CHAPTER I

INTRODUCTION

Chitin

Chitin, an insoluble linear β-(1→4)-linked polymer of *N*-acetyl-D-glucosamine (GlcNAc), is one of the most abundant polysaccharides in nature (Figure 1). It is the main component in the cuticles of crustaceans, insects, and, mollusks and in the cell wall of fungi (Muzzarelli, 1977). Chitin content is different in various organisms (Table 1). Chitin can be synthesized by some unicellular organisms such as diatom and protozoa starting from glucose (Figure 2).

Natural chitin has different structures depending on its source. By molecular conformation, chitin is classified into 3 groups: α-chitin, β-chitin, and γ-chitin (Figure 3). Alpha-chitin, a tightly packed antiparallel strand form, is presented in wide range of organisms such as crustaceans, insects, and fungi. The β-chitin, a parallel strand form, is a rare form that is found in 4 sources: the spines of polychaete *Aphodite*, the pen of the squid *Loligo*, the tubes of *Pogonophora*, and the spines of certain marine diatoms. The last form is γ-chitin, a mixed form of parallel and antiparallel, that has been reported from the stomach lining of *Loligo*.

Chitin, like cellulose, is insoluble in ordinary solvents. It has crystalline structure with some fiber-forming potential. Strong acid, fluoroalcohols, and certain hydrotropic salt solution can dissolve chitin, but can degrade chitin and inconvenient to use.

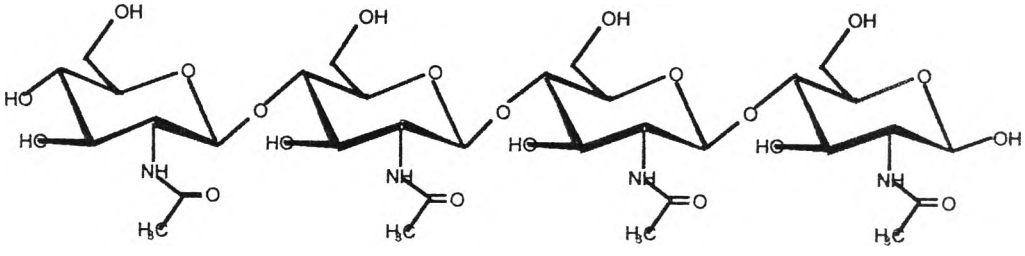


Figure 1. Chitin is a β-(1→4) linked homopolymer of *N*-acetyl-D-glucosamine.

Table 1. Chitin content (%) in different organisms (Muzzarelli, 1977).

Sources of chitin	(%) chitin	conformation of chitin
Crustacea		
Crangon (shrimp)	69.1	α-chitin
Alaskan (shrimp)	28.0	
Insect		
May beetle	16.0	
Pieris (sulfur butterfly)	64.0	
Colcoptera (beetle)	27-35	α-chitin
Diptera (true fly)	54.8	
Bombyx (silk worm)	44.2	
Calleria (wax worm)	33.7	
Molluscan organ		
Squid, skeletalpen	41	
Oyster shell	3.6	β-chitin
Clamshell	6.1	
Fungi		
Aspergillus niger	42.0	
Aspergillus phoenicis	23.7	
Pennicilium notatum	18.5	
Histoplasma capsulatum	25.8	α-chitin
Histroplasma farciminosum	40.0	
Mucor rouxii	44.5	
Mortierella vinacea	22.0	

