The 16S rRNA amplified was sequenced. The result 1537 bp is shown above. The primer used for sequencing is shown in color.

```
CLUSTAL X (1.83) multiple sequence alignment

Serratia sp.TU09      -AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAG
S. marcescens         GAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAG
                       ************************************************************
Serratia sp.TU09      CGGTAGCACGGGGGAGCTTGCTCCCTGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTG
S. marcescens         CGGTAGCACAGGGGAGCTTGCTCCCTGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTG
                      ********* ************************************************
Serratia sp.TU09      GGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTC
S. marcescens         GGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTC
                      ************************************************************
Serratia sp.TU09      GCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTA
S. marcescens         GCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTA
                     ************************************************************
Serratia sp.TU09      GCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGA
S. marcescens         GCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGA
                      ************************************************************
Serratia sp.TU09      CCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
S. marcescens         CCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
                      ************************************************************
Serratia sp.TU09      TTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGT
S. marcescens         TTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGT
                      ************************************************************
Serratia sp.TU09      TGTAAAGCACTTTCAGCGAGGAGGAAGGTGGTGAACTTAATACGTTCATCAATTGACGTT
S. marcescens         TGTAAAGCACTTTCAGCGAGGAGGAAGGTGGTGAACTTAATACGCTCATCAATTGACGTT
                      ******************************************** ***************
Serratia sp.TU09      ACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCA
S. marcescens         ACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCA
                      ************************************************************
Serratia sp.TU09      AGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTG
S. marcescens         AGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTG
                      ************************************************************
Serratia sp.TU09      AAATCCCCGGGCTCAACCTGGGAACTGCATGTTGAAACTGGCAAGCTAGAGTCTCGTAGA
S. marcescens         AAATCCCCGGGCTCAACCTGGGAACTGCAT-TTGAAACTGGCAAGCTAGAGTCTCGTAGA
                      ****************************** ****************************
Serratia sp.TU09      GGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGG
S. marcescens         GGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGG
                      ************************************************************
Serratia sp.TU09      CGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAG
S. marcescens         CGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAG
                      ************************************************************
Serratia sp.TU09      GATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGA
S. marcescens         GATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGA
                      ************************************************************
Serratia sp.TU09      GGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTT
S. marcescens         GGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTT
                      ************************************************************
Serratia sp.TU09      AAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAT
S. marcescens         AAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAT
                      ************************************************************
```

```
Serratia sp.TU09      GCTACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTAGCAGAGATGCTTTGGT
S. marcescens         GCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTTCCAGAGATGGATTGGT
                      ** ************************************  ********  *****
Serratia sp.TU09      GCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGT
S. marcescens         GCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGT
                      ************************************************************
Serratia sp.TU09      TGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCTTTTGTTGCCAGCGGTTCGGCCGGGA
S. marcescens         TGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTCGGCCGGGA
                      ********************************* ************************
Serratia sp.TU09      ACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCCAAGTCATCC
S. marcescens         ACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTC-AAGTCATC-
                      ************************************************ ********
Serratia sp.TU09      ATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGTATACAAAGAGAAGCGACC
S. marcescens         ATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGTATACAAAGAGAAGCGACC
                      ************************************************************
Serratia sp.TU09      TCGCGAGAGCAAGCGGACCTCATAAAGTACGTCGTAGTCCGGATTGGAGTCTGCAACTCG
S. marcescens         TCGCGAGAGCAAGCGGACCTCATAAAGTACGTCGTAGTCCGGATTGGAGTCTGCAACTCG
                      ************************************************************
Serratia sp.TU09      ACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCC
S. marcescens         ACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCC
                      ************************************************************
Serratia sp.TU09      CGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGC
S. marcescens         CGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGC
                      ************************************************************
Serratia sp.TU09      TTAACCTTCGGGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGTGAAGTCGTAACAA
S. marcescensa        TTAACCTTCGGGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGTGAAGTCGTAACAA
                      ************************************************************
Serratia sp.TU09      GGTAACCGTAGGGGAACCTGCGGCTGGATCACCTCCTT
S. marcescens         GGTAACCGTAGGGGAACCTGCGGCTGGATCACCTC---
                      ***********************************
```

Figure 3.3 Nucleotide sequence alignment of 16S rRNA of *Serratia* sp.TU09 compare with *Serratia marcescens* (AB061685)

(Where * denotes identical nucleotide and Red color indicated nucleotides that are different from *Serratia* sp.TU09)

classified this bacteria, as *Burkholderia cepacia* and *Serratia liquefaciens*. Nevertheless, the 16S rRNA gene of this bacteria was more similarity to *Serratia marcescens*. We decided to classify this strain as *Serratia* sp.TU09.

Scanning electron microscopy (SEM) of colloidal chitin and β–crystalline chitin

To study the structural nature of the substrates to be use in our experiment, SEM analysis was employed. Scanning electron micrographs of colloidal chitin showed an amorphous and porous character of colloidal chitin (Figure 3.4, A. and B.). In contrast, scanning electron micrographs of β–crystalline chitin showed fibrous crystalline character of β–crystalline chitin (Figure 3.4, C. and D.).

Production and purification of CHI60

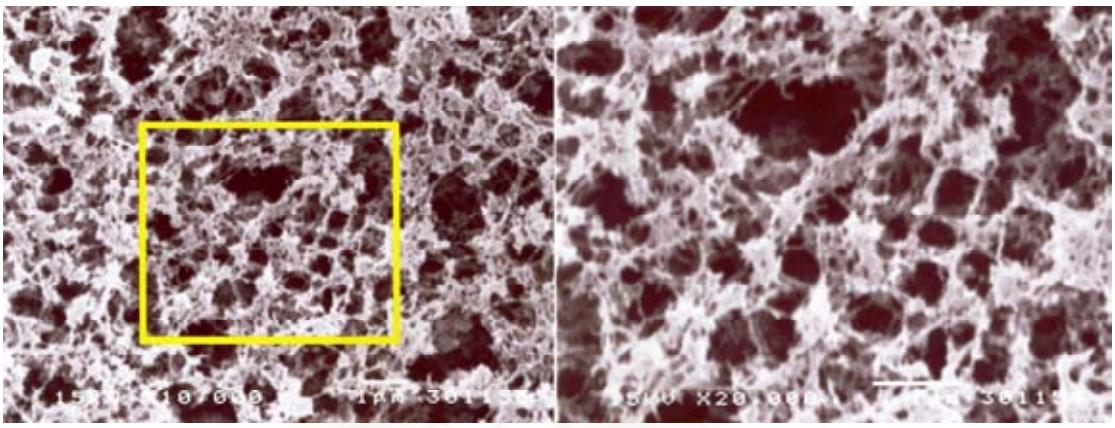
Wild type (WT) CHI60 was expressed in *E. coli* cells carrying a plasmid encoding *chi60* gene and purified from the culture medium. CHI60 purification was accomplished by ultrafiltration (MW cut off 30,000 Da), then CHI60 was further purified by DEAE-cellulose column. The chitinase activity was found in unbound protein, the profile of DEAE-cellulose chromatography showed in Figure 3.5. Then, unbound protein from DEAE-cellulose chromatography was purified by chitin affinity adsorption. The protein from each of the purification steps of CHI60 was analyzed by SDS-PAGE (shown in Figure 3.6).

Characterization of CHI60

Effect of ionic strength on the hydrolyzing activity of CHI60

The effect of ionic strength on the activity of WT CHI60 was determined on soluble substrate, partially *N*-acetylated chitin (PNAC), amorphous substrate (colloidal chitin) and crystalline substrate (β - crystalline chitin). The increase in ionic strength did not significantly affect the hydrolyzing activity of WT CHI60 on PNAC (Figure 3.7A). The activity of WT CHI60 on colloidal chitin was significantly enhanced at lower ionic strength (0 - 0.25 M sodium chloride), however, further increase in ionic strength (0.5-2 M sodium chloride) significantly reduced the activity (Figure 3.7B). In contrast, the activity of WT CHI60 on β - crystalline chitin slightly

A. **B.**



C. **D.**

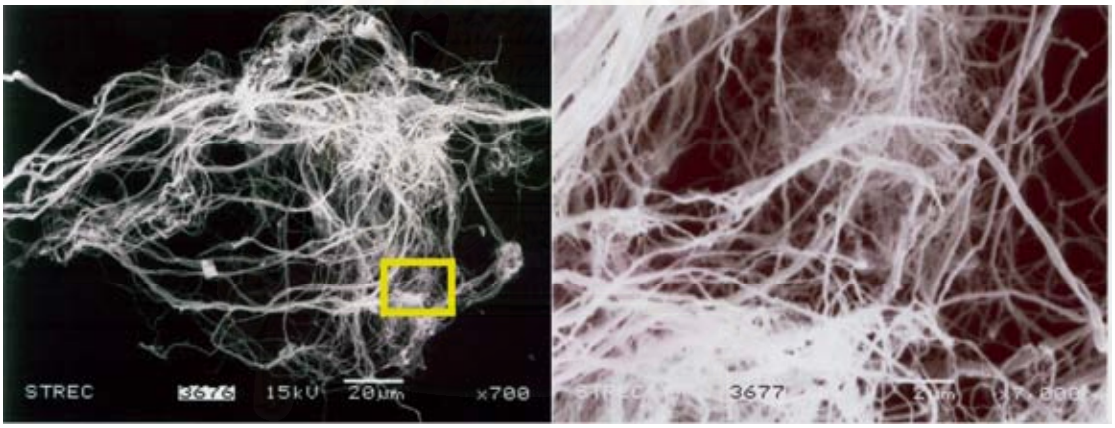
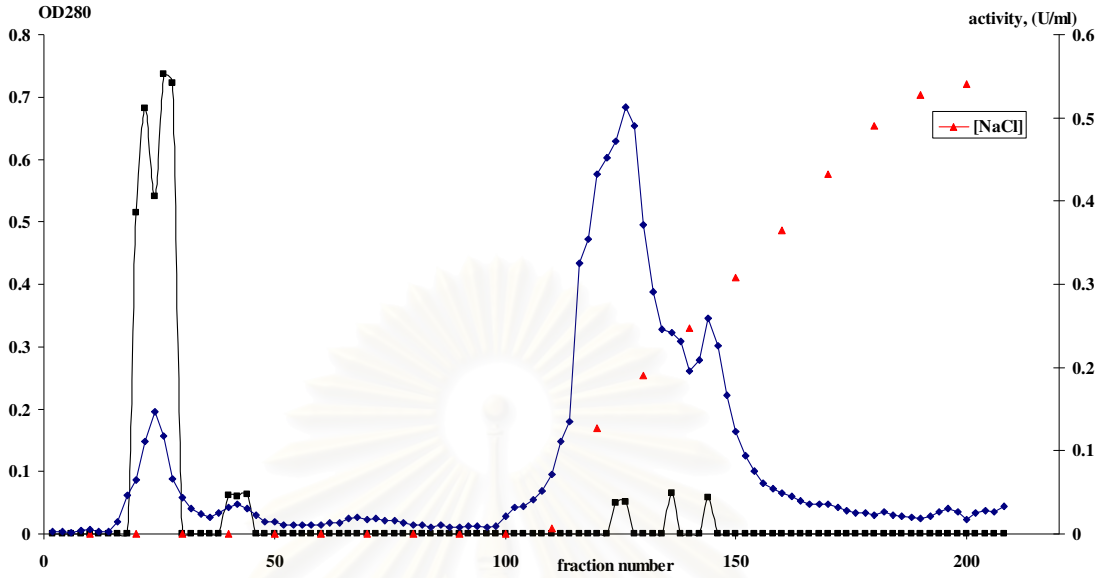


Fig. 3.4 Scanning electron micrographs (SEM) of colloidal chitin (A) and β – crystalline chitin (B)

The colloidal chitin are shown in A. and B. with close ups x10,000 and x20,000 magnification, respectively and β–crystalline chitin are shown in C. and D. with close ups x700 and x7,000 magnification, respectively.



♦ A280, ▲ [NaCl], ■ activity

Figure 3.5 Partial purification of *Serratia* sp.TU09 CHI60 by DEAE-cellulose column

The concentrated enzyme was applied to DEAE-cellulose column and washed with 20 mMTris-HCl pH 7.0 until A280 decrease to base line. Gradient elution of bound protein was made by 0-1 M NaCl in the same buffer at the flow rate of 0.5 ml/min.

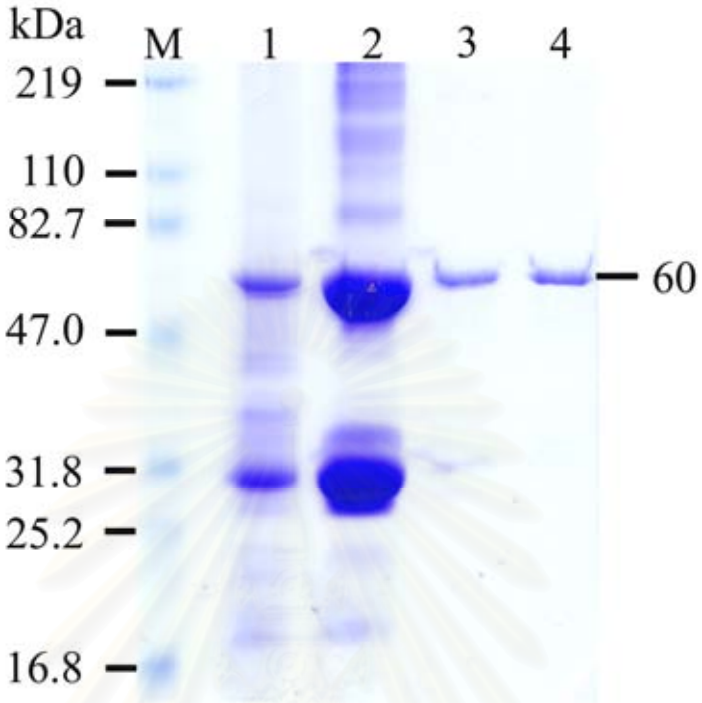


Figure 3.6 SDS-polyacrylamide gel electrophoresis analysis of the wild-type CHI60 Chitinase proteins produced in *E. coli* DH5α cells were purified from the culture supernatant. Protein staining of the SDS-polyacrylamide gel with Coomassie Brilliant Blue R-250 is shown.

