# CHAPTER I

# INTRODUCTION

## 1. Chitin and chitosan

Chitin is the second most abundant organic compound in the nature. It is a polymer of unbranched polymer of β-(1,4)-linked 2-acetamido-2-deoxy-D-glucose (*N*-acetyl-D-glucosamine, GlcNAc) residue. It may be regarded as a derivative of cellulose, the most abundant organic compound, in which the hydroxyl group (-OH) at the second carbon position of the pyranose ring is replaced in chitin by an acetamide group (-NHCO-$CH_3$) (**Figure 1.1**). Chitin present in the exoskeleton of marine invertebrate and in cell walls of insect, fungi and yeasts. It, like cellulose in plant, acts as supportive and protective materials for biological living systems. Chitin may be produced at approximately 1 X $10^9$ metric tons annually in the world.

Chitosan is derived from chitin by deacetylation in the presence of alkaline. Therefore, chitosan is a copolymer consisting of β-(1→4)-2-acetamido-2-deoxy-D-glucose (GlcNAc) and β-(1→4)-2-amino-2-deoxy-D-glucose (D-glucosamine, GlcN) units with the latter usually exceeding 70%.[1]

In invertebrates, chitin occurs in a close association with water insoluble proteins which had to be removed in a production of chitin. Structure of chitin determined by polarized light and electron microscope indicated that chains of chitin usually orientated in a high degree of order.[2] X-ray diffraction was the first to show the crystalline nature of chitin.[3] The chitin chains are assembled into microfibrils forming into a crystalline structure *via* inter- and intramolecular hydrogen bonds. A comparison of X-ray data for chitin from different sources had revealed the existence, in nature, of three polymeric forms; α, β, and γ forms.[4] Most chitin including those from insects, fungi and crustaceans are classified as the α-form. While the rare second form known as β-chitin has been found in four sources: the spines of certain marine diatoms, the spine of the polychaete *Aphrodite*, the tubes of *Pogonophora*, and the

pen of squid *Loligo*. The last form, γ-chitin, has been reported from stomach lining of *Loligo* and probably in coelenterates.[3]

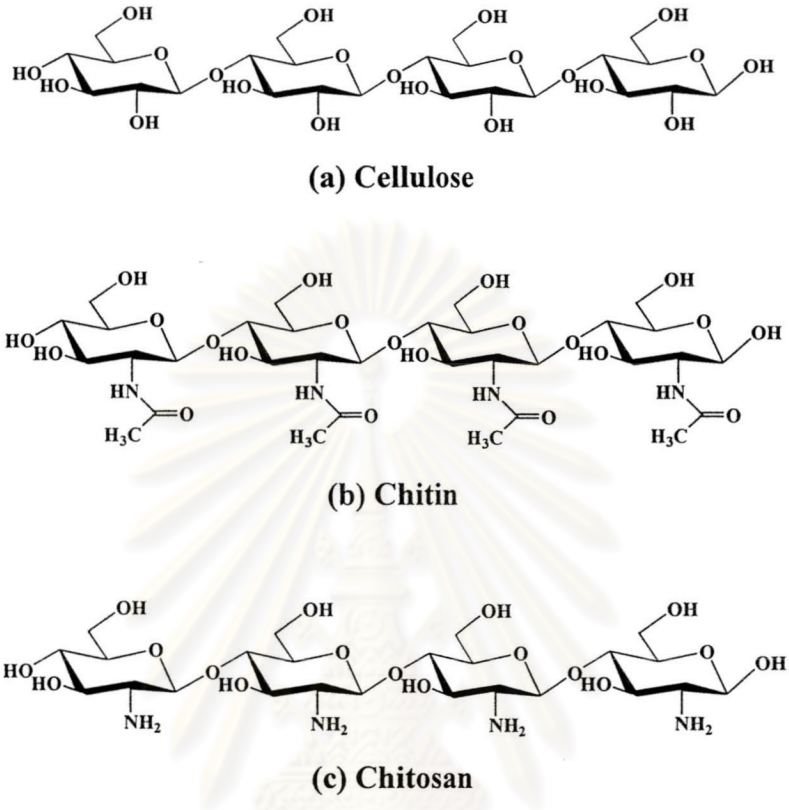



**Figure 1.1** Structures of (a) cellulose, (b) chitin and (c) chitosan

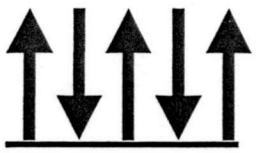
The structure refined for α-chitin either by X-ray diffraction[5] or linked atom least-square procedure[4] revealed an antiparallel of two adjacent polysaccharide chains (**Figure 1.2**). Half of the hydroxyl groups of sugar ring are bonded to amidic carbonyl groups within the same stack of chain and half are bonded to hydroxyl group between the adjacent stacks. The existence of this intersheet bonding is probably responsible for the stability of the α-chitin structure, specifically its inability to swell in water.