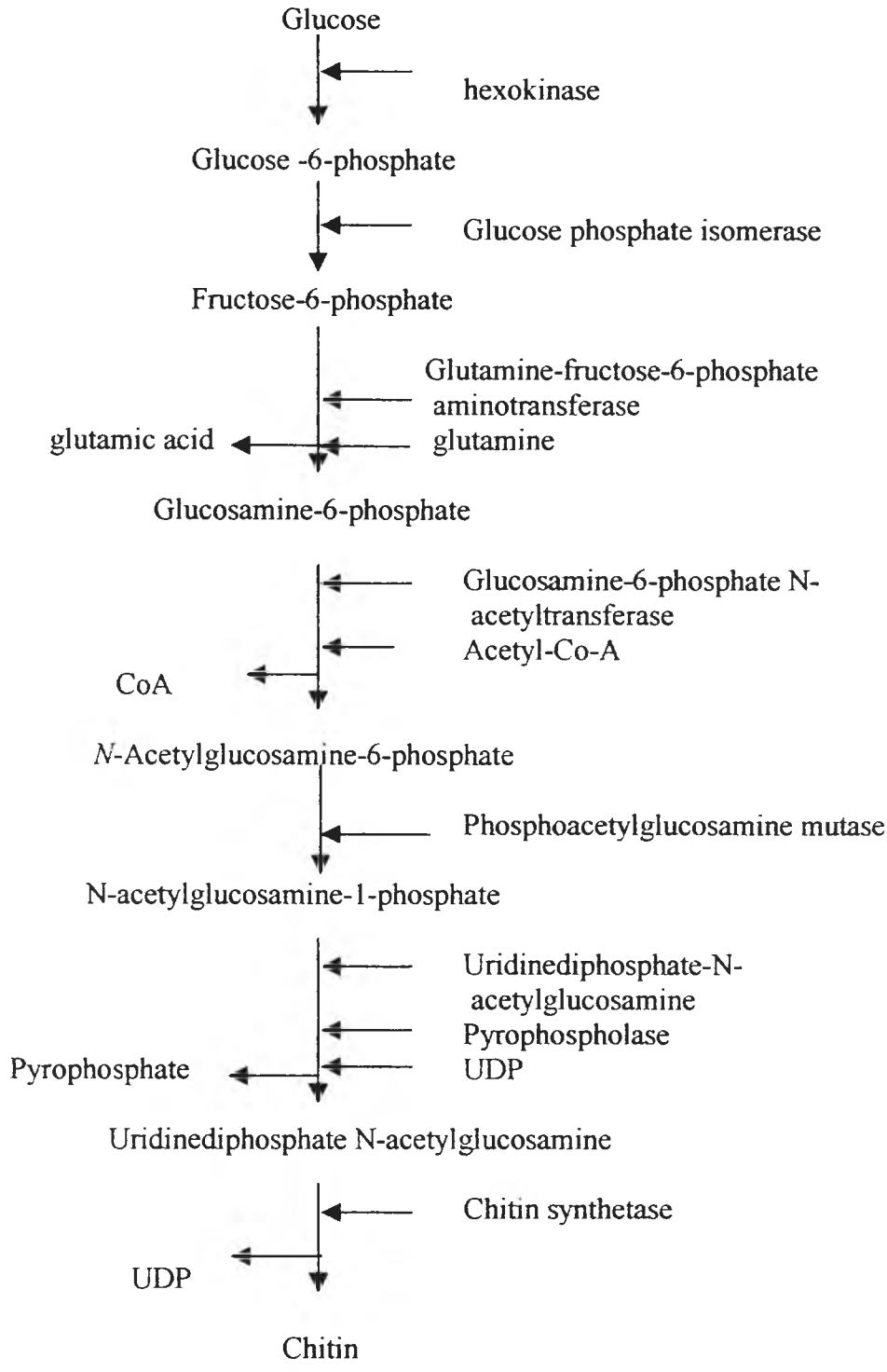
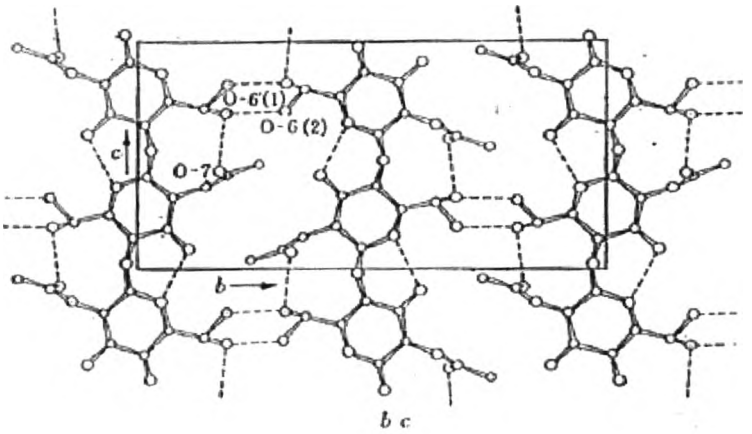
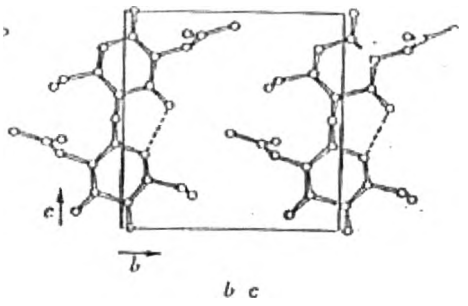


Figure 2. Chitin synthesis pathway from unicellular organisms (Muzzarelli, 1977).



α -chitin (antiparallel)



β -chitin (parallel)

Figure 3. Crystal structure of α-Chitin and β-Chitin (Muzzarelli, 1977).

Commercial chitin is now produced from crab and shrimp shells by treatment with dilute NaOH and HCl solution (Figure 4). Chitosan, a deacetylate form of chitin, is produced by deacetylating chitin with concentrated NaOH at elevated temperature.

Chitosan

Chitosan, a deacetylate form of chitin, is a copolymer of β-(1→4)-2-acetamido-D-glucose and β (1→4)-2-amino-D-glucose, deacetylation of chitin (Figure 5). Up to now, only a few studies on molecular conformation have been reported, so there are still no simple model describing the molecular conformation of chitosan. Chitosan is different from chitin at the second carbon of glucose molecule, there is a acetamide group on chitin chain and amino group on chitosan chain. Because of this, chitosan has different characteristic from chitin such as molecular weight and solubility. Solid chitin is insoluble in weak acid, while chitosan dissolves in weak acid. Acetic and formic acid are two of the most widely used weak acid for dissolving chitosan.

Commercial chitosan is produced from seashell materials by deacetylating chitin with concentrated NaOH at elevated temperature. The quality and properties of chitosan products varies widely depending on the manufacturing process.

Chitin and Chitosan Application

Chitin/chitosan have some characteristic that can be applied to industry.