Lane M: molecular mass standards

Lane 1: crude CHI60

Lane 2: partially purified CHI60 obtained by ultrafiltration

Lane 3: partially purified chitinase by DEAE-cellulose

Lane 4: purified chitinase by chitin affinity adsorption

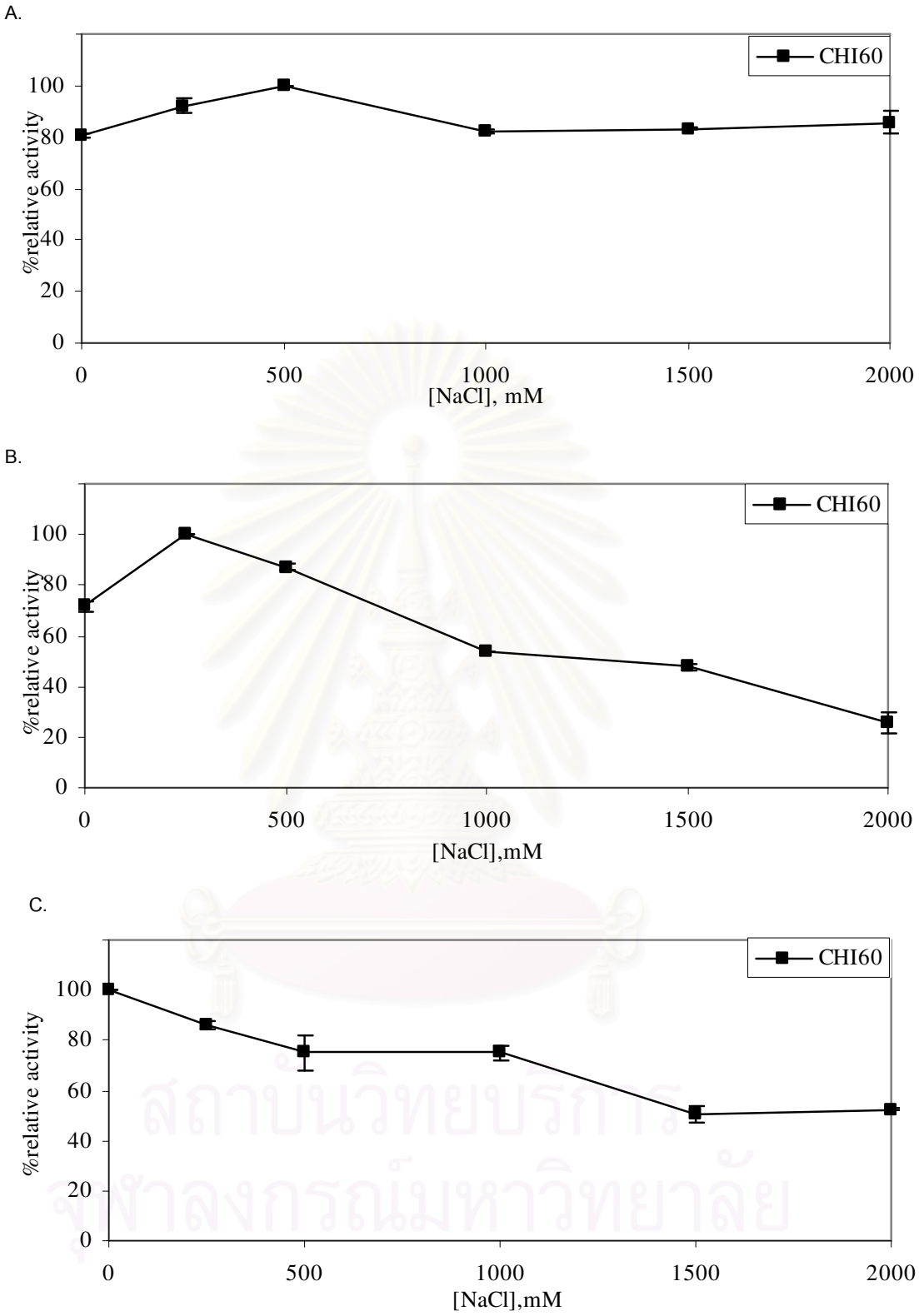


Figure 3.7 Hydrolysis of PNAC (A); colloidal chitin (B) and β-chitin (C) by wild-typeCHI60. ■, wild-type CHI60, Reaction mixtures contained 1 mg (dry weight) of substrate and either 0.5 mg/ml (A) or 1 mg/ml (B) or 10 mg/ml (C). Reactions were performed at 37 °C, and the amount of reducing sugar generated was monitored.

decreased as the ionic strength was increased from 0 - 2 M sodium chloride (Figure 3.7C).

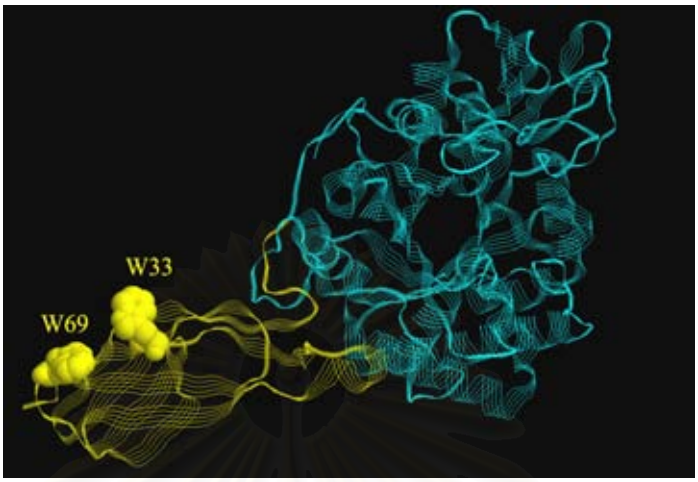
Site-directed mutagenesis

To study the importance of exposed surface Trp residues on the activity, mode of catalysis, product size distribution as well as the mechanism of hydrolysis, mutants with mutations of these exposed Trp residues were constructed. We mutated the exposed Trp residues in the Trp track on the N-terminal domain and the catalytic domain of *Serratia* sp.TU09 CHI60, Trp-33, Trp-69, Trp-245, as well as two Trp residues in the catalytic domain Trp-275 and Tyr-418 (Figure 3.8). The site-directed mutagenesis of the aromatic residues to Phe, Tyr or Ala was carried out. The mutant *Chi60*, single point mutants (W33F, W33Y, W69F, W69Y, W245F, W245Y, W275A, W275F and Y418A) and double point mutants (W33F/W69Y, W33Y/W245F, W33F/W245F, W33Y/W245Y and W69Y/W245Y), were sequenced. The entire sequence of all *chi60* mutants were checked to confirm the mutation and that no other mutations were created during PCR amplification.

Production and purification of CHI60 mutants

To characterize the mutant CHI60 each of the mutants each was expressed and purified. Fourteen CHI60 mutants, W33F, W33Y, W69F, W69Y, W245F, W245Y, W275A, W275F, Y418A, W33F/W69Y, W33Y/W245F, W33F/W245F, W33Y/W245Y and W69Y/W245Y, were expressed in *E. coli* DH5α cells carrying the plasmid encoding each of the mutated *chi*60 gene. The expressed mutant CHI60 was purified from the culture supernatant. All CHI60 mutants were produced at a activity level similar to that at which wild-type (WT) CHI60 was produced. The mutated chitinase proteins were purified by ultrafiltration (30 kDa cut off), DEAE-cellulose column, and chitin affinity adsorption, respectively. The purification profile from DEAE-cellulose column of mutants was similar to WT CHI60. The partial purification of Y418A mutants by DEAE-cellulose used as example from mutants (shown in Figure 3.9) and the proteins from this purification was analyzed by SDS-PAGE (shown in Figure 3.10). The purified chitinase of all mutants were analyzed by

A.



B.

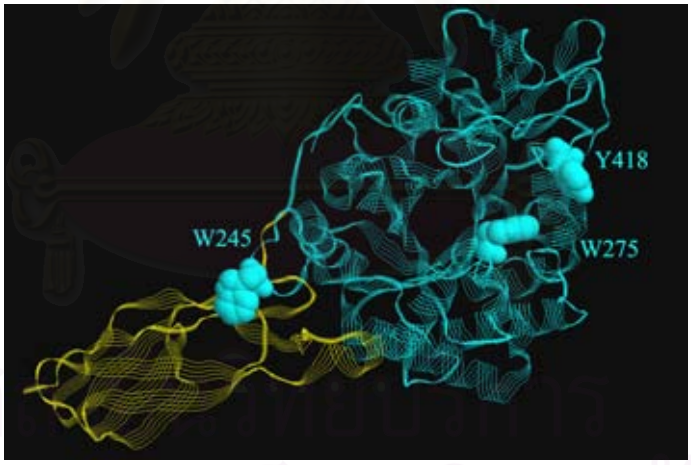
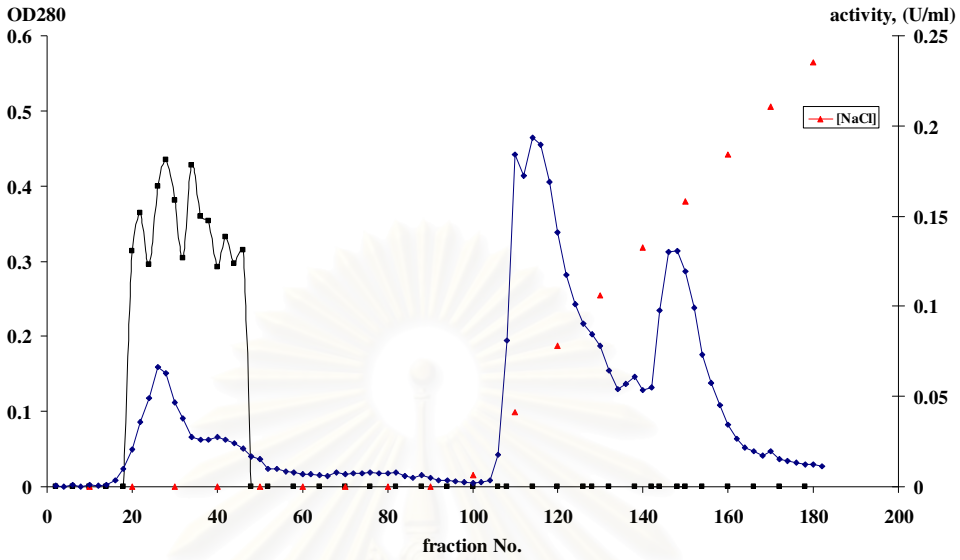


Figure 3.8 Positions of aromatic residues in *Serratia* sp.TU09 CHI60

The catalysis domain is shown in cyan and N-terminal domain in yellow. *Serratia* sp.TU09 CHI60 with the side chains of aromatic residues in the catalytic domain and the N-terminal domain that were mutated, are labeled with cyan (A) and yellow (B) letters, respectively.



♦ A280, ▲ [NaCl], ■ activity

Figure 3.9 Partial purification of Y418A mutants by DEAE-cellulose column

The concentrated enzyme was applied to DEAE-cellulose column and washed with 20 mMTris-HCl pH 7.0 until A280 decrease to base line. Gradient elution of bound protein was made by 0-1 M NaCl in the same buffer at the flow rate of 0.5 ml/min.

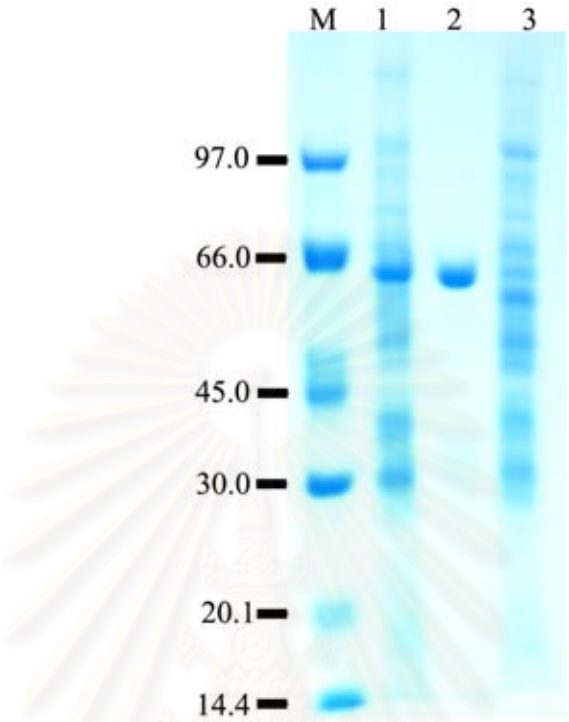


Figure 3.10 SDS-PAGE analysis of purification step of Y418A by DEAE-cellulose column

Lane M: protein standard marker

Lane 1: concentrated protein

Lane 2: unbound protein by DEAE cellulose column

Lane 3: bound protein by DEAE cellulose column

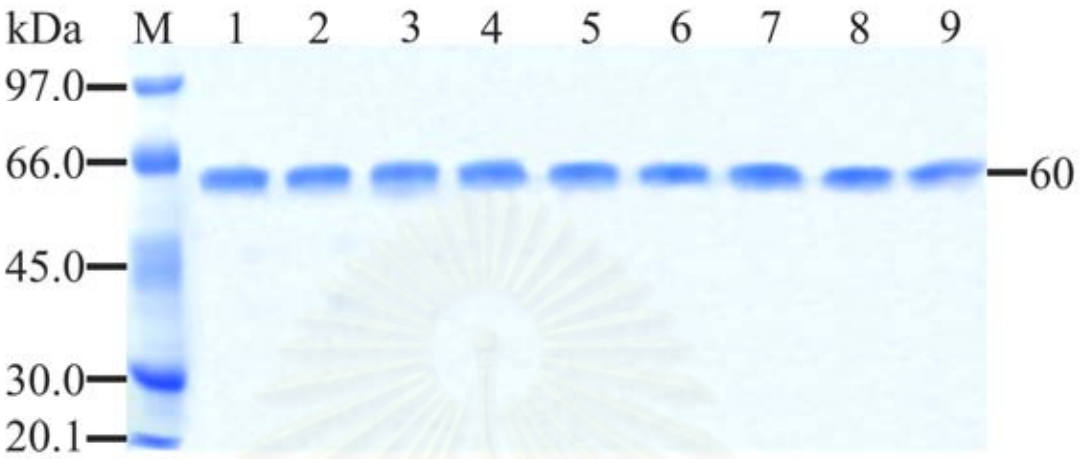


Figure 3.11 SDS-polyacrylamide gel electrophoresis analysis of single point mutated chitinases in the catalytic domain and N-terminal domain

Chitinase proteins produced in *E. coli* DH5α cells were purified from the culture supernatant. Protein staining of the SDS-polyacrylamide gel with Coomassie Brilliant Blue R-250 is shown.

Lane M: molecular mass standards

Lane 1: purified W33F

Lane 2: purified W33Y

Lane 3: purified W69F

Lane 4 : purified W69Y

Lane 5: purified W245F

Lane 6: purified W245Y

Lane 7: purified W275A

Lane 8: purified W275F

Lane 9: purified Y418A

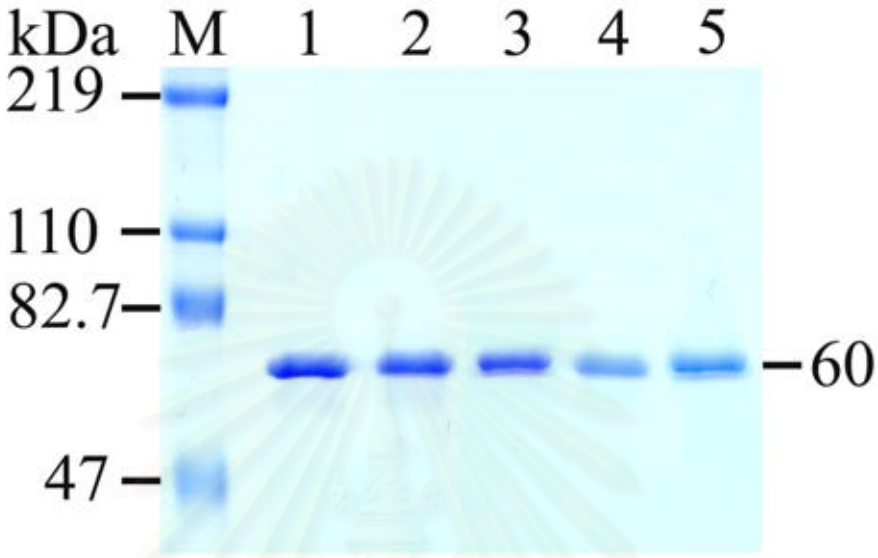


Figure 3.12 SDS-polyacrylamide gel electrophoresis analysis of double point mutated chitinases

Chitinase proteins produced in *E. coli* DH5α cells were purified from the culture supernatant. Protein staining of the SDS-polyacrylamide gel with Coomassie Brilliant Blue R-250 is shown.

Lane M: molecular mass standards

Lane 1: purified W33F/W69Y

Lane 2: purified W33F/W245F

Lane 3: purified W33Y/W245Y

Lane 4: purified W33Y/W245F

Lane 5: purified W69Y/W245Y

SDS-PAGE (shown in Figure 3.11 and 3.12). The result showed that all mutant proteins were expressed and can be purified to a single protein band with the correct molecular weight of 60 kDa.

Effect of temperature on the hydrolyzing activity of WT CHI60 and its mutants on various substrate

To determine the activity and ability to hydrolyze various substrates, WT and mutant CHI60 chitinolytic activity at 37°C and 50°C and hydrolytic activity ratio of 37°C/50°C was measured on PNAC, colloidal chitin and β crystalline chitin. The results are shown in Table 3.2.

On PNAC, the hydrolytic activity ratio of 37°C/50°C of single mutants which reduced hydrophobic interaction between substrate and enzymes can be categorized into 2 groups. Firstly, the hydrolytic activity ratio of 37°C/50°C of W33F, W69F, W69Y and W245Y (0.41-0.53) was lower than WT CHI60. Secondly, the hydrolytic activity ratio of 37°C/50°C of W33Y and W245F (0.59-0.61) was similar to WT CHI60 (0.61). The hydrolytic activity ratio of 37°C/50°C of the 5 double mutations (0.39-0.52) was lower than WT CHI60. Furthermore, the hydrolytic activity ratio of 37°C/50°C of single mutants within the catalytic domain, and enzymes can be divided into 3 groups. Firstly, the hydrolytic activity ratio of 37°C/50°C of Y418A (0.39) was lower than that of WT CHI60, secondly, the hydrolytic activity ratio of 37°C/50°C of W275A (0.76) was higher than that of WT CHI60 and finally, the hydrolytic activity ratio of 37°C/50°C of W275F (0.56) was similar to WT CHI60.

On colloidal chitin, the activity ratio of 37°C/50°C of single mutants which reduced hydrophobic interaction between substrate and enzyme can be categorized into 2 groups. Firstly, the hydrolytic activity ratio of 37°C/50°C of W33Y and W69F (0.60-0.64)was lower than WT CHI60 (0.71). Secondly, the hydrolytic activity ratio of 37°C/50°C of W33F, W69Y, W245F and W245Y (0.76-1.02) was higher than WT CHI60. The hydrolytic activity ratio of 37°C/50°C of the double mutations can be categorized into 2 groups. First, the hydrolytic activity ratio of 37°C/50°C was lower than that of WT CHI60, W33F/W69Y and W33Y/W245F (0.52-0.61). Second, the hydrolytic activity ratio of 37°C/50°C was higher than that of WT CHI60, W33F/W245F (0.76) and third, the hydrolytic activity ratio of 37°C/50°C was similar to WT CHI60, W33Y/W245Y and W69Y/W245Y (0.72). Moreover, the hydrolytic

activity ratio of 37°C/50°C of the single mutants in the catalytic domain, W275A and Y418A (0.59-0.61) was lower than WT CHI60, but the hydrolytic activity ratio of 37°C/50°C of W275F (0.70) was similar to WT CHI60.

On β-chitin, the hydrolytic activity ratio of 37°C/50°C of single mutants which reduced hydrophobic interaction between substrate and enzyme can be categorized into 3 groups. The first group, hydrolytic activity ratio of 37°C/50°C of W33Y (0.44) was lower than that of WT CHI60 (0.63). The second group, hydrolytic activity ratio of 37°C/50°C of W33F, W69F and W245Y (0.84-1.27) was higher than that of WT CHI60. The third group the hydrolytic activity ratio of 37°C/50°C was similar to WT CHI60, W69Y and W245F (0.62-0.63). The hydrolytic activity ratio of 37°C/50°C of the double mutations, W33F/W69Y and W33Y/W245F (0.4-0.56), was lower than WT CHI60, but the hydrolytic activity ratio of 37°C/50°C of W33F/W245F, W33Y/W245Y and W69Y/W245Y (0.71-0.94) was higher than WT CHI60. The hydrolytic activity ratio of 37°C/50°C of the single mutants, W275A, W275F and Y418A (0.68-4.18) in the catalytic domain was lower than WT CHI60.

Interestingly, at Trp245 position, the hydrolytic activity ratio of 37°C/50°C of W245F on soluble substrate (PNAC) and crystalline substrate (β-crystalline chitin) was similar to WT CHI60, but on amorphous substrate (colloidal chitin) was higher than WT CHI60. Moreover, the hydrolytic activity ratio of 37°C/50°C of W245Y on insoluble substrate, amorphous and crystalline chitin was higher than WT CHI60 (0.76 and 1.27, respectively). However, the hydrolytic activity ratio of 37°C/50°C of W33Y/W245F on all substrates had the same value in range of 0.52-0.56.

Effect of ionic strength on the hydrolyzing activity of CHI60 mutants

Effect of ionic strength on the hydrolyzing activity of single point mutants in the catalytic domain and N-terminal domain to reduce hydrophobic interaction

The effect of ionic strength on the hydrolyzing activity of mutations on PNAC, colloidal chitin and β-crystalline chitin was studied. The effect of ionic strength on

Table 3.2 The hydrolytic activity ratio of 37°C /50°C of wild-type CHI60 and its mutants on various substrate

Ratio of Temperature (37°C/50°C) 0 M NaCl			
Substrate	**PNAC**	**Colloidal chitin**	**β-chitin**
CHI60	0.61	0.71	0.63
W33F	0.51	0.81	0.84
W33Y	0.59	0.60	0.44
W69F	0.38	0.64	0.91
W69Y	0.53	0.79	0.63
W245F	0.61	1.02	0.62
W245Y	0.41	0.76	1.27
W33F/W69Y	0.40	0.61	0.40
W33F/W245F	0.39	0.76	0.94
W33Y/W245Y	0.39	0.72	0.71
W33Y/W245F	0.52	0.52	0.56
W69Y/W245Y	0.42	0.72	0.77
W275A	0.76	0.59	0.75
W275F	0.56	0.70	4.18
Y418A	0.39	0.67	0.68

the activity of single point mutants, W33F, W33Y, W69F, W69Y, W245F and W245Y, on PNAC with increasing ionic strength was similar to WT CHI60 (Figure 3.13A). However, on colloidal chitin, the effect of ionic strength on the enzymatic activity was slightly reduced for all single point mutants when compared to WT CHI60 (Figure 3.13B). On β - crystalline chitin, the effect of ionic strength on the activity of W69F, W69Y, W245F, and W245Y was slightly lower than WT CHI60, while the effect of ionic strength on the activity of W33F and W33Y was slightly higher than WT CHI60, as we increase the ionic strength (Figure 3.13C).

Effect of ionic strength on the hydrolyzing activity of single point mutants in the catalytic domain

The effect of ionic strength on the hydrolyzing activity of mutations on PNAC, colloidal chitin and β-crystalline chitin was studied. The effect of ionic strength on the activity of single point mutants, W275A, W275F and Y418A in the catalytic domain on PNAC with increasing ionic strength was similar to WT CHI60 (Figure 3.14A). However, on colloidal chitin, the effect of ionic strength on the enzymatic activity was slightly reduced for all single point mutants when compared to WT CHI60 (Figure 3.14B). On β-crystalline chitin, the effect of ionic strength on the activity of Y418A was slightly lower than that of CHI60, while the effect of ionic strength on the activity of W275A and W275F was slightly higher than WT CHI60, as we increase the ionic strength (Figure 3.14C).

Effect of ionic strength on the hydrolyzing activity of double point mutants in the catalytic domain and the N-terminal domain

The effect of ionic strength on the hydrolyzing activity of 5 double point mutants (W33F/W69Y, W33Y/W245F, W33F/W245F, W33Y/W245Y and W69Y/W245Y) on PNAC, colloidal chitin and β-crystalline chitin was studied. The effect of ionic strength on the activity of W33F/W69Y, W33Y/W245F, W33F/W245F, W33Y/W245Y and W69Y/W245Y on PNAC with increasing ionic strength was similar to WT CHI60 (Figure 3.15A). However, on colloidal chitin, the

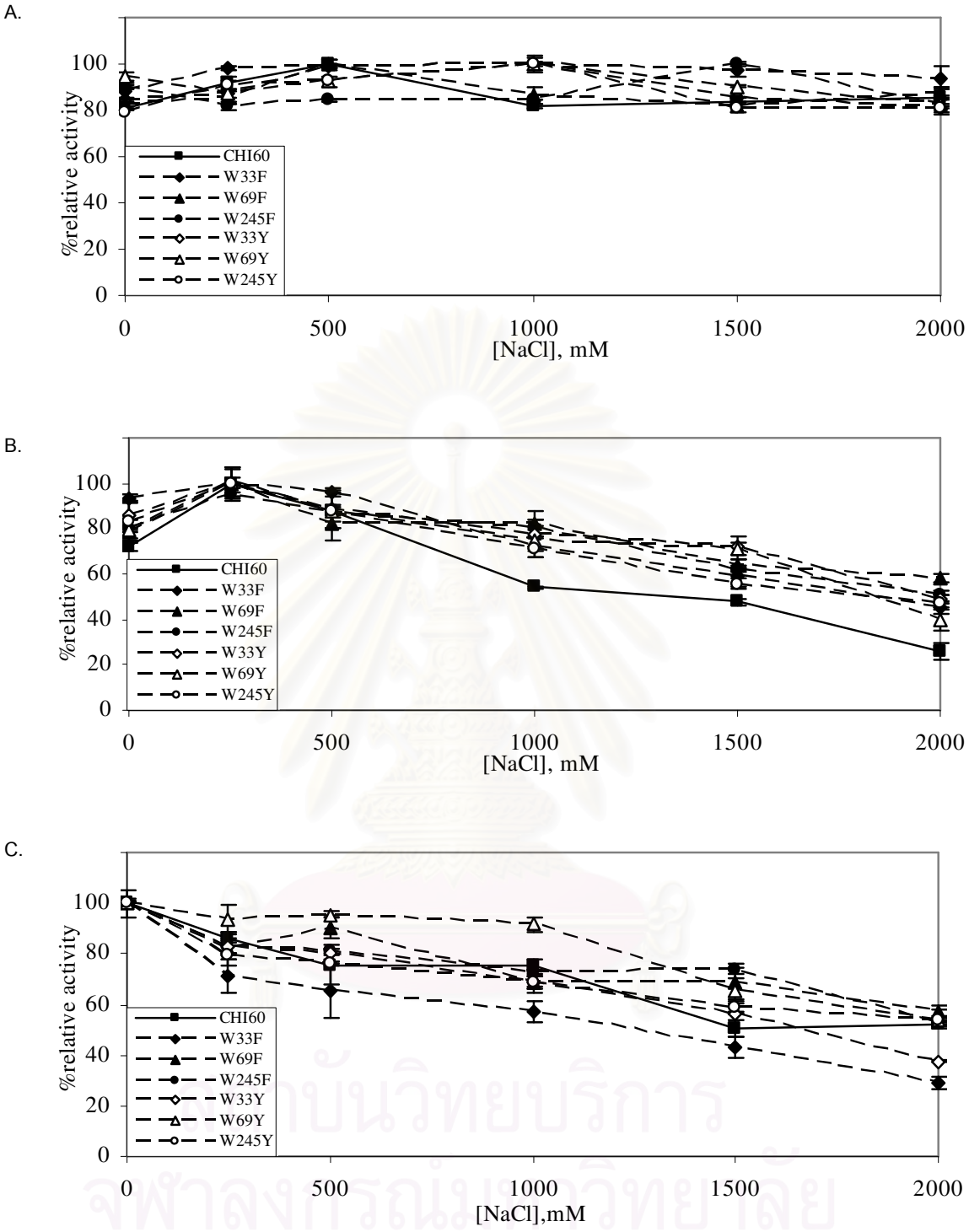


Figure 3.13 Hydrolysis of PNAC (A); colloidal chitin (B) and β-chitin (C) by wild-type CHI60 and single point mutant CHI60 in the N-terminal domain. ■, wild-type CHI60; ♦, W33F; ◊, W33Y; ▲, W69F; Δ, W69Y; ●,W245F; ○, W245Y. Reaction mixtures contained 1 mg (dry weight) of substrate and either 0.5 mg/ml (A) or 1 mg/ml (B) or 10 mg/ml (C) of each chitinase. Reactions were performed at 37 °C, and the amount of reducing sugar generated was monitored.