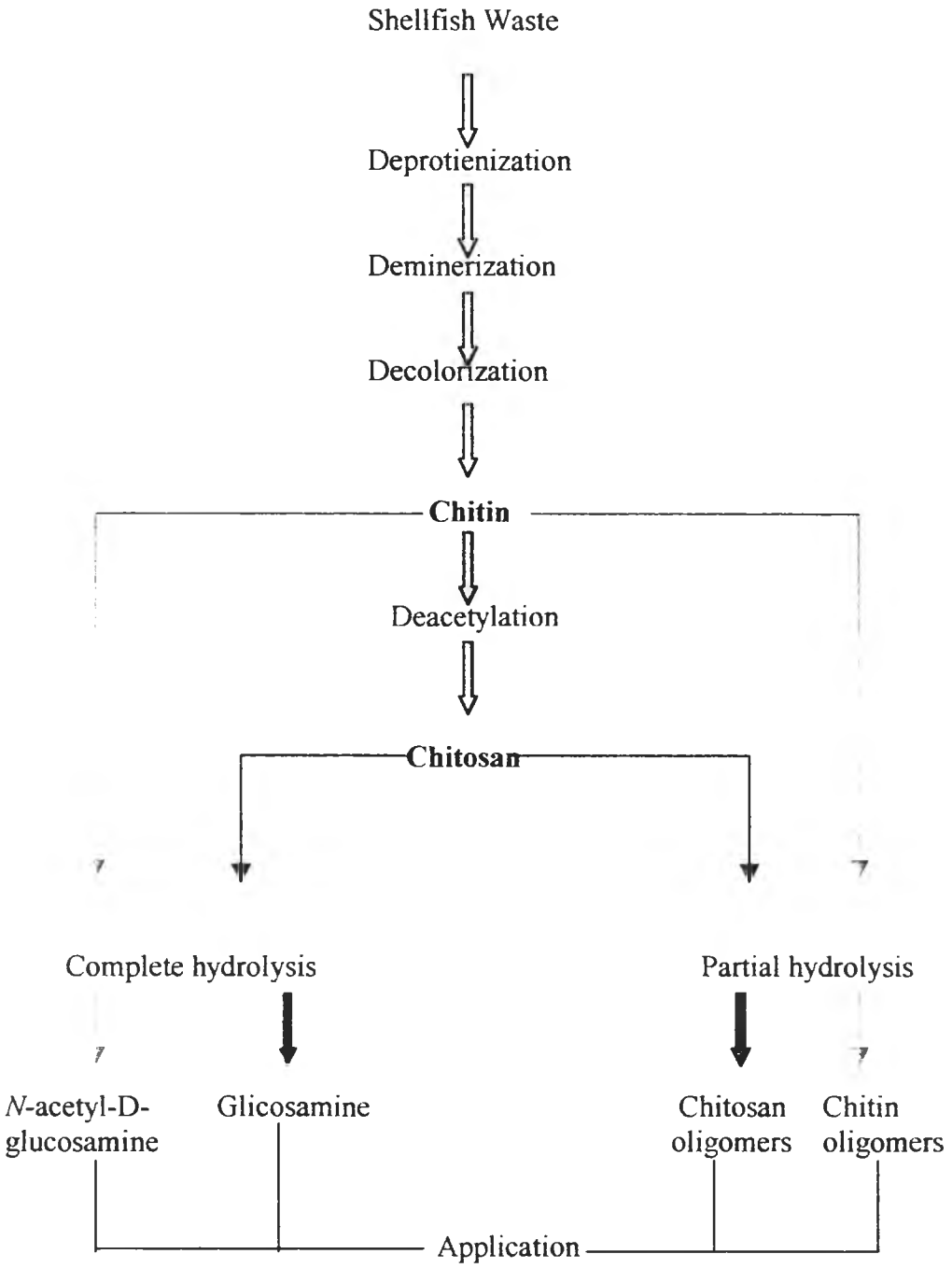


Figure 4. Simplified flow chat of preparation of chitin, chitosan, and their oligomers from shellfish waste (Shahidi *et al.*, 1999).

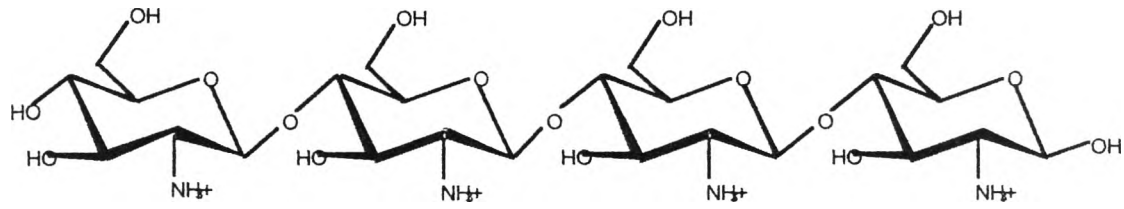


Figure 5. Chitosan structure.

These characteristics are:

(a) Biologically renewable natural resources

(b) Almost non toxic

(c) Changeable in molecular weight and conformation

(d) Able to manufacture into gels, beads, fibers, colloids, and films.

Chitin chitosan and their derivatives are used in various fields such as biotechnology, cosmetics, agricultural, food, medicine and environment (Table 2).

Chitinase

Chitinase (EC 3.2.1.14) is the key enzyme that catalyses the hydrolysis of chitin. This enzyme is found in a great variety of organisms including bacteria, yeast, fungi, plants, insects, crustaceans, and some vertebrates (Watanabe, 1992).

For the chitin-containing organisms such as crustaceans, insects, yeast, and fungi, chitinase is important for their growth. In crustaceans and insects, chitinase acts by degrading the exoskeleton chitin in cuticle or shell for ecdysis. Yeast chitinase is required all separation during growth and fungal chitinase is important for apical growth and branching of hyphae (Watanabe, 1995).

For the organisms those do not contain chitin as their structural component such as bacteria, plants and animals, chitinase seem to play important roles in defense and metabolizing of chitinase materials. Bacteria produce chitinase to digest chitin

Table 2. Current practical uses of chitin, chitosan, and their derivatives.

Area of Application	Example
Agriculture	-Plant seed coating -Fertilizer
Biotechnology	- Enzyme immobilization - Chromatrography
Cosmetic	- Moisturizer
Food Industry	- Color stabilization - Emulsify agent - Dietary fiber
Medicine	- Dressing material for burns and skin lesions of human and animals
Paper	- Surface treatment - Photographic Paper
Wastewater Treatmant	- Recovery metal of ions, pesticides and phenols - Removal of dye

and utilize it primarily as carbon and energy source (Watanabe, 1993). In plants, chitinase is produced and accumulated in response to microbial infections and is thought to be involved in the defense of plant against infections (Inbar, 1991). Fish and mammals also use chitinase for defense.

Chitinase can be devided into 2 groups, exochitinase and endochitinase following by substrate cleaving. Exochitinase digests substrate by non-specific cleaving, while endochitinase digests substrate from non-reducing end to reducing end.

Chitinase is one of the enzymes involved in the breakdown of sugar-based polymers. These enzymes have been cataloged, based on amino acid sequences, into nearly 50 families of glycohydrolases (Henrissat, 1993). Chitinase are found primarily in two families of glycohydrolases, family18 and 19.

Family 18 chitinase is the largest chitinase family, which is found in eukaryotes and prokaryotes. It contains several runs of conserved amino acids (Figure 6). The three dimensional structures of several members of this family have been solved, including plant chitinase from Para-rubber tree, hevamine (Figure 7) and bacterial chitinase from *Serratia marcescens* (Figure 8). The core of this enzyme is an eight-stranded α/β barrel. That is eight strands of parallel of β-sheet laid down with an alpha helix as return stroke. The eight strands of sheet bend into barrel structure with the helices forming a ring toward the outside (Robetus and Monzingo, 1999).