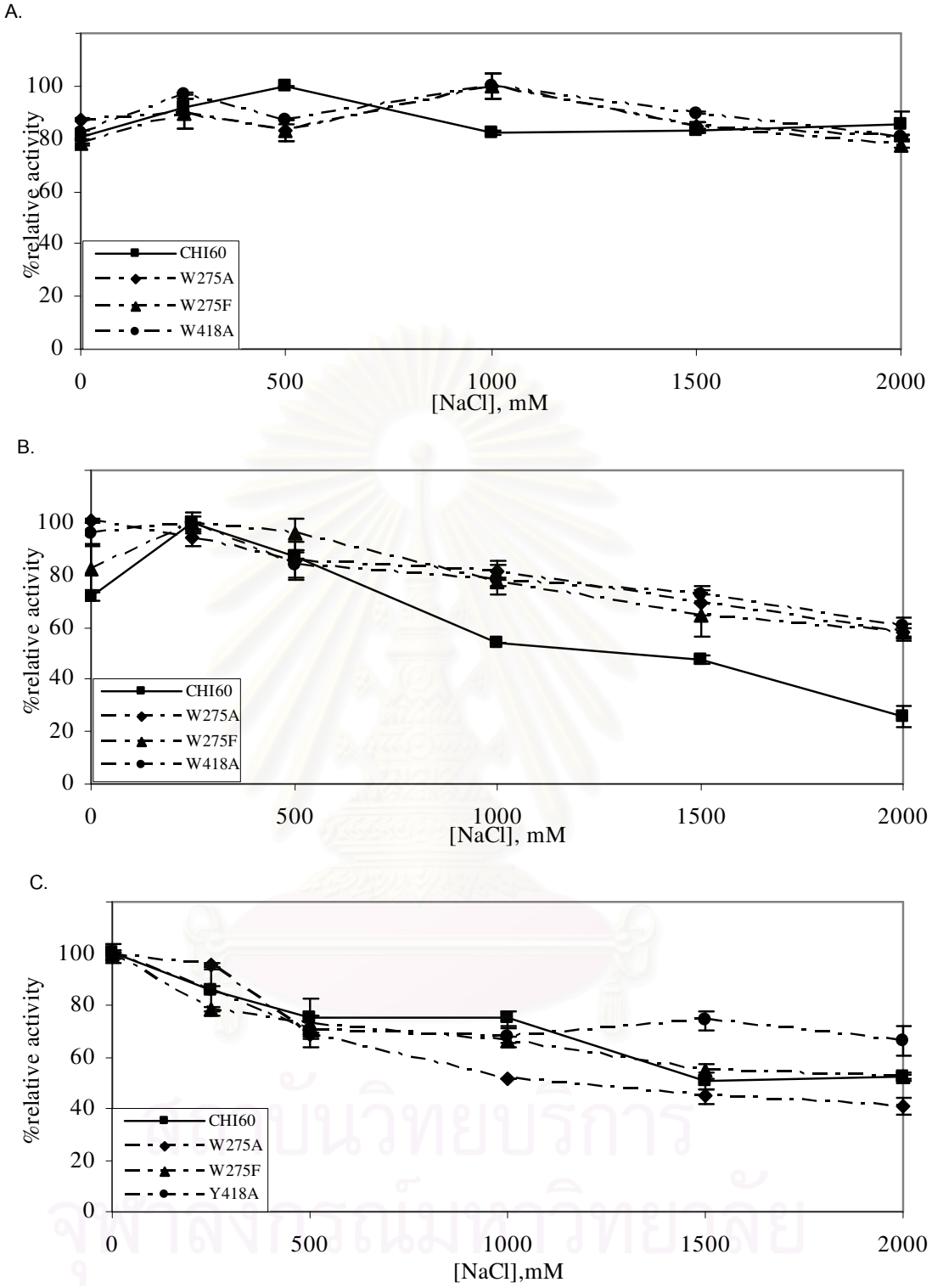


Figure 3.14 Hydrolysis of PNAC (A); colloidal chitin (B) and β-chitin (C) by wild-type CHI60 and single point mutant CHI60 in the catalytic domain. ■, wild-typeCHI60; ♦, W275A; ▲, W275F; ●, Y418A. Reaction mixtures contained 1 mg (dry weight) of substrate and either 0.5 mg/ml (A) or 1 mg/ml (B) or 10 mg/ml (C) of each chitinase. Reactions were performed at 37 °C, and the amount of reducing sugar generated was monitored.

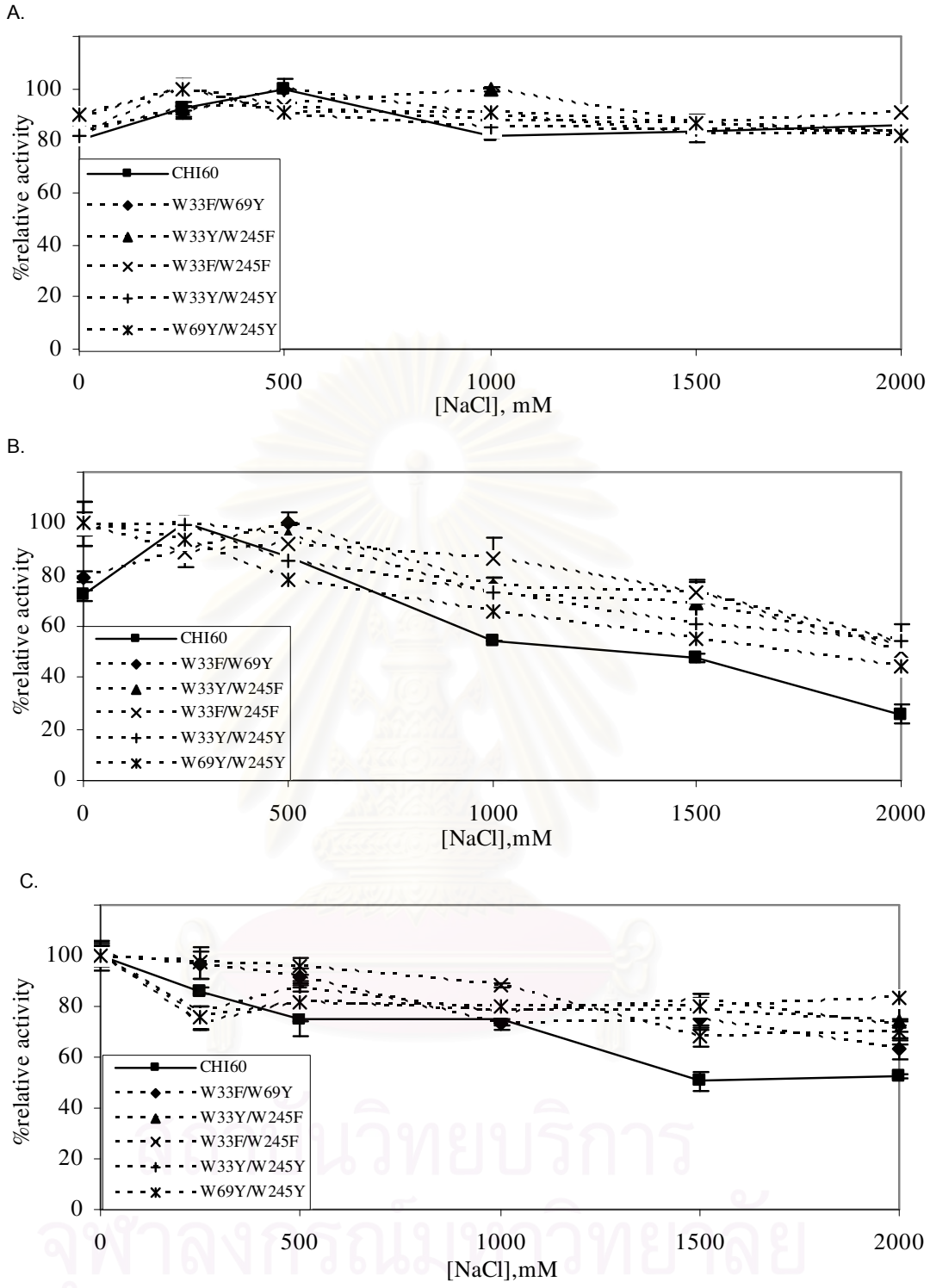


Figure 3.15 Hydrolysis of PNAC (A); colloidal chitin (B) and β-chitin (C) by wild-type CHI60 and double point mutant CHI60. ■, wild-typeCHI60; ♦, W33F/W69Y; ▲, W33Y/W245F; x, W33F/W245F; +, W33Y/W245Y; *,W69Y/W245Y. Reaction mixtures contained 1 mg (dry weight) of substrate and either 0.5 mg/ml (A) or 1 mg/ml (B) or 10 mg/ml (C) of each chitinase. Reactions were performed at 37 °C, and the amount of reducing sugar generated was monitored.

effect of ionic strength on the enzymatic activity was slightly reduced for all double point mutants when compared to WT CHI60 (Figure 3.15B). On β-crystalline chitin, the effect of ionic strength on the activity of double point mutants was slightly lower than WT CHI60, while the effect of ionic strength on the activity of W33F was slightly higher than WT CHI60, as we increase ionic strength (Figure 3.15C).

The degradation products analysis of WT CHI60 by Thin layer chromatography (TLC)

The hydrolytic product of colloidal chitin from WT CHI60 were analyzed by TLC. When incubation at 37°C, we observed dimer as a major product and monomer as a minor product in both absent NaCl and present 1 M NaCl. However, we found dimer as a major product and monomer, trimer and tetramer as a minor products at 50°C. At 50°C temperature with 1 M NaCl, dimer and monomer were detected as a major and a minor product, respectively, but trimer and tetramer were not found (Figure 3.16).

Analyses of the degradation products of Chitinase by HPLC

Hydrolytic product of WT CHI60 and its mutants were examined using colloidal chitin as the substrate at different NaCl concentration, at 37°C. The salt was removed from the reaction mixtures prior TLC analysis by using activated charcoal column. Colloidal chitin was hydrolyzed by WT CHI60 and mutants, single and double point mutation produce dimer ($(NAG)_2$) as a major product and monomer (NAG) and trimer ($(NAG)_3$) as a minor product in all NaCl concentration, except, W275F which produces monomer and dimer as a major product and only small amount of trimer at 0-2 M NaCl (0.42-0.52). The HPLC chromatogram of colloidal chitin hydrolyzed by CHI60 and W245Y mutant at 37°C in various NaCl showed in Figure 3.17 and 3.18. used as example from this experiment. The relative sugar production of WT CHI60, and its mutants was shown in Table 3.3.

Moreover, the relative sugar product of monomer of W245Y was increased from 0.008 to 0.26, and the relative sugar product of W33F/W245F was increased from 0.1 to 0.26 with increasing ionic strength (to 2 m NaCl), too. In contrast, the

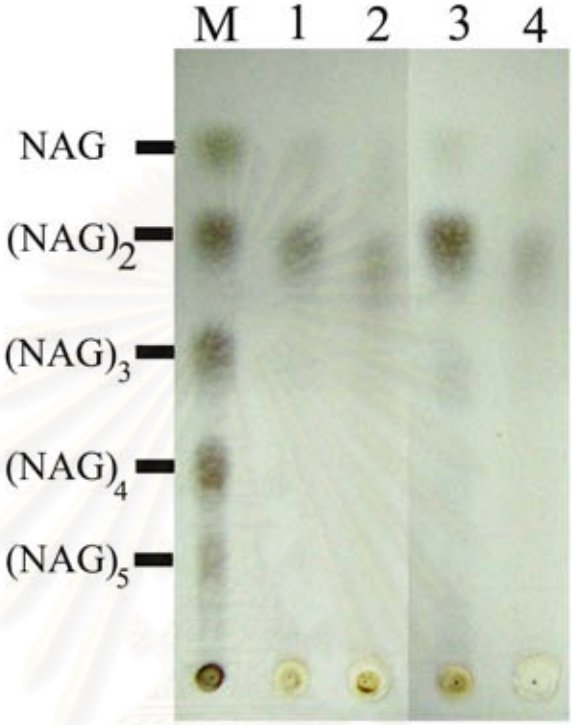


Figure 3.16 Thin layer chromatographic analysis of product from hydrolysis of colloidal chitin by WT CHI60.

Lane M: Chitooligosaccharide standard

Lane 1: hydrolysate by WT CHI60 at 37°C

Lane 2: hydrolysate by WT CHI60 at 37°C with 1 M NaCl.

Lane 3: hydrolysate by WT CHI60 at 50°C

Lane 4: hydrolysate by WT CHI60 at 50°C with 1 M NaCl.

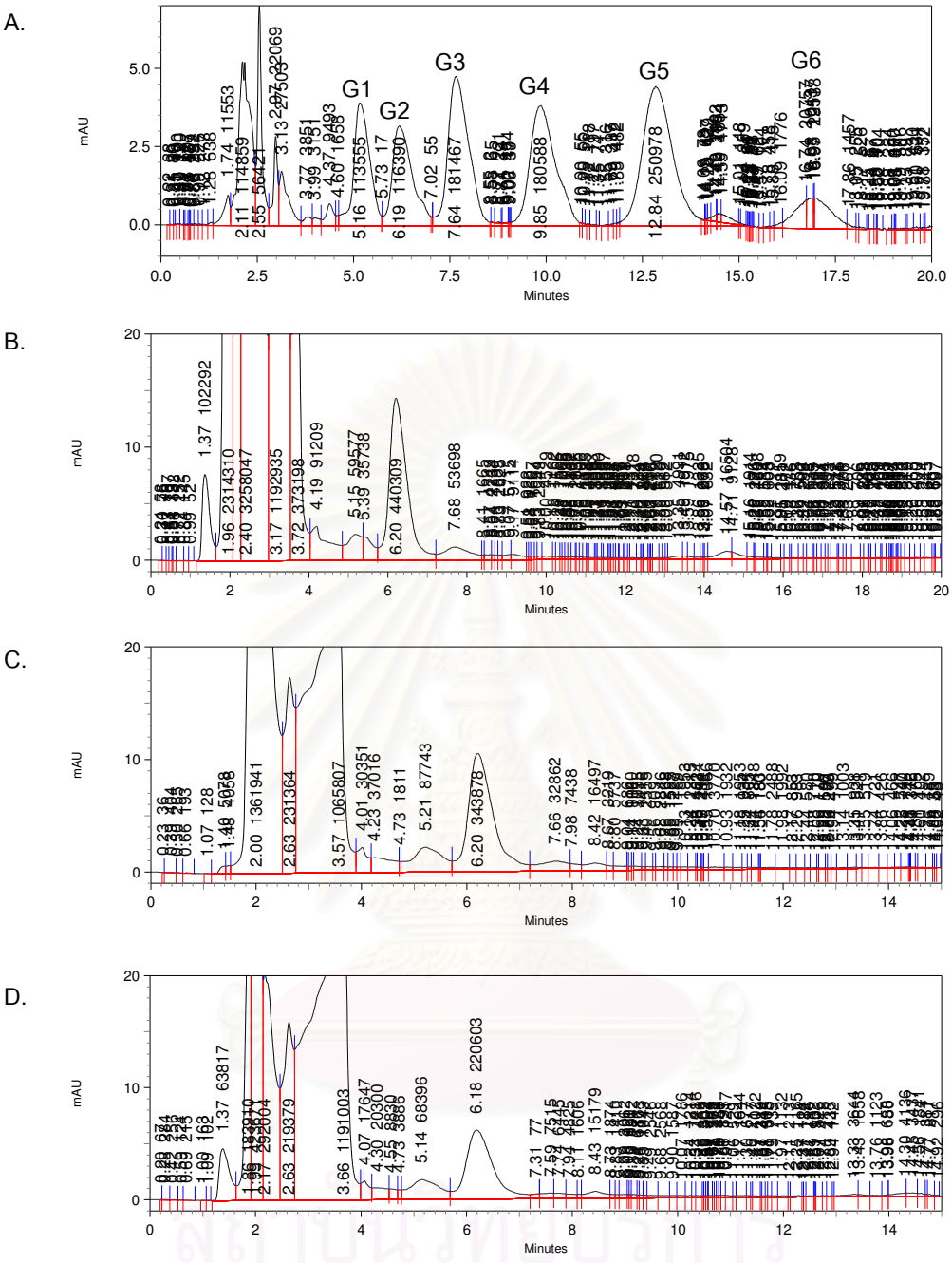


Figure 3.17 Determination of product size of CHI60 at various ionic strength

HPLC chromatogram of colloidal chitin hydrolyzed by CHI60 at 37°C for 1 hour.

A. *N*-acetyl-chitooligosaccharide standards, G1: monomer, G2: dimmer, G3: trimer, G4: tetramer, G5: pentamer and G6: hexamer

B. hydrolysate by WT CHI60 without NaCl.

C. hydrolysate by WT CHI60 with 1 M NaCl.

D. hydrolysate by WT CHI60 with 2 M NaCl

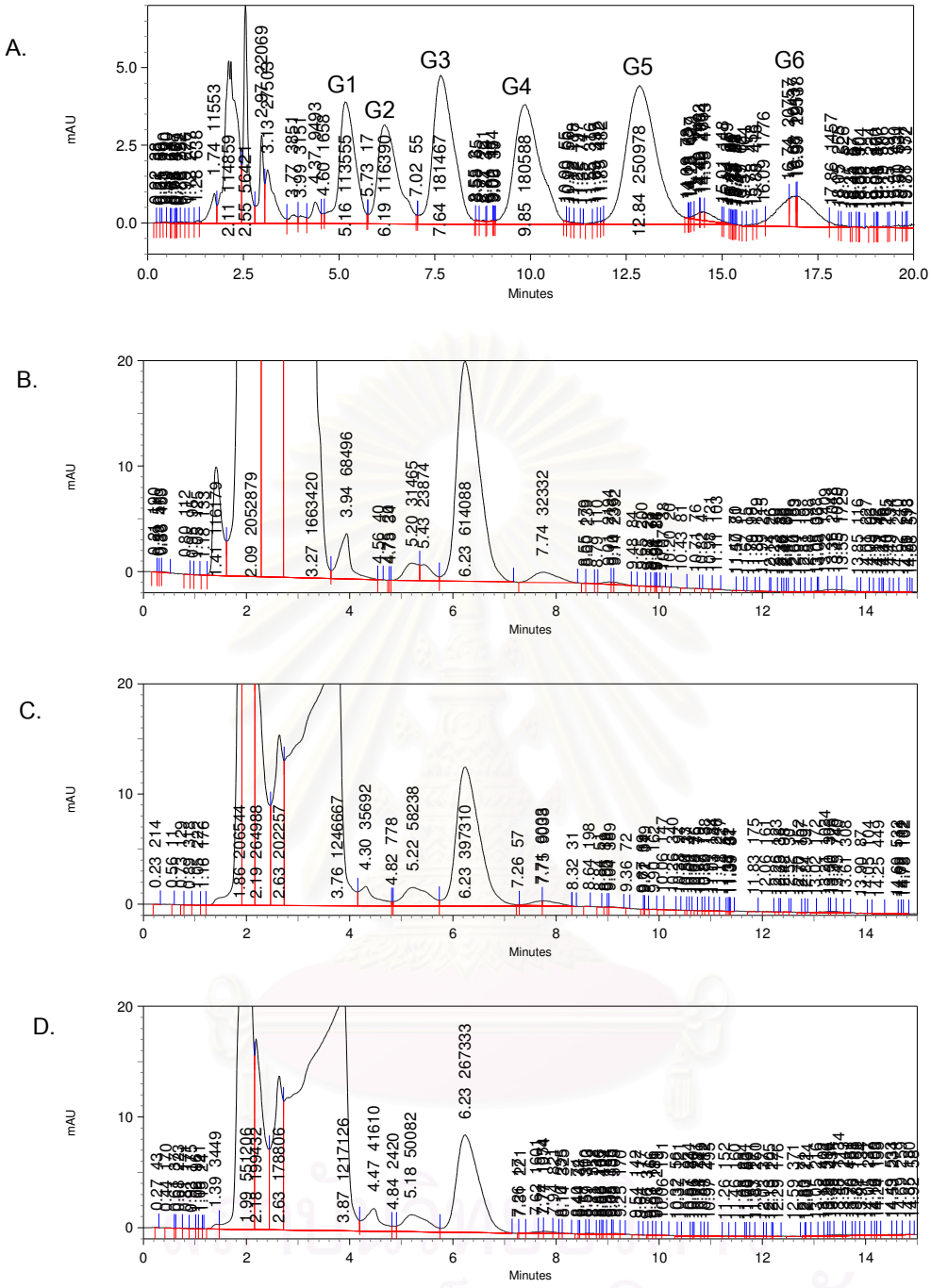


Figure 3.18 Determination of product size of W245Y at various ionic strength

HPLC chromatogram of colloidal chitin hydrolyzed by CHI60 at 37°C for 1 hour.

A. *N*-acetyl-chitooligosaccharide standards, G1: monomer, G2: dimmer, G3: trimer, G4: tetramer G5: pentamer and G6: hexamer

B. hydrolysate by W245Y without NaCl.

C. hydrolysate by W245Y with 1 M NaCl.

D. hydrolysate by W245Y with 2 M NaCl

relative sugar product of monomer of W69Y was decreased from 0.25 to 0.06, although, the relative sugar product of dimer was increased from 0.7 to 0.93 with increasing ionic strength (to 2 m NaCl).

To investigate the mode of catalysis the $(NAG)_2/(NAG)_3$ ratios were studied. The $(NAG)_2/(NAG)_3$ ratios as colloidal chitin degradation of WT CHI60 was higher with increasing ionic strength (0-2 M NaCl) (Table 3.4).

The $(NAG)_2/(NAG)_3$ ratio of the single mutant, single mutants which reduced hydrophobic interaction between substrate and enzyme can be categorized into 3 groups. The first group, the $(NAG)_2/(NAG)_3$ ratio of W69F and W245F (46-75) was slightly higher than WT CHI60 (35). The second group,W33F, W69Y and W245Y (135-367) was higher than WT CHI60 at high ionic strength and was at least 4 fold higher than WT CHI60 the $(NAG)_2/(NAG)_3$ ratios of. The third group, the $(NAG)_2/(NAG)_3$ ratio of W33Y (32.2) was similar to WT CHI60.

The $(NAG)_2/(NAG)_3$ ratio of the double mutants can be categorized into 2 groups. The first group, the $(NAG)_2/(NAG)_3$ ratio of W69Y/W245Y (42) was slightly higher than WT CHI60 (35) at high ionic strength. The last group, W33F/W69Y, W33Y/W245F, W33F/W245F and W33Y/W245Y (183-324) was higher than WT CHI60 at high ionic strength and was at least 5 fold higher than WT CHI60.

The $(NAG)_2/(NAG)_3$ ratio of the single mutants within the catalytic domain can be categorized into 2 groups. Firstly, the $(NAG)_2/(NAG)_3$ ratio of W275F (7.2) was lower than WT CHI60 (35) at high ionic strength, secondly, the $(NAG)_2/(NAG)_3$ ratios of W275A and Y418A (294-306) was higher than WT CHI60 at high ion strength.

Interestingly, the $(NAG)_2/(NAG)_3$ ratios of all mutants was increased from 10-369), except the $(NAG)_2/(NAG)_3$ ratios of W275F was 8.3, 40.5 and 7.2 at 0, 1 and 2 M NaCl, respectively.

The $(NAG)/(NAG)_3$ ratio of all mutants was decreased from 10 to 0.8 with increasing in ionic strength, except The $(NAG)/(NAG)_3$ ratio of W69Y was increased from 3-16 as we increase ionic strength 0-2 M NaCl.

Table 3.3 The relative sugar products of wild-type CHI60 and its mutants on colloidal chitin at various ionic strength

Concentration	0 M NaCl			1 M NaCl			2 M NaCl		
Relative sugar production	NAG/ tolal $(NAG)_{1,2,3}$	$(NAG)_2$/ tolal $(NAG)_{1,2,3}$	$(NAG)_3$/ tolal $(NAG)_{1,2,3}$	NAG/ tolal $(NAG)_{1,2,3}$	$(NAG)_2$/ tolal $(NAG)_{1,2,3}$	$(NAG)_3$/ tolal $(NAG)_{1,2,3}$	NAG/ tolal $(NAG)_{1,2,3}$	$(NAG)_2$/ tolal $(NAG)_{1,2,3}$	$(NAG)_3$/ tolal $(NAG)_{1,2,3}$
CHI60	0.19	0.72	0.08	0.32	0.63	0.06	0.37	0.61	0.017
W33F	0.21	0.76	0.04	0.20	0.78	0.02	0.27	0.73	0.002
W33Y	0.33	0.65	0.03	0.40	0.59	0.02	0.44	0.55	0.017
W69F	0.13	0.85	0.02	0.16	0.82	0.02	0.33	0.66	0.009
W69Y	0.25	0.70	0.05	0.05	0.94	0.01	0.06	0.93	0.007
W245F	0.15	0.80	0.06	0.17	0.80	0.03	0.23	0.75	0.016
W245Y	0.08	0.87	0.04	0.22	0.76	0.02	0.26	0.74	0.003
W33F/W69Y	0.28	0.69	0.04	0.21	0.79	0.01	0.35	0.65	0.002
W33F/W245F	0.10	0.83	0.07	0.12	0.86	0.03	0.26	0.74	0.004
W33Y/W245Y	0.10	0.82	0.08	0.19	0.80	0.01	0.12	0.88	0.004
W33Y/W245F	0.25	0.73	0.02	0.17	0.83	0.00	0.24	0.76	0.003
W69Y/W245Y	0.14	0.83	0.03	0.18	0.79	0.03	0.20	0.78	0.019
W275A	0.29	0.68	0.03	0.27	0.72	0.01	0.40	0.60	0.002
W275F	0.42	0.51	0.06	0.47	0.52	0.01	0.52	0.42	0.059
Y418A	0.12	0.85	0.03	0.20	0.77	0.03	0.25	0.75	0.003

Table 3.4 The ratio of the hydrolytic products of wild-type CHI60 and its mutants on colloidal chitin at various ionic strength

Concentration	o M NaCl		1 M NaCl		2 M NaCl	
Ratio of sugar production	$(NAG)_2$/ NAG	$(NAG)_2$/ $(NAG)_3$	$(NAG)_2$/ NAG	$(NAG)_2$/ $(NAG)_3$	$(NAG)_2$/ NAG	$(NAG)_2$/ $(NAG)_3$
CHI60	3.8	8.6	2.0	10.9	1.6	35.0
W33F	3.7	21.2	3.9	52.2	2.7	367.1
W33Y	2.0	24.1	1.5	33.4	1.3	32.2
W69F	6.4	48.6	5.0	38.8	2.0	74.8
W69Y	2.8	14.6	17.9	104.8	15.3	134.5
W245F	5.3	14.2	4.7	28.7	3.2	46.3
W245Y	10.4	19.8	3.5	45.5	2.8	280.8
W33F/W69Y	2.5	19.0	3.8	99.6	1.8	323.5
W33F/W245F	8.4	12.3	7.3	32.2	2.9	182.9
W33Y/W245Y	7.9	10.1	4.2	91.8	7.5	206.3
W33Y/W245F	2.9	36.6	5.0	239.5	3.2	223.4
W69Y/W245Y	5.8	27.2	4.5	26.6	3.9	41.6
W275A	2.3	19.6	2.7	55.8	1.5	306.1
W275F	1.2	8.3	1.1	40.5	0.8	7.2
Y418A	7.1	27.9	3.8	28.7	3.1	293.1

CHAPTER IV

DISCUSSION

Identification of bacterial strain TU09

Characteristic of strain TU09 lies between *marcescens* and *liquefaciens* specie. Bacteria TU09 can fermentative produce the acid from D-xylose and arabinose, which is an unique feature of the *liquefaciens* specie. On the other hand, *marcescens* specie cannot fermentatively produce acid from D-xylose and adonitol. That is the biochemical characteristics, which is a different between two *Seratia* species, *marcescens* and *liquefaciens* specie.

16S rRNA gene of strain TU09 was *in vitro* amplified. The nucleotide sequence of 16S rRNA gene was compared to those of others, deposited in GenBank. Sequence with most similarly with 16S rRNA gene of strain TU09 was 16S rRNA gene from *S.marcescens,* with 99% identity, next was uncultured bacterium with 99% identity. However there were only 7 nucleotide sequences of 16S rRNA gene of *S. liquefaciens* deposited in Genbank, which might be the reason that we did not find a match of our sequence with to *liquefaciens* specie. When we align the two 16S rRNA gene sequences of *S. liquefaciens* (DQ123840) and strain TU09, it shows the overall sequence similarity of 96%. The alignment was show in Appendix G. We decided to called this bacteria strain was *Serratia* sp. TU09.

Production and purification of CHI60

WT CHI60 was expressed in *E.coli* cells carrying a plasmid encoding *chi60* gene and their derivatives. CHI60 and derivatives were purified from the culture supernatant. For chitinase purification, CHI60 and derivatives was concentrated by ultrafiltration (cut off 30,000 Da), then the partial purified crude enzyme was applied to DEAE-cellulose column. In this purification step chitinase was not bound to the column and can be detected in the unbound fractions. The unbound protein from DEAE-cellulose was purified by chitin affinity adsorption. This purification system is very easy and efficient for the purification of crude chitinase.

CHI60 posses both exo- and endo-type activity

The activity of WT CHI60 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 us to distinguish each of the activity of the enzyme, exo-and endo-type. Soluble substrates can freely associate and dissociate with the active site of CHI60, therefore the activity determined on this substrate would reflect the amount of active catalytic domain present in the reaction. This activity can also indicate the endo-type activity of CHI60 that is independent of the substrate binding Trp residues. It has been shown that mutations of the Trp residues that are involved in substrate binding did not affect the activity of the enzyme on soluble substrates (Uchiyama *et al*., 2001). Amorphous substrate was used to determine the endo-type activity of the enzyme. Colloidal chitin where chitin was solubilized and partially hydrolyzed in concentrated acid, giving an amorphous structure was used, which can be hydrolyzed effectively by endo-chitinase. The enzyme must associate on chitin, hydrolyze then dissociate from the initial site to the next hydrolytic site, since it can not move processively along the entangle chitin strands of amorphous chitin. β–Crystalline chitin was use to study the processive exo-type mode of enzyme. Since in β–crystalline chitin the chitin strands are arranged in a parallel unidirectional manner. Moreover, in β–crystalline chitin there are very few intermolecular hydrogen bindings between the strands which made it a good substrate for exo-chitinase.