	130	170
Chi - ci	LSIGGWTY SPNF	FDGI DIDWEYPED
Chi - th	LSIGGWTWSTNF	FDGI DIDWEYPAD
Chi - aa	LSIGGWTWSTNF	FDGI DIDWEYPAD
Chi - sm	PSIGGWTL SDPF	FDGVDIDWEFPGG

Figure 6. Conserved amino acid of chitinase family 18: *Coccidiodes immitis* (ci), *Trichoderma harzianum* (th), *Aphanocladium album* (aa) and *Serratia marcescens* (sm) (Robert and Monzingo, 1999).

Figure 7. Crystal structure of chitinase from Para-rubber tree, (PDB code 1HVN).

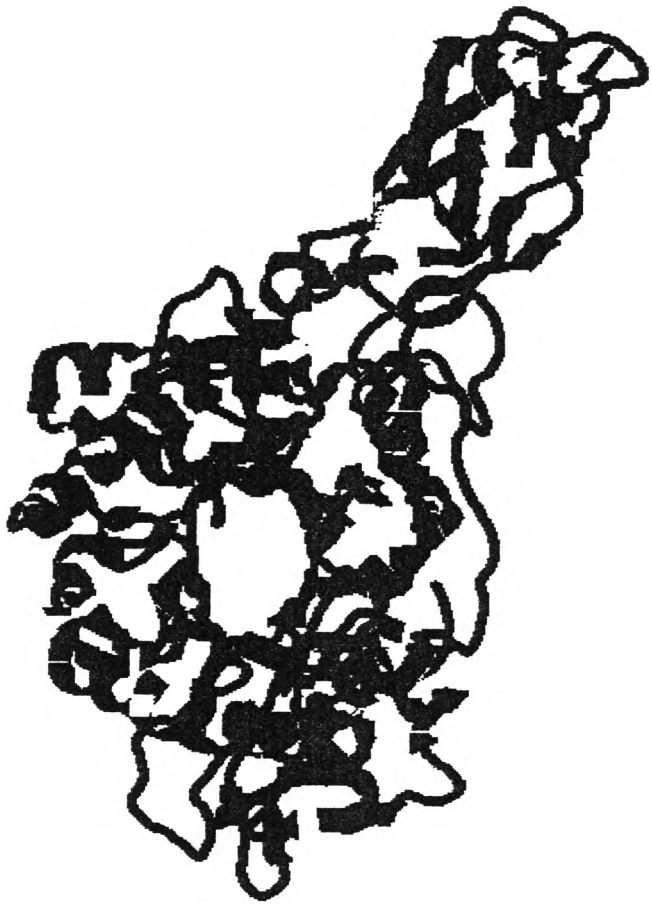


Figure 8. Crystal structure of chitinase from *Serratia marcescens* (PDB code 1EHN).

Family 19 chitinase is commonly found in plants. The section of conserved sequence for the family 19 chitinase, shown in Figure 9 is represented by barley, potato and pea chitinase. One sample of this family for which the x-ray structure has been solved is *Hordeum valgare* (barley). This structure has a 1.8 A resolution. It has 10 α-helical segments, and one three-stranded β-sheet. An analysis of the protein shows that many hydrophobic residues conserved in the family 19 form the core of the protein. In a similar fashion, those polar residues conserved within the family tend to line the large cleft in the enzyme, which is presumed to be the substrate binding and catalytic site. The nature of these conserved residues justified the notion that barley chitinase is a reasonable representative for other enzymes of family 19 where nonpolar residues in the core control folding and those residues responsible for enzyme activity are conserved in the active site.

Bacterial Chitinase

Bacterial chitinase has been reported in many species of bacteria such as *Aeromonas* sp. (Ueda, M., *et al.*, 1995), *Bacillus circulan* WL-12 (Watanabe, *et al.*, 1990), *Bacillus thruringiensis* (Tantimavanich, *et al.*, 1997), and *Serratia marcescens* BJ200 (Brurberg, *et al.*, 1982).

Chitinase encoding genes have been cloned from a wide range of bacteria species (Table 3). Chitinase activities expressed in recombinants clones were localized in both the periplasmic fraction and extracellular fraction. There are few studies reporting chitinase activities from periplasmic fraction. Chitinase from *Acinetobacter* sp. WC17 when expressed in *E.coli.* is localized in the periplasmic

Chi-hv KREVAAFLAQTSHETTGGWATAPDGAFAWGYCFKQERGASSDYCTPSAQWPCAPGK

Chi-st KREI AAFFAQTSHETTGGWASAPDGAFAWGYCFLR ERGASSDYCPPSAQWPCAPGK

Chi-at KKEVAAFFGQTSHETTGGWATAPDGAFAWGYCFKQEQGASSDYCEPSAQWPCAPGK

Chi-ps KREI AAFLGQTSHETTGGWPTAPDGPYAWGYCFLR EQGASSDYCQPSAQWPCAPGK

Figure 9. Conserve amino acid of family 19 chitinase.

Hordeum valgare (hv) (Barley)

Solanum tubersorum (st) (Potato)

Arabidopsis thaliana (at) (Pea)

Pisum sativum (ps) (Pea)

Table 3 Molecular cloning of chitinase genes

Species	Host	plasmid	DNA insert sizes	References
Aeromonas sp. No. IOS-24	*E'. coli*	pUC18	8.0	Shiro, *et al.*, 1996
Aeromonas eaviae	*E'.coli*	pBJuescript 11 SK	4.5	Sitrit, *et al.*, 1995
Alleromonas sp strain 0-7	*E-coli*	pUC18	5.0	Tsujibo, *et al.*, 1993
Bacillus circulans WL-12				
Chitinase Al	*E. coli*	pKK233-3	4.0	Watanabe, *et al.*, 1990
Chitinase C	*E'. coli*	PUC19	2.8	Watanabe, *et al.*, 1995
Clostridium parapuirifictim				
Chitinase B	*E. coli*	pmwI 19	5.1	Morimoto, *et al.*, 1997
Streptomyces thermoviolaceus OPC-520				
Chi 30	*E. coli*	pUW1-219	3.4	Tsujibo, *et al.*, 2000
Serratia marcescens BU 2000				
Chitinase B	*E.coli*	pBR325	9.2	Brurberg, *et al.*, 1995
Streptomyces griseus HUT 603 7				
Chitinase Cl	*E. coli*	PUC 119	1.7	Ohno, *et al.*, 1996

space where it was secreted in the original strain (Hong *et al.*, 1995). Most chitinase are exported, but some remain in the periplasmic space.