The amount of enzyme which yields equal activity on PNAC was used to hydrolyzed β-chitin and 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 the interaction between CHI60 to each of the substrates. Our results in figure 3.7 demonstrated that CHI60 can hydrolyze both amorphous and crystalline chitin well suggesting that CHI60 has both endo- and exo-mode of hydrolysis depending on type of substrate it hydrolyses. Since the interaction between the enzyme and substrate is the hydrophobic interaction between the sugar ring and Trp residues, we should be able to influence the mode of hydrolysis of CHI60 by modulating the binding affinity of CHI60 to its substrate. This can be accomplished by two approaches. Firstly, the interaction can be modulated by

changing of the ionic strength of the environment using salt gradients. Secondly, it can be modulated by reducing the interaction by generating derivatives of CHI60 with mutations on the Trp residues in the Tryptophan-track, where the Trp residues were change to Phe or Tyr residues to reduce the interaction of the enzyme with the substrate. Therefore in our experiments we used different substrates, soluble, amorphous, and crystalline, for hydrolysis at ionic strength from 0-2 M NaCl. CHI60 was first studied then the mutants were used to confirm our hypothesis.

CHI60 uses an endo-mode when it hydrolyzes soluble chitin (PNAC) involving only the catalytic domain and this activity is independent of the Trp residues that are involved in substrate binding. This fact can be clearly demonstrated by our result in figure 3.7, that the increase in the ionic strength of the reaction mixture did not influence how the enzyme works on this substrate. Moreover, we would show later that CHI60 derivatives with mutations in the Tryptophan-track, Trp69, Trp33, and Trp 245, either single or double mutants shows similar results as those of WT CHI60.

CHI60 uses both endo- and exo-mode when it hydrolyzes amorphous chitin. As we increase the ionic strength the endo-type activity decreases since the enzyme would remain bound to the substrate and cannot dissociate to a new hydrolytic site, therefore a decrease in activity should be observed at high salt concentration. Our results in figure 3.7 demonstrated that CHI60 behaves as we have expected showing high activity at low salt concentration and decreasing activity as we increase the salt concentration.

CHI60 uses only exo-mode when it processively hydrolyzes β-crystalline chitin. When the ionic strength was increase by addition of salt the enzyme continues to work at about 80%-60% from 0.25-2.0 M NaCl.

However, previous study by Horn and co-worker (2006), has shown that *Serratia marcescens* produces multiple enzyme, ChiA, ChiB, and ChiC, which favor different mode of catalysis, exo- or endo-type. However, 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 was able to hydrolyze both amorphous and crystalline chitin equally well, indicating that it posses both exo- and

endo-type activity. Our results confirm that CHI60 has both endo- and exo-hydrolysis mode depending on the type of substrate it hydrolyses.

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 hydrolyzed and the enzyme moves symmetrically two GlcNAc 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).

From our results, the mode and mechanism of how of CHI60 hydrolyses was proposed base on type of substrate and processivity of this enzyme (Figure 4.1). For soluble chitin, the substrate can diffuse into catalytic site independent of the chitin binding domain. Therefore, activity of CHI60 was not affected by ionic strength. For insoluble chitin, CHI60 uses N-terminal domain for substrate binding. In amorphous chitin, CHI60 cannot processively hydrolyze chitin chain. At low ionic strength, CHI60 binds and hydrolyzes chitin chain then CHI60 must dissociate to next cleavage site and hydrolyzes again. At high ionic strength, the enzyme cannot dissociate from the substrate, therefore, a reduction in activity was observed. In β-chitin, at low ionic strength, enzyme can processively hydrolyze without releasing from the substrate. At high ionic strength, enzyme retains its activity therefore its activity was not affected by high ionic strength.

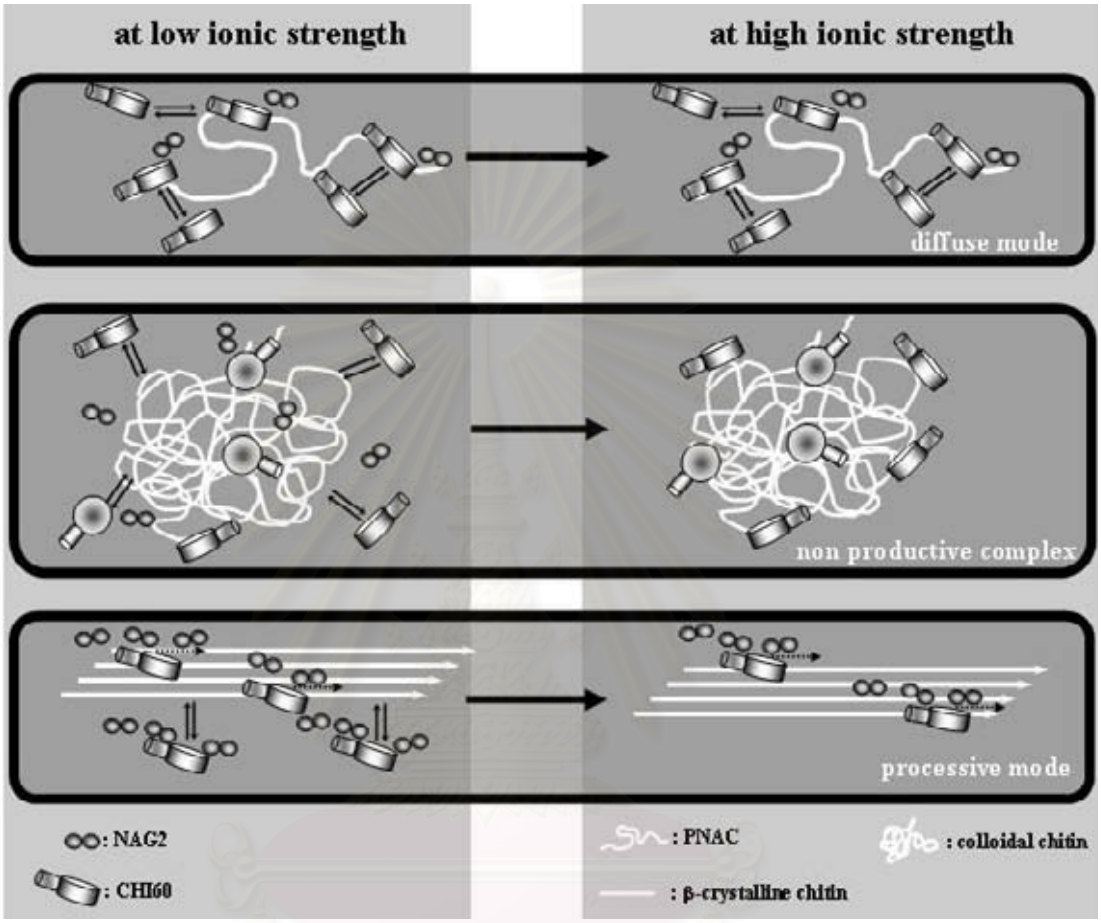


Figure 4.1 Model for various substrates hydrolysis by CHI60.

Site-directed mutagenesis

Selection of aromatic residues for reduction of hydrophobic interaction

Many Family 18 proteins contain a second domain, namely the chitin-binding domain, which is considered to maintain the enzyme bound to the insoluble substrate so that the reaction can be progressive. The N-terminal domain of CHI60 is located at its N-terminus and has a fibronectin type III fold (Perrakis *et al*., 1994). The N-terminal domain contains two tryptophan residues (W33 and W69) which, along with two additional residues (W232 and W245) on the catalytic domain beyond the −5 site of the cleft, appear to be positioned correctly to correspond to binding sites for additional odd-numbered GlcNAcs. Mutagenesis of W33 and W69 at the N-terminal domain of ChiA revealed that they are important for the degradation of crystalline *β*-chitin, but not for the degradation of oligosaccharides (Uchiyama, *et al*., 2001). Likewise, W232 and W245 on the catalytic domain of ChiA (Figure 1.7A) are selective in helping to degrade only the natural chitin polymer (Watanabe *et al*., 2001 and Uchiyama, *et al*., 2001). In the catalytic domain, chitooligosaccharide interacts with three aromatic residues, Y418 and F396 at the +2 site and W275 at the +1 site. Although Tyr418 appears to mark the end of the cleft, it does not interfere with the extension of the reducing end beyond the +2 site. W275 and Phe396 from opposite sides of the cleft, stacking against the hydrophobic faces of the GlcNAcs. In contrast, chitooligosaccharide lie flat in the cleft and every other GlcNAc in this region has its hydrophobic face aligned with an aromatic amino acid in the cleft floor [−1 (W539), −3 (W167), −5 (W170), and W33 and W69 on the chitin-binding domain were spaced appropriately to correspond to the −11 and −13 sites), whereas the sugars in the even-numbered subsites (−2 and −4) have their hydrophobic surfaces exposed.

Aronson and his co-worker (2003) was observed that the four-subsite binding of pentamer ($(NAG)_5$) of ChiA was the mode of action of the native Family 18 chitinases during the hydrolysis and cleaved between subsite (+2 to -2) to produce trimer and dimer. NAG residues at the reducing end of the substrate are not bound to the enzyme during hydrolysis. Partial hydrolysis of ChiA produced trimer ($(NAG)_3$) and dimer ($(NAG)_2$) as a major product and monomer (NAG) and tetramer($(NAG)_4$) as a minor product. But W275A mutant hydrolysed the *β*-anomer of the $(NAG)_5$ substrate, smaller amounts of $(NAG)_4$ and NAG were produced more than ChiA.

Our laboratory has two main interests, firstly to understanding the mechanism of how the exposed tryptophan track functions on binding and guiding the substrate into the catalytic cleft and, secondly, to study the possible role of these residues on the mode of catalysis, the endo- and exo-type activity, of CHI60. Therefore, we have constructed mutant CHI60 with single and multiple point mutations at the exposed tryptophan residues on the N-terminal domain of CHI60. These mutants were use for the studying of the mode of catalysis, and their role in binding and guiding the substrate during processive hydrolysis, as well as the possible effect on the product size.

We have mutated three aromatic amino acid residues, W33, W69 and W245. To reduce hydrophobic interaction between the enzyme (CHI60) and substrates and also modulate the enzyme–substrate interaction by vary ionic strength and temperature. Furthermore, we have mutate two aromatic amino acid residues, W275 and Y418 within the catalytic cleft because early reports has shown that mutations at W275 can shift the product size

We have successfully mutated Trp residues in the exposed tryptophan track at the N-terminal domain and the catalytic domain of *Serratia* sp.TU09 CHI60, Trp-33, Trp-69, Trp-245, Trp-275 and Tyr-418 to smaller aromatic residues Phe or Tyr, or Ala (Figure 3.7). The single point mutants are W33F, W33Y, W69F, W69Y, W245F, W245Y, W275A, W275F and Y418A. The double point mutants are W33F/W69Y, W33Y/W245F, W33F/W245F, W33Y/W245Y and W69Y/W245Y.

Expression and purification of CHI60 mutants

The fifteen CHI60 mutants, W33F, W33Y, W69F, W69F, W69Y, W245F, W245Y, W275A, W275F, Y418A, W33F/W69Y, W33Y/W245F, W33F/W245F, W33Y/W245Y and W69Y/W245Y, were produced in *E. coli* DH5α cells carrying each of the plasmid encoding mutated *chi*60 gene and purified from the culture supernatants. All CHI60 mutants were produced at a level similar to that at which wild-type (WT) CHI60 was produced. Purification profile of mutated chitinase proteins was similar to purification profile of WT CHI60.

Effect of ionic strength on the hydrolyzing activity of CHI60 mutants

Effect of ionic strength on the hydrolyzing activity of single point mutants in the catalytic domain and N-terminal domain

The effect of ionic strength on the activity of single point mutants, W33F, W33Y, W69F, W69F, W245F and W245Y in the N-terminal domain and the catalytic domain, on PNAC with increasing ionic strength was similar to WT CHI60 (Figure 3.13A). The result indicated that ionic strength did not effect of the activity of mutants on PNAC and the enzyme retains its activity through out ionic strength range studied.

However, on colloidal chitin, the result shows that mutations of Trp to Phe, or Tyr which would reduce the hydrophobic interaction between enzyme and substrate appeared to shift the activity profile of the mutants higher than that of the WT CHI60 at high ionic strength. This indicates the mutated enzyme was not affected by the high ionic strength as much as the WT CHI60. The weaker binding of these mutants to its substrate allows it to function better at high ionic strength. In other words, the reduction in the enzyme substrate interaction will allow the enzyme to dissociate and re-associate with the substrate better in the mutants than WT CHI60 at high ionic strength. Therefore we would observe higher activity of the mutants at high ionic strength than WT CHI60, as shown in Figure 3.13B.

On β- crystalline chitin, the result shows that at high ionic strength, the reduced enzyme-substrate interaction of mutants also allow the enzyme to be more active than WT CHI60. This may possibly due to the lower interaction between the substrate and the enzyme, which would allow the enzyme to move along the substrate more efficiently at high ionic strength. Surprisingly, for W33F and W33Y mutant, the activity of the enzyme was lower than that of WT CHI60 at high ionic strength. This result suggested that W33 may play an important role in the processivity of the enzyme. Mutation of this residue may render the enzyme to be less processive, either by reducing the ability of the enzyme to stay on the substrate or its ability to move along the chitin strand.

Effect of ionic strength on the hydrolyzing activity of single point mutants in the catalytic domain

Previous reports have not suggested any role of the Trp residues within the catalytic cleft to be involved in substrate binding. Therefore we did not expect that mutations of these residues would have a different effect by ionic strength from WT CHI60. On the contrary, our result on the effect of ionic strength of the single point mutants, W275A, W275F and Y418A in the catalytic domain was affected by the change in ionic strength.

On PNAC the activity of single point mutants, W275A, W275F and Y418A in the catalytic domain was not affect by ionic strength and the mutants remain active on PNAC. In contrast, at high ionic strength, the enzymatic activity of W275A, W275F and Y418A on colloidal chitin was less affected, and their activity was greater than that WT CHI60. When β-crystalline chitin was used as the substrate, the result demonstrated that the activity of Y418A was affected less than WT CHI60 at high ionic strength. However, the activity of W275A and W275F was more strongly affected than that of WT CHI60, with increasing ionic strength. Therefore, Trp275 in the catalytic domain might play an important role in processivity of CHI60. It may also be possible that this residue plays a structural role, which stabilizes the structure of the catalytic cleft at high ionic strength.