The gene encoding extracellular chitinase from *Aeromonas* sp. strain O-7 was cloned to *E.coli*. The two recombinant chitinase was also not secreted into growth medium but accumulated in the periplasmic space (Tsujibo *et al.*, 1993). These reports suggest that appropriate signal peptide must be used for optimal secretion of chitinase from *E. coli*.

Crystalline Chitin Degrading Chitinase

A few studies of crystalline chitin degrading chitinase have been reported. Crystalline chitin degrading chitinase was isolated and characterized from marine bacterium *Aeromonas hydrophila* H-2330 by using flake chitin as a substrate. This strain secreted five chitinases and β-*N*-acetyl-D-glucosaminidase. The main chitinase, ChiA, was purified and characterized. The optimum pH of ChiA was 5-8 and the activity was inhibited by Hg^{2+} and Fe^{3+}. Molecular weight of ChiA was approximately 62 kDa (Hiraka, *et al.*, 1997)

Aromatic residues in catalytic and on the surface of enzyme were also shown the importance for crystalline chitin hydrolysis. There was a study of the importance of four exposed aromatic residues in crystalline chitin hydrolysis by Chitinase A from *Serratia marcescenes* 2170. They were Trp-69 and Trp-33 in N-terminal domain and Trp-245 and Phe-232 in catalytic domain, which are linearly aligned with the deep catalytic cleft. To investigate the importance of these

residues in the binding activity and hydrolyzing activity against insoluble chitin, site-directed mutagenesis to alanine was carried out. The substitution of Trp-69, Trp-33 or Trp-245 significantly reduced the binding activity to both highly crystalline chitin and colloidal chitin while Phe-232 did not affect the binding activity. In contrast, the substitution of any one of the four residues significantly reduced the hydrolyzing activity against β-chitin microfibrils. None of the mutation reduced the hydrolyzing activity against soluble substrate. These results predict that four exposed aromatic residues on the surface of the N-terminal molecule are essential determinants for crystalline chitin hydrolysis (Uchiyama, *et al.*, 2001).

Chitinase Mechanism

Chitinase acts by hydrolytically cleaving the β-glycosidic linkages between GlcNAc residues. In generally, the hydrolysis can occur in one of two ways either with a retention of anomeric configuration of the product or with an inversion. Family 19 chitinase hydrolyzes chitin giving an inversion from product, while family 18 chitinase giving a retention form.

Barley chitinase, one of member of family 19 chitinase has an inversion mechanism. It proceeds through a positively charged oxacarbonium intermediate, which has a distort geometry (Figure 10). The catalysis of barley chitinase involved two acidic residues, Glu 89 acting as a base to polarize the attacking water molecule, whereas Glu 67 act as an acid to protonate O_4 of leaving sugar. This mechanism is thought to be a double displacement type. First, there is bond breaking between the sugars involving protonation of the leaving group alcohol and

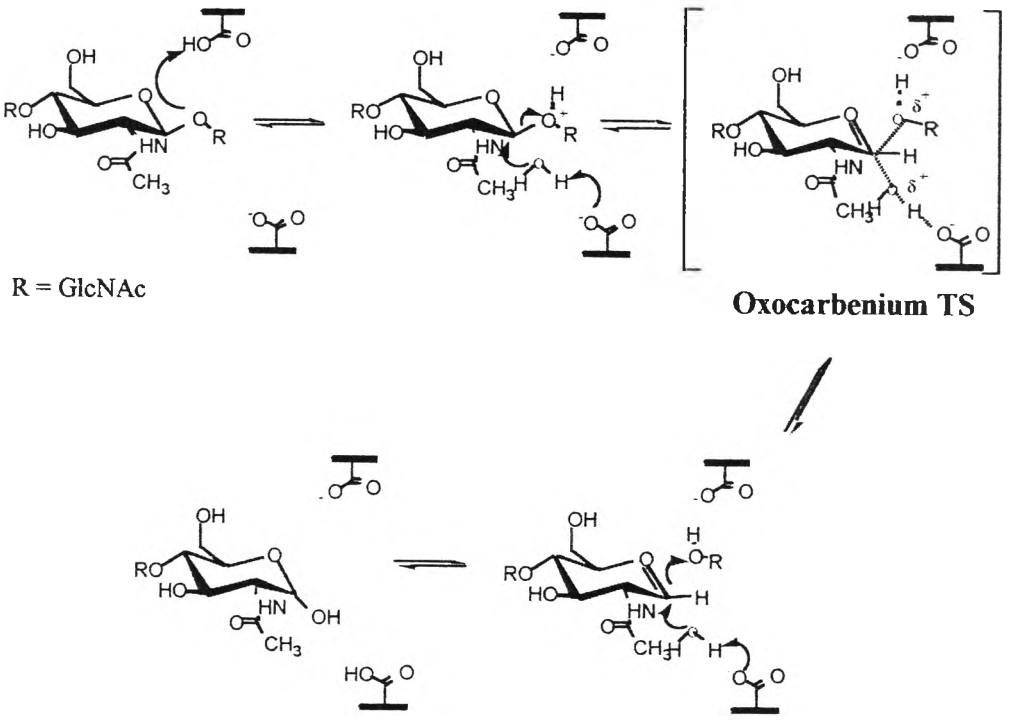


Figure 10. Inversion mechanism of family 19 chitinase (Hart *et al.*, 1995).

stabilization of the positively charged intermediate by Glu 89. This intermediate is then attacked by water molecule, which replaces the leaving sugar group (Robertus and Manzingo, 1999). The substrate-binding cleft of barley chitinase is an extensive one, and it has been hypothesized to contain at least six-sugar ring. Normally, it prefers to bind and cleave this hexasaccharides into two trisaccharides at the position between third and fourth sugar residues (Figure 11) (Robertus and Manzingo, 1999).