Effect of ionic strength on the hydrolyzing activity of double point mutants

We hypothesize that by making double mutations we could further reduce the interaction between the enzyme and substrate, therefore elevating the effect that we observed earlier in the mutants with single mutations. Our result demonstrated that the effect of ionic strength on the activity of W33F/W69Y, W33Y/W245F, W33F/W245F, W33Y/W245Y and W69Y/W245Y on PNAC with increasing ionic strength was not affect by ionic strength and the mutants remain active. On colloidal chitin, the effect of ionic strength on the enzymatic activity of 5 double point mutants with increasing ionic strength was similar to the activity profile of all single point mutants. This suggested that further mutation to reduce the interaction did not have

much effect on amorphous substrates. On the other hand, for β- crystalline chitin, the activity of all double point mutants was slightly higher than the activity profile of all single point mutants at high ionic strength. This would suggest that, although further reduction in the interaction did not effect the enzymatic activity on amorphous substrates, the reduce interaction of double point mutants can have further a positive effect on crystalline substrates. So, the double mutation may increase the processively hydrolyzed β- crystalline chitin at high ionic strength by allowing the enzyme to move along the substrate more efficiently. Since the enzyme-substrate interaction of the double mutant is lower than WT CHI60 and single mutant.

The degradation products analysis of WT CHI60 by Thin layer chromatography (TLC)

To further confirm the mode of hydrolysis of the WT and mutant enzymes, we studied the product size distribution when an amorphous substrate was used. On amorphous substrate the enzyme would be forced to hydrolyze the substrate via the endo-mode., which would give multiple size products. However, by increasing the ionic strength, we should be able to force the enzyme to shift to the exo-mode by reducing the dissociation of the enzyme from the substrate. This would also result in the shift in the size of the product from the multiple size oligosaccharide to a more uniform disaccharide product.

Furthermore we found that at 50°C, CHI60 used endo- mode more than at 37°C, and in the absent of NaCl in the reaction, CHI60 produced mixture of chitooligosaccharide (monomer, dimer, trimer and tetramer) at 50°C. At 37°C CHI60 produced almost exclusively dimer and minor monomer. The higher endo-mode of CHI60 at 50°C was probably due to the higher kinetics energy of the molecules at higher temperature, which resulted in the decrease in the enzyme-substrate binding. However, in the present 1 M NaCl in the reaction, CHI60 produced dimer as a major product and monomer as a minor product at both temperatures. These implied that at 50°C, the endo-mode of CHI60 was reduced while the exo-mode of CHI60 was enhanced by increasing in ionic strength, due to the higher enzyme-substrate interaction of CHI60 at high ionic strength. However, the result from this TCL experiment was inconclusive; therefore we used HPLC analysis for further quantitative of product.

Analyses of the degradation products of Chitinase by HPLC

Hydrolytic product of WT CHI60 and its mutants were examined using colloidal chitin as the substrate at different NaCl concentration, at 37°C. Colloidal chitin was hydrolyzed by WT CHI60 and all mutants (single and double point mutation) producing dimer as a major product and monomer and trimer as a minor product, in all NaCl concentration. Surprisingly, W69Y at this same hydrolytic condition produced almost exclusively dimer, see table 3.3.

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.

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.

The $(NAG)_2$ /$(NAG)_3$ ratio is interesting because it can give information concerning the substrate-binding modes and/or processivity. Therefore, processivity may be assessed by studying the $(NAG)_2$ /$(NAG)_3$ ratios in product mixtures. Degradation of chitin that resulted in a low $(NAG)_2$ /$(NAG)_3$ ratios indicate that the enzyme had a nonprocessive endo-mode, but high $(NAG)_2$ /$(NAG)_3$ ratios indicate that enzyme had a processive exo-mode.

These result showed that the $(NAG)_2$ /$(NAG)_3$ ratio of colloidal chitin degradation by WT CHI60 was higher with increasing ionic strength. The result

demonstrated that the exo-mode of CHI60 was enhanced and the substrate was processively hydrolyzed with increasing ionic strength.

In addition, the $(NAG)_2$ /$(NAG)_3$ ratios in Table 3.4 indicated that the single point mutants, W33F, W33Y, W69F, W69F, W245F, W245Y W275A and Y418A in the N-terminal domain and the catalytic domain had enhancing exo-mode when degrading colloidal chitin with increasing ionic strength. However, the reduced $(NAG)_2$ /$(NAG)_3$ ratio of W275F may be caused by the higher flexibility of W275F which resulted in the lost of processivity of this mutant. Moreover, the $(NAG)_2$ /$(NAG)_3$ ratios at 2 M NaCl of the 5 single point mutants (W33F, W69Y, W245Y, W275A and Y418A) was at least 4 fold higher than WT CHI60.

Interestingly, the $(NAG)_2$ /$(NAG)_3$ ratio of 5 double point mutants (W33F/W69Y, W33Y/W245F, W33F/W245F, W33Y/W245Y and W69Y/W245Y) showed $(NAG)_2$ /$(NAG)_3$ ratios higher than WT CHI60 with increasing ion strength. These result explained that the all double point mutants has higher processivity than WT CHI60. Furthermore, the $(NAG)_2$ /$(NAG)_3$ ratios at 2 M NaCl of the 4 double point mutants (W33F/W69Y, W33Y/W245F, W33F/W245F and W33Y/W245Y) was at least 5 fold higher than WT CHI60.

While these experiments clearly show that we can enhance the processivity by increasing ionic strength and the mutation of the Trp in the substrate binding site can further modulate the ratio of products.

Effect of temperature on the hydrolyzing activity of WT CHI60 and its mutants on various substrate

The ratio of the activity of the enzyme at 37°C versus 50°C could further help us understand the enzyme substrate binding and the change in the kinetics of the reaction. The difference in the activity ratio of 37°C/50°C of the mutants comparing to WT CHI60 would give us some answers on how the enzyme works at the optimum temperature. A reduction of in the activity ratio of 37°C/50°C would suggest an improvement in the enzymatic activity at higher temperature, which would suggest higher processivity and hydrolytic efficiency. On the other hand, an increase in the activity ratio of 37°C/50°C, would suggest that the enzyme might lost its processivity. BY using various substrates this activity ratio of 37°C/50°C could also shed us some

light in the mechanism and kinetics of the enzyme. The comparison of this ratio among the different mutant would further elucidate the role and importance of each of the Trp residue at each of the position on the enzyme.

The activity ratio of 37°C/50°C of the single and double point mutations on PNAC was lower than that of WT CHI60, except the activity ratio of 37°C/50°C of W245Y which was similar to WT CHI60.

Moreover, the activity ratio of 37°C/50°C of the single point mutants in catalytic domain (W275A, W275F and Y418A) on PNAC was lower than WT CHI60, except the activity ratio of 37°C/50°C of W275A was higher than WT CHI60. The mutation of W275F and Y418A may help to increase the kinetics of the enzymatic hydrolysis at higher temperatures. These mutants may result in a reduction of the enzyme product interaction, which may facilitate the dissociation of the product from the catalytic cleft.

On colloidal chitin, the result of the activity ratio of 37°C/50°C of the single point mutations showed that W245F had a unexpected high value. This would indicate that the mutation of Trp at position 245 to Phe greatly reduce the activity of the enzyme at higher temperature. This residue was reported to be the residue needed to guide the substrate into the catalytic cleft, therefore mutation of this residue to Phe may prevent the substrate from interring the catalytic cleft resulting in the reduction in the activity of the mutated enzyme.

Furthermore, the activity ratio of 37°C/50°C of the single mutants W275A, in the catalytic domain was slightly lower than WT CHI60. This may be a result from its higher kinetics and/or the reduction of the enzyme product interaction, which may facilitate the dissociation of the product from the catalytic cleft as we observe before when PNAC was used as substrate.

On β-chitin, the activity ratio of 37°C/50°C of single mutants which reduced hydrophobic interaction between substrate and enzyme can be categorized into 3 groups. Firstly, the activity ratio of 37°C/50°C of W33Y that was lower than that of WT CHI60. Secondly, the activity ratio of 37°C/50°C of W33F, W69F and W245Y that was higher than that of WT CHI60 which may due to the lost in processivity from weaker binding at higher temperature. Thirdly, the activity ratio of 37°C/50°C of W69Y was similar to WT CHI60.

The activity ratio of 37°C/50°C of the single mutants in the catalytic domain was higher than that of WT CHI60. These result suggested that these mutation lowered the kinetics of the enzyme at higher temperature, especially W275F.

CHAPTER V

CONCLUSION

In this study we pay more attention on CHI60 from *Serratia* sp. TU09. The two main objectives of this research are to study the role of the exposed Trp residues in the tryptophan track functions on binding and guiding the substrate into the catalytic clef and, secondly, to study the possible role of these residues on the mode of catalysis both endo- and exo- type activity mode by site directed mutagenesis. We successfully mutated the amino acids in Tryptophan track, 9 single point mutants (W33F, W33Y, W69F, W69Y, W245F, W245Y, W275A, W275F and Y418A) and 5 double point mutants (W33F/W69Y, W33Y/W245F, W33F/W245F, W33Y/W245Y and W69Y/W245Y).

To investigate an effect of an ionic strength on catalytic activity of CHI60 on soluble, amorphous and crystalline chitin, we varied an ionic strength of the CHI60 reaction in the range of 0 to 2 M NaCl. From this result we found that CHI60 capable of hydrolyzing by both of endo- and exo-mode, depending on type of substrate and ionic strength of the reaction which provide us a novel information to purpose the mode of action of CHI60 on various substrates.

From Trp mutation, we found that W33 in the N-terminal domain and W275 in the catalytic domain may influence in processivity of CHI60. When double point mutations were performed to reduce the interaction of the enzyme and crystalline substrate, they may increase the processively hydrolyzed □-crystalline chitin at high ionic strength. While we constructed the mutants, W33F, W69F and W245Y, the activity of these mutants on β-chitin significantly decreased at high temperature due to they lost their processivity. W245 play an important role in insoluble chitin hydrolysis.

From an amorphous chitin hydrolysis, we found that the endo-mode of CHI60 was reduced while the exo-mode of CHI60 was enhanced by increasing in ionic strength. This result suggested that the shift in the size of the product from the multiple size of oligosaccharides to a more uniform disaccharide product may modulate by changing in temperature and ionic strength of the reaction.

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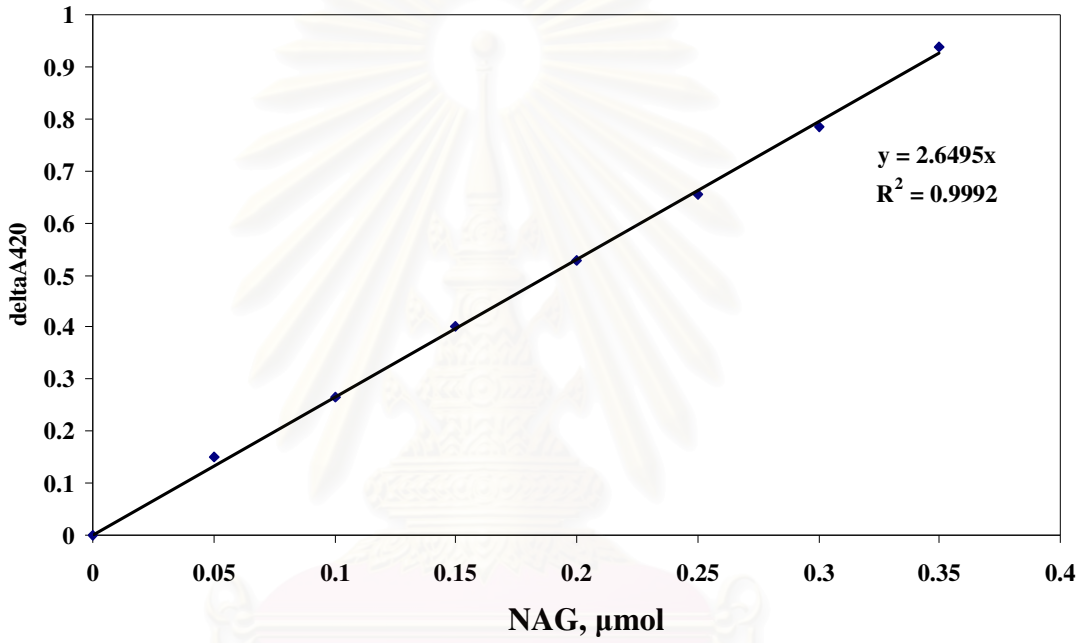
APPENDICES

APPENDIX A

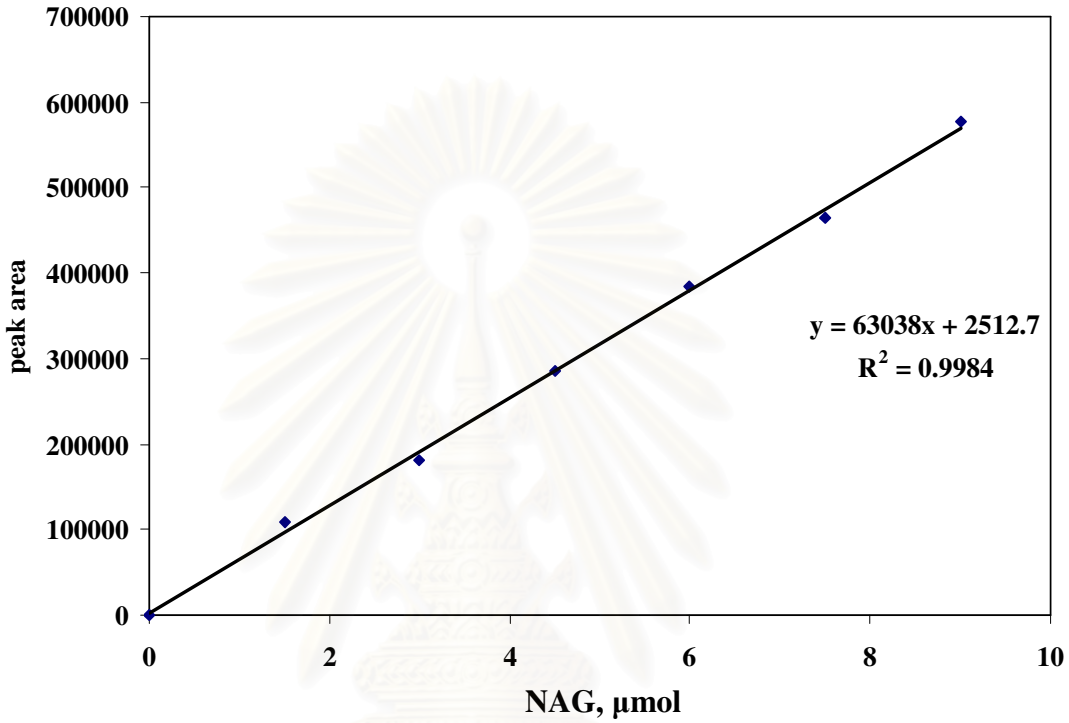
Standard curve for NAG determination by colorimetric method.

Standard curve for NAG was made by morintoring the absorbance at 420 nm of standard concentration NAG according to the Schale's method.