Family 18 chitinase has not been studied as extensively as those have from family 19. It appears that this family of enzymes operates by retaining mechanism. X-ray crystal structure of two family 18 chitinases reveals no second acidic residue in active site capable of stabilizing the oxocarbonium ion in the active site of the enzyme. Thus, neither the single nor double displacement mechanism is consistent with the observed structure and hydrolysis products. An increasing body of experimental and theoretical evidence points to an oxalozine ion intermediate formed to anchimeric assistance by the neighboring N-acetyl group (Figure 12) as being the likely mechanism for family 18 chitinase (Robertus and Manzingo, 1999).

Chitosanase

Chitosanase (EC 3.2.1.132) is an enzyme that catalyzes the hydrolysis of glycosidic bonds in chitosan. Most chitosanases are found in microorganisms and a few are found in plants.

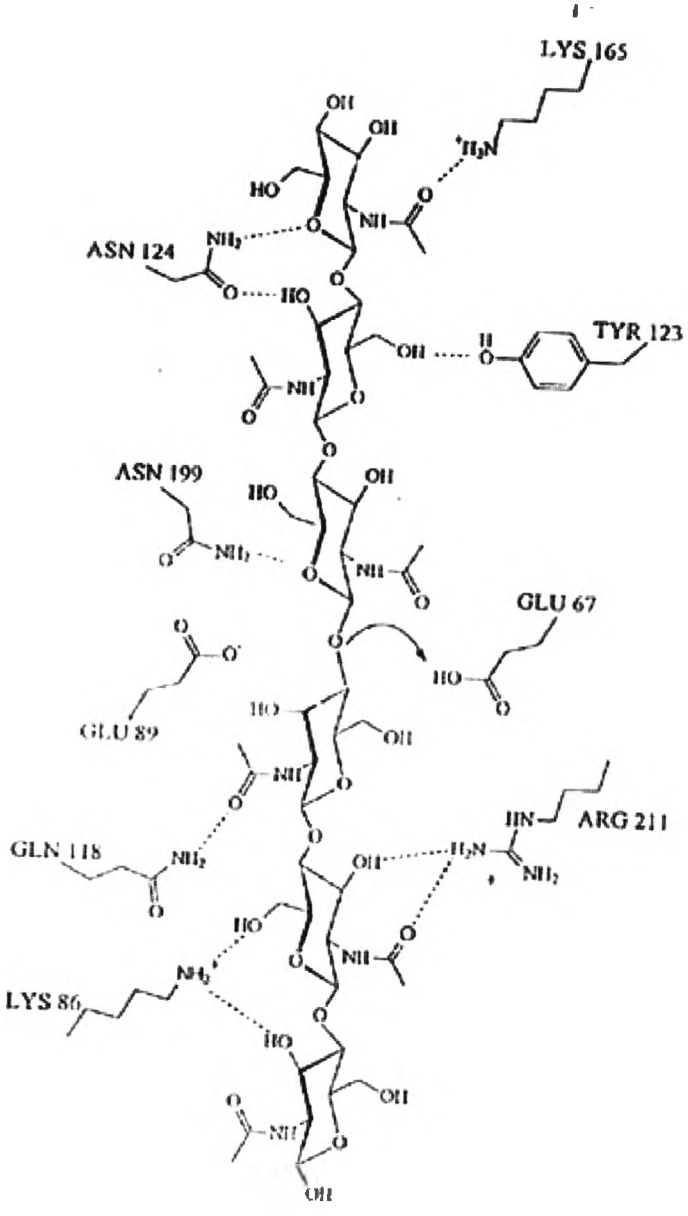


Figure 11. Hypothetical binding of chitin hexamer to barley chitinase (Robertus and Manzingo , 1999).

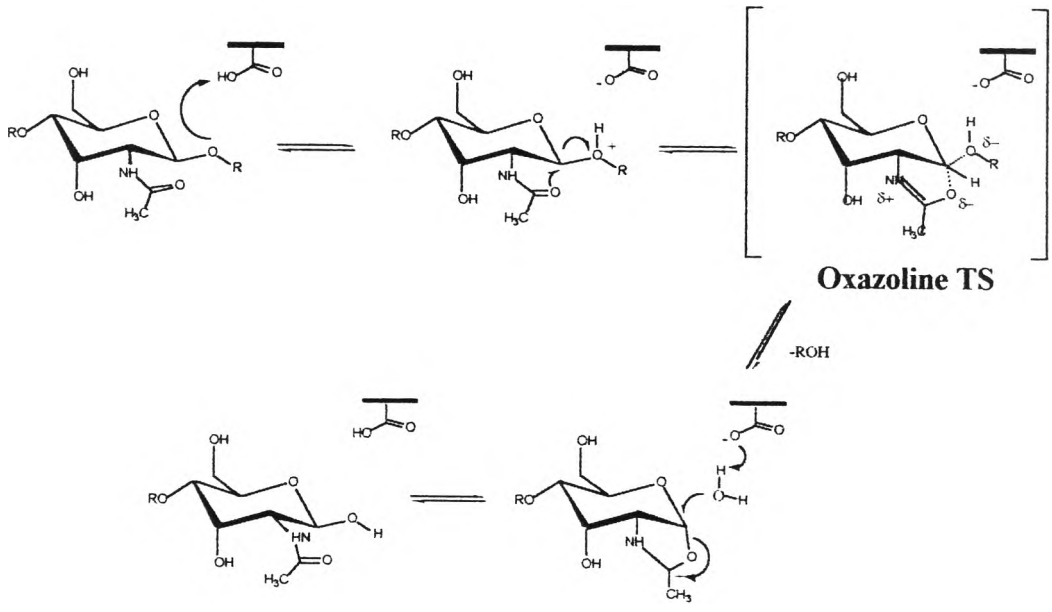


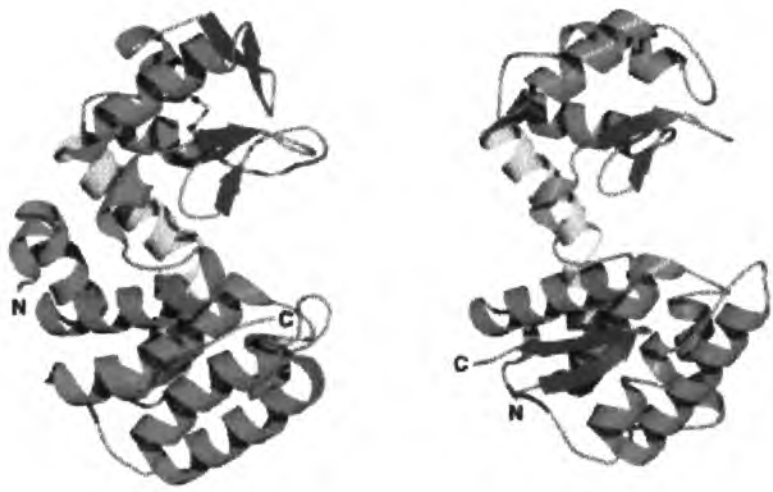
Figure 12. Retaining mechanism of family 18 chitinase (Hart *et al.*, 1995).

Microbial chitosanases are classified in 3 subclasses based on the specificity of the cleavage positions for partially deacetylated chitosan (Saito *et al*, 1999). Subclass I chitosanases hydrolyse glycosidic linkages between GlcN-GlcN and GlcNAc-GlcN (Fukamoto *et al.*, 1992). One of the members of this subclass is chitosanase from *Streptomyces* sp. N174 (Ohtakara *et al.*, 1992). Subclass II chitosanases such as *Bacillus* sp No. 7-M chitosanase can cleave only GlcN-GlcN linkages where as *Bacillus circulans* MH-K1 chitosanase in subclass III can cleave both of GlcN-GlcN and GlcN-GlcNAc linkages (Watanabe *et al.*, 1996).

Some microorganisms can produce both chitosanse and chitinase, such as Chlorella virus PBCV-1 (Ettenjl *et al.*, 1999), *Clostridia* sp. (Kopecny and Hodrova, 2000) and *Bacillus* sp. X-b (Helisto *et al.*, 2001). Bacillus sp. X-b secreted a complex of hydrolytic enzymes composed of chitinase, chitosanase, and protease. After partially purified, chitinase showed two major bands with molecular masses of 46,000 and 35,000 Dalton on

Two strains of *Clostridia* isolated from the rumen fluid of sheep with a potential antagonist toward anaerobic fungi produced chitinolytic enzymes including two chitinase, chitosanase, N-acetylglucosaminidase, and chitin deacetylase. These enzymes are found mainly in the extracellular fraction.

Few studies on three-dimention structures of chitosanases have been reported. The crystal structure of chitosanases from *Streotomyces* sp. N174 (N174 chitosanase) and *Bacillus circulans* MH-K1 (MH-K1 chitosanase) has been solved (Figure 13). The overall folding of MH-K1 chitosanase is similar to *Streptomyces*



A B

Figure 13. Crystal structure of chitosanase from *Streptomyces* sp. N-174 and *Bacillus circulans* MH-K1 (Saito, *et al.*, 1999). A is *Streptomyces* sp. MH-K1 chitosanase and B is *Bacillus circulans* N174 chitosanase. The backbone helices are shown in the yellow. The protuding roofs of the cleft are shown in pink.

sp. N174 chitosanase except three regions that are different. First, in the N-terminal region, MH-K1 chitosanase has two additional helices, which are helix 1 and helix 2 that were 16 residues longer than N174 chitosanases. Second, in the top region of the upper domain, MH-K1 chitosanase has two strands (β4 and β5) following to α6 helix whereas N174 chitosanase has only 5 helix. Third, the secondary structures were completely different at C-terminal region of both chitosanses, a helical structures α14 in MH-K1 chitosanase and two β-sheets in N174 chitosanase (Saito, *et al.*, 1999).

Chitosanase Mechanism

Chitosanase act by hydrolytically cleaving the glycosidic linkages in chitosan chain. The interaction between the cleft of the enzyme and the substrate, on hexamer chitosan sugar, was specified only at three subsites between sugar residues 3, 4, and 5.

The substrate was cleaved between sugar residue 4 and 5 sugar by two catalytic residues. Chitosanases are glycosyl hydrolases, which were classified into two by the groups according to mechanisms, retaining and inverting of the anomeric carbon products (Saito, *et.al*, 1999).

N174 chitosanase from *Streptomyces* sp., one of the member of subclass I chitosanase, is an inverting enzyme. The two catalytic residues are Glu22 and Asp40. Glu22 was found to act as a proton donor, incorporation with Asp40, there

by activating the attacking of water molecule to the anomeric carbon of glucoamine residue in substrate.

MH-K1 chitosanase from *Bacillus circulans*, the member of subclass III chitosanase, also has an inversion mechanism. Its catalytic residues are Glu37 and Asp55. Glu37 fixed on long central helix may act as a general base to polarize the attacking water molecule.

Chitin Deacetylase

Chitin deacetylase (E.C.3.5.1.41) is the enzyme that catalyzes the conversion of chitin to chitosan by deacetylation of N-acetyl-D-glucosamine residues. The presence of this enzyme activity has been reported in several fungi, insects and bacteria.

In Zygomycetes, a group of fungi accumulate chitin in cell wall and use chitin deacetylase for removing N-acetyl group from nascent chitin for the biosynthesis of chitosan. Deuteromycetes, which most members are plant pathogen fungi, also produce and secret chitin deacetylase during plant infection. In bacteria, chitin deacetylase has been reported in *Clostridium* sp., which was isolated from the rumen fluid of sheep.

Chitin deacetylases were purified and characterized from many microorganisms, such as *Mucor rouxii* (Tsigos *et al.*, 1999), *Aspergillus nidulans*

(Alfaonso *et al.*, 1995), *Collectrichum lindemuthianum* (Tsigos and Bouriotis, 1995) and *Vibrio alginolyticus* H-8 (Miwa, *et al.*, 1997).