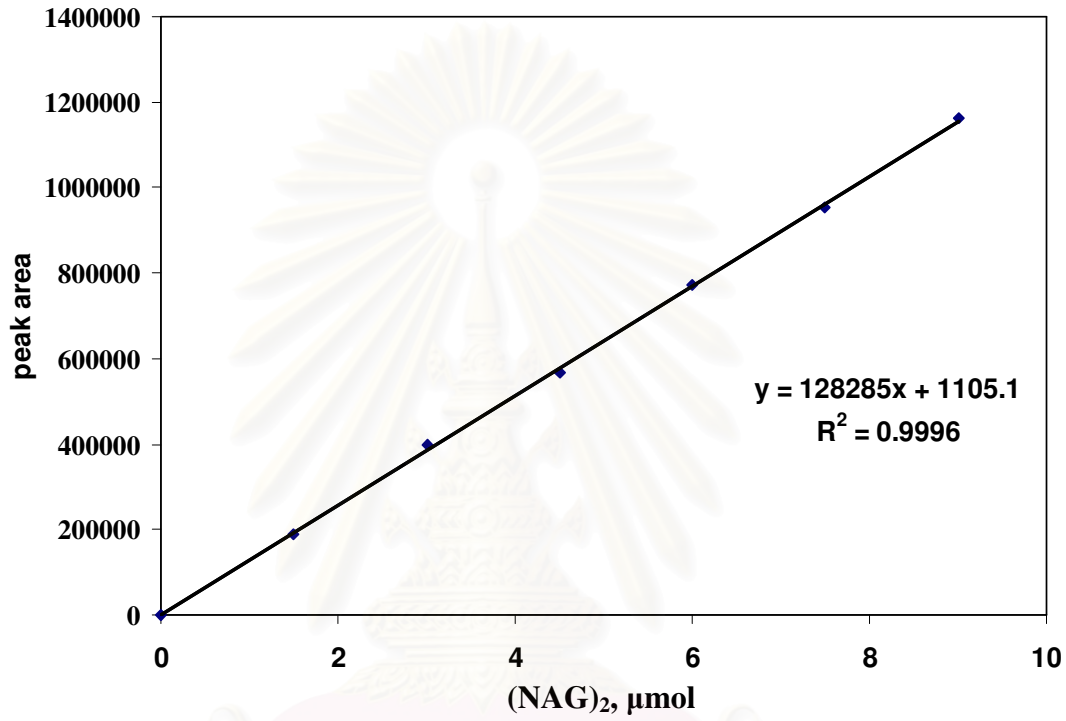
APPENDIX B

Standard curve for NAG determination by HPLC



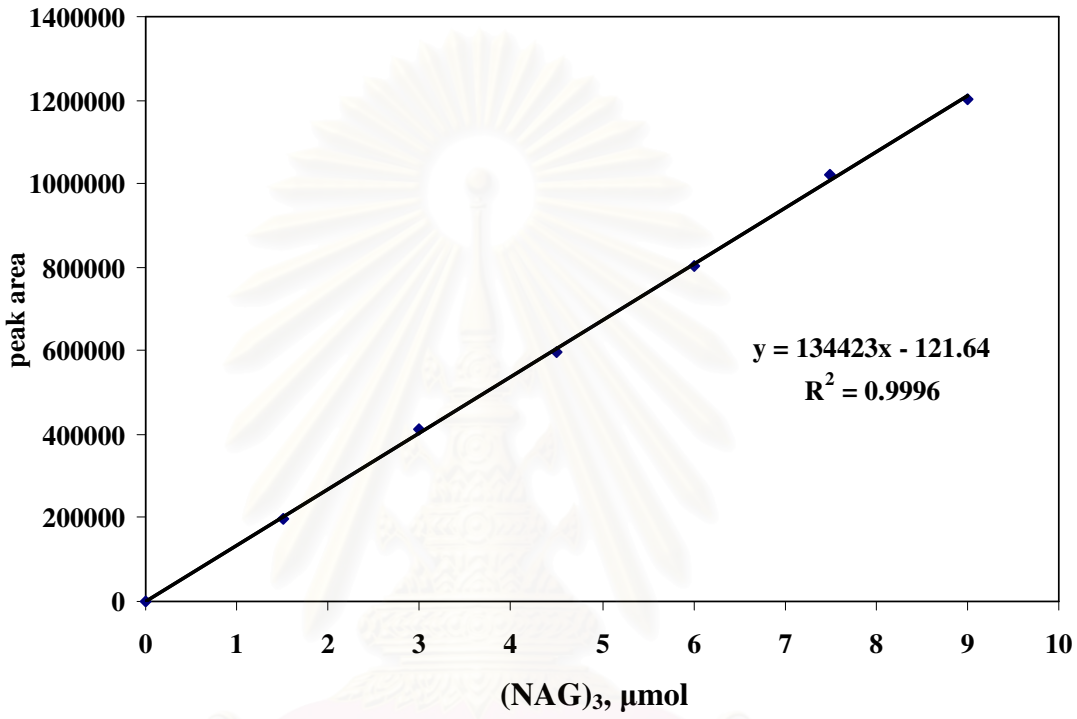
APPENDIX C

Standard curve for $(NAG)_2$ determination by HPLC



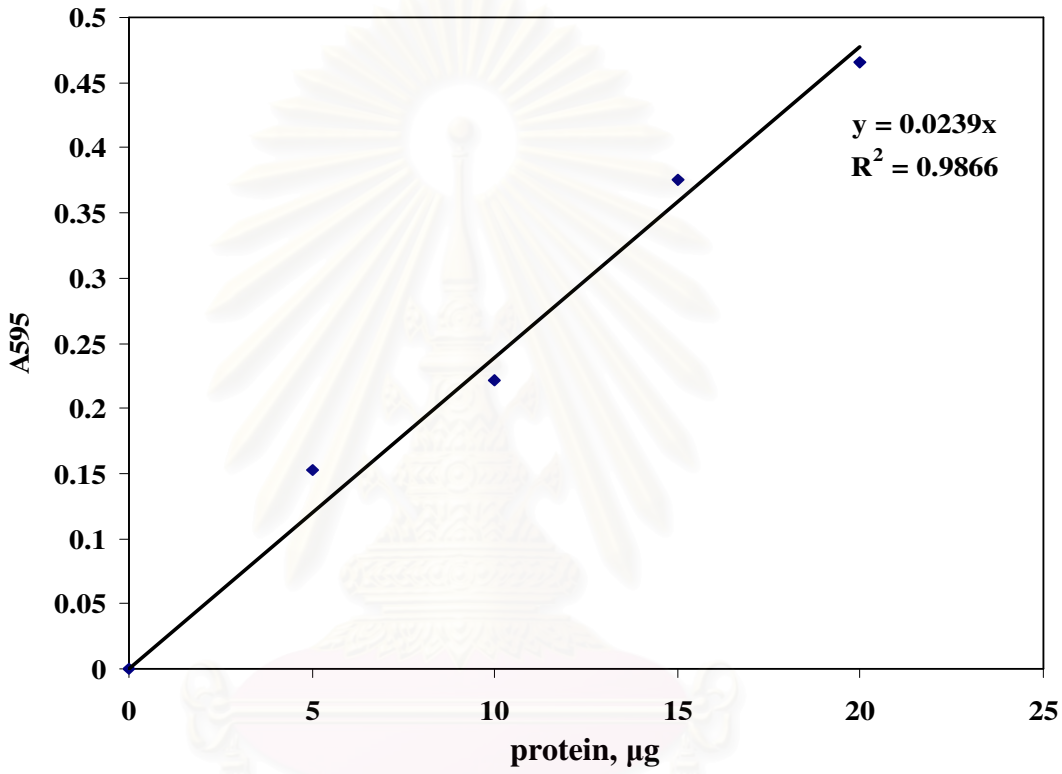
APPENDIX D

Standard curve for $(NAG)_3$ determination by HPLC



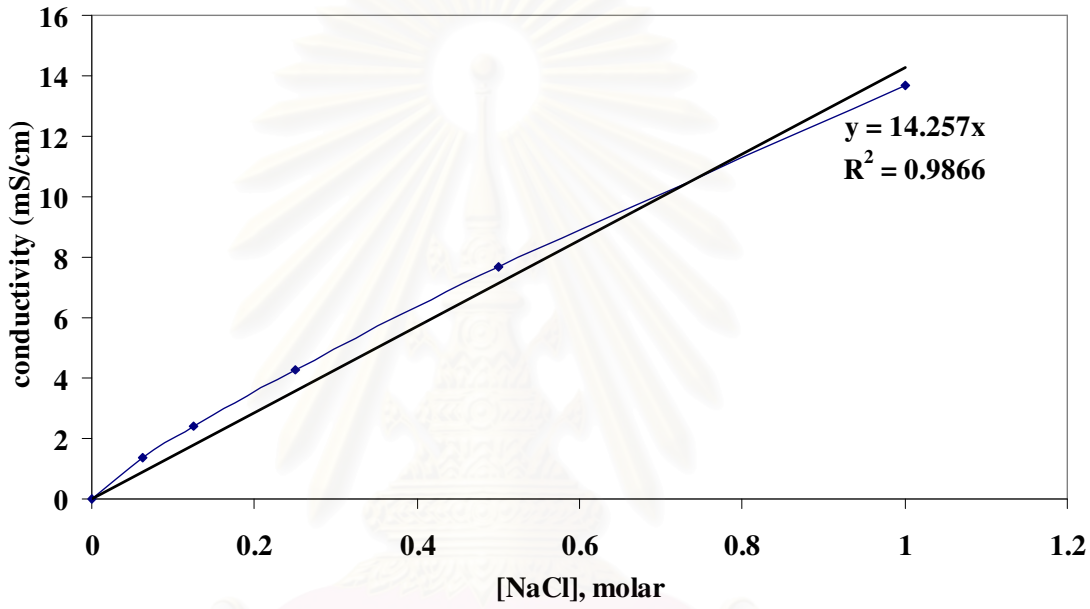
APPENDIX E

Standard curve for protein determination by Bradford's Method (Bradford, 1976) using bovine serum albumin as a standard.



APPENDIX F

Standard curve for conductivity of 0-1 M NaCl in 20mM Tris–HCl buffer pH 7.0



APPENDIX G

Nucleotide alignment of 16S rRNA gene from *Serratia* sp. TU09 and *Serratia lique faciens* (DQ123840) by CLUSTAL X (1.83).

Where * denotes the identical sequence.

```
16Scomplete     AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAGC
DQ123840        AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGC
                **************************************** ******************

16Scomplete     GGTAGCACGGGGGAGCTTGCTCCCTGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGG
DQ123840        GGTAGCACAGGAGAGCTTGCTCTCTGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGG
                ******** ** ********** ************************************

16Scomplete     GAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCG--CATAACGT
DQ123840        GAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCG--CATAACGT
                ************************************************  ********

16Scomplete     CGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATT
DQ123840        CTACGGACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATT
                *    ******** ****************** ****************************

16Scomplete     AGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATG
DQ123840        AGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATG
                ************************************************************

16Scomplete     ACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
DQ123840        ACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
                ************************************************************

16Scomplete     ATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGG
DQ123840        ATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGG
                ************************************************************

16Scomplete     TTGTAAAGCACTTTCAGCGAGGAGGAAGGTGGTGAACTTAATACGTT-CATCAATTGACG
DQ123840        TTGTAAAGCACTTTCAGCGAGGAGGAAGGGTTCAGTGTTAATAGCAC-TGTGCATTGACG
                ****************************       ******       *  ********

16Scomplete     TTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTG
DQ123840        TTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTG
                ************************************************************

16Scomplete     CAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATG
DQ123840        CAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATG
                ************************************************************

16Scomplete     TGAAATCCCCGGGCTCAACCTGGGAACTGCATGTTGAAACTGGCAAGCTAGAGTCTCGTA
DQ123840        TGAAATCCCCGCGCTTAACGTGGGAACTGCAT-TTGAAACTGGCAAGCTAGAGTCTTGTA
                ************ *** *** ************ ********************** ***

16Scomplete     GAGGGGGG-TAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGG
DQ123840        GAGGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGG
                ******** ************************************************

16Scomplete     TGGCGAAGGCGGCCCCCTGGA---CGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGC
DQ123840        TGGCGAAGGCGGCCCCCTGGA---CAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGC
                ********************   * ************************************

16Scomplete     AAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTGC
DQ123840        AAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGACTTGGAGGTTGTGC
                ********************************************* *************

16Scomplete     CCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGC
DQ123840        CCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGC-TGGGGAGTACGGCCGC
                ********************************** ********* ****************

16Scomplete     AAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAA
DQ123840        AAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAA
                ************************************************************

16Scomplete     TTCGATGCTACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTAGCAGAGATGC
DQ123840        TTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAATTCGCTAGAGATAG
                ******** ************************************ *    ******

16Scomplete     TTTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTG
DQ123840        CTTAGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTG
                 ** ************************************************************

16Scomplete     AAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCTTTTGTTGCCAGCG-GTTCG
DQ123840        AAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGCGTAAT
                ****************************************** ************* **

16Scomplete     GCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCCAA
DQ123840        GGCGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTC-AA
                * ********************** ********* ********************** **

16Scomplete     GTCATCCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGTATACAAAGAGA
DQ123840        GTCATC-ATGGCCCTTACGAGTAGGGCTACACACGTGCCACAATGGCGTATACAAAGAGA
                ****** ****************************** ********************

16Scomplete     AGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTACGTCGTAGTCCGGATTGGAGTCTG
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DQ123840          AGCGAACTCGCGAGAGCAAGCGGACCTCATAAAGTACGTCGTAGTCCGGATCGGAGTCTG
                  ***** ****************************************** ********
16Scomplete       CAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAAT
DQ123840          CAACTCGACTCCGTGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAAT
                  ************ **********************************************
16Scomplete       ACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGT
DQ123840          ACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGT
                  ************************************************************
16Scomplete       AGGTAGCTTAACCTTCGGGAGGGCGCTT-ACCACTTTGTGATTCATGACTGGGGTGAAGT
DQ123840          AGGTAGATTAACCTTCGGGAGGGCGCTT-ACCACTTTGTGATTCATGACTGGGGTGAAGT
                  ****** ******************** ******************************
16Scomplete       CGTAACAAGGTAACCGTAGGGGAACCTGCGGCTGGATCACCTCCTT
DQ123840          CGTAACAAGGTAACCGTAGGGGAACCTGCGGCT-------------
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BIOGRAPHY

Miss. Kamontip Kuttiyawong was born on April, 19th, 1976 in Mahasarakham, Thailand. She graduated with a Bachelor Degree in Chemistry, Faculty of Science, Silapakorn University in 1997 and a Master Degree in Biochemistry, Faculty of Science, Chulalongkorn University in 2001. She has enrolled in the Ph.D. Biochemistry Program since 2002.