Vibrio alginolyticus H-8 isolated from mud of Hamana Lake can produce both of chitinase and deacetylases extracellularly. From the enzyme production, deacetylases secretions started at the log phase and became maximum at early stationary phase. Then it decreased gradually and disappeared at 36 hours while chitinase was stable throughout the cultivation period. These deacetylases were different from other chitin deacetylases, because they had substrate specificity with only two unit of GluNAc while general chitin deacetylase had a broad substrate specificity for N-acetyl-chitooligosaccharides.

Because of the important role of chitin deacetylase converting chitin to chitosan, this enzyme is now of great interest. Normally, chitosan is produced from chitin in a harsh thermochemical procedure, which posed environmental problems. Moreover, it leads to broad and heterogeneous ranges of products. The uses of chitin deacetylase for preparation of chitosan polymer and oligomer could be improved and overcome most of these drawbacks.

Mode of Action of Chitin Deacetylase

Mode of action of chitin deacetylase has been studies on both chitin and chitosan oligomer. Chitin deacetylase will bind with substrate polymer to form an active enzyme-polymer complex and catalyses in three pathways (Tsigos, *et al.*, 2000).

1. Single chain

Chitin deacetylase will bind with substrate chain and catalyses the reaction toward the end of the chain. It does not bind with another substrate until it reaches the end of the first chain.

2. Multiple chain

The enzyme bind with the substrate to form an active complex and catalyses the hydrolysis of one acetyl group before it dissociates and forms a new active complex with another polymer chain

3. Multiple attack

The enzyme forms the enzyme-polymer complex and further catalyses the hydrolysis of several acetyl groups before it dissociates and forms a new active complex with another polymer chain.

Chitin Hydrolysis

Chitin, chitosan, and their derivatives were used in many applications. For some applications, which need to use low molecular weight of chitin and chitosan, there are 2 methods for hydrolyzing them: acid hydrolysis and enzymatic hydrolysis. Acid hydrolysis hydrolyses chitin and chitosan with inorganic acids and gives the product of various sizes. On the other hand, enzymatic hydrolysis with chitin and chitosan hydrolyses chitin and chitosan and gives products of the same size (Figure 14).

In this study, we are interested in chitinase because it is a key enzyme to change chitin in nature to chitin oligomers. In general, chitin in nature is in crystalline form, which is not a good substrate for most chitinase. Thus the in efficient acid hydrolysis method was used to prepare chitin oligomers. This experiment aim to search for high crystalline chitin degrading chitinase to develop an enzyme for chitin oligomer preparation from natural crystalline chitin, which will allow uniform products and friendly environmental method for the production of oligosaccharide.

In this work, we first screened for crystalline chitin degrading chitinase from bacteria in soil of Thailand. After characterized some properties of enzyme, a suitable isolate was chosen and chitinase-encoding gene was cloned into *E. coli* by using pBluescript SK^- as vector. Phenotype screening was used for selected recombinant clones. Recombinant plasmid from positive clones were cut with *Pst*I to determine the size of insert fragments and retransformed to another host for ensuring the result. Chitinase activities from recombinant clones were detected by using SDS-PAGE.

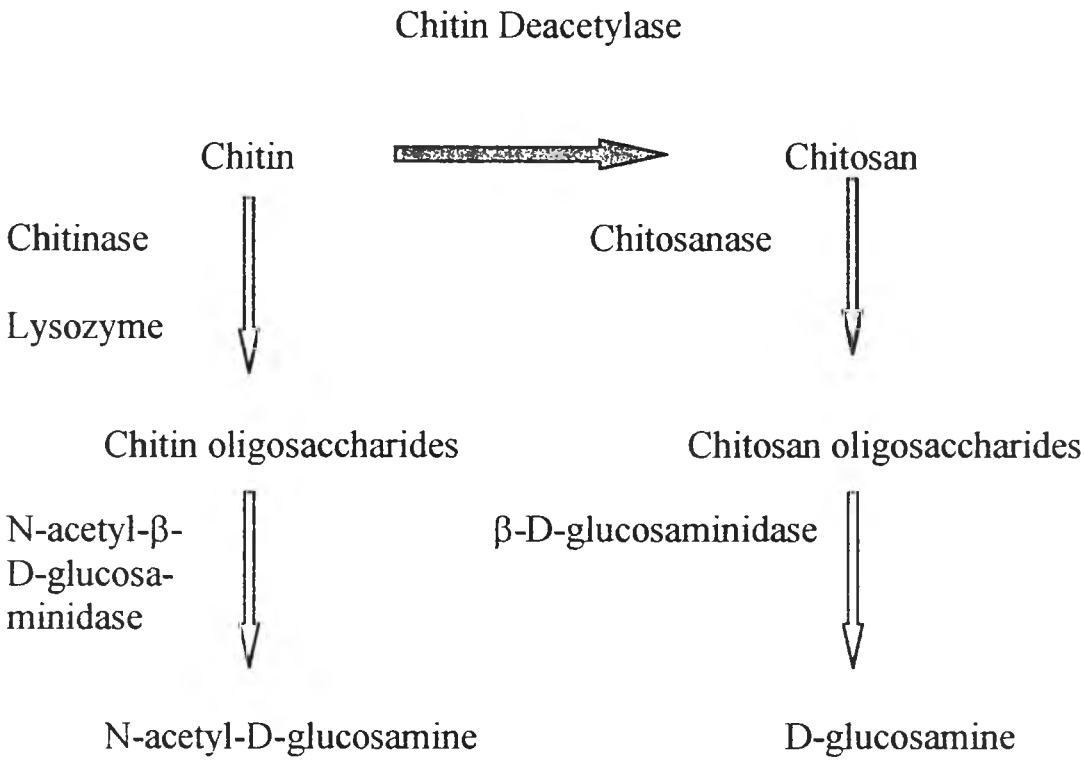


Figure 14. Enzymes for hydrolysis of chitin and chitosan (Goodsen, 1997).