The β-chitin is characterized by a parallel arrangement of the polysaccharide chain (**Figure 1.3**). In this arrangement, there are no hydrogen bonds between the adjacent stacks. Thus, β-chitin is easily swollen by intercalation of water molecules between the stacks of chitin chains. In this regard, it is interesting that β-chitin is found exclusively in aquatic organisms.[6] The differences between two forms are slight, however, the α-form is more stable that β-chitin can be converted to the α-

chitin by treatment with anhydrous formic acid or strong nitric acid but no known means to date by which this transformation can be reversed.[3,4] The infrared spectra of α-chitin and β-chitin are also essentially similar. It is probable that α-chitin and β-chitin do not differ in any essential chemical manners, since both are readily hydrolyzed by chitinase from a number of sources.[3] The third form, γ-chitin, is a mixture of antiparallel and parallel arrangements of chitin chains.

Chitin is a by-product or a waste from crab, shrimp and squid processing industries. However, isolation and preparation of chitin from other marine invertebrate shells have taken place.[7,8] Chitin and chitosan offer wide range of applications, including clarification and purification of water and beverages, applications in pharmaceuticals and cosmetics, as well as agriculture, food and biotechnological uses.[9,10] Recent efforts for the use of chitin and chitosan have intensified since efficient utilization of marine biomass resources has become an environmental priority. Early applications of chitin and chitosan include a treatment of wastewater and heavy metal adsorption agent in industry, immobilization of enzyme and cells, resin for chomatography, functional membrane in biotechnology, seed coating and animal feed in agriculture, artificial skin, absorbable surgical suture, controlled releasing material for pharmaceutical agents, and wound healing accelerator in the medical field. However, chitin and chitosan have been developed as new physiological materials lately since possess antitumor activity by immuno-enhancing antibacterial activity, hypocholesterolemic activity, and antihypertensive action.[9]

Although chitin and chitosan are known to have very interesting physiological properties, but there is doubt concerning their level of absorption in human intestine, their high molecular weights and highly viscous nature may restrict their in-vivo uses. Because most animal intestines, especially human gastrointestinal tract, do not possess enzyme such as chitinase and chitosanase which can directly degrade the β-glucosidic linkage in chitin and chitosan. Recently, studies have attracted interest to converting chitin and chitosan to their monomer and oligomer (**Figure 1.4**). The monomers and oligomers of chitin and chitosan have low viscosity due to their small molecular weight and short-chain lengths that allows them to be readily soluble in neutral aqueous solution and absorbed in the in vivo system



**α-Chitin**

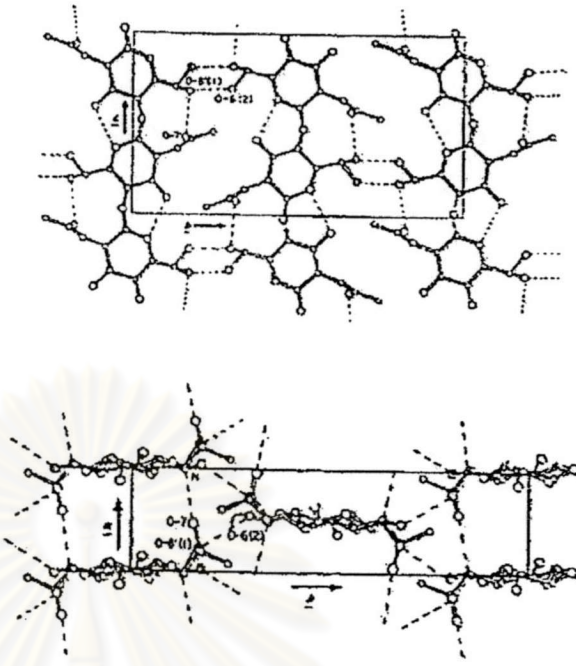
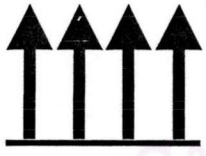


**Figure 1.2** Diagrammatic illustration with arrangement as antiparallel and X-ray crystal structure of hydrogen bond linkage between to C=O···NH group of **α-chitin**.



**β-Chitin**

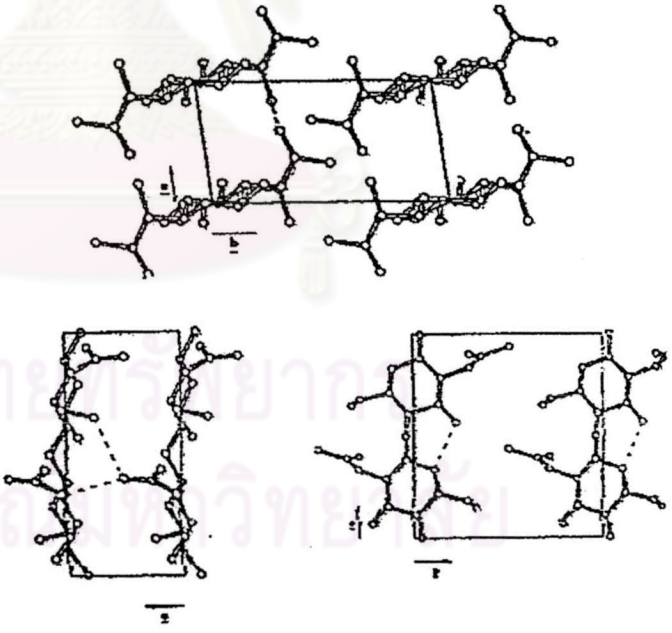


**Figure 1.3** Diagrammatic illustration with arrangement as parallel and X-ray crystal structure of hydrogen bond linkage between to C=O···NH group of **β-chitin**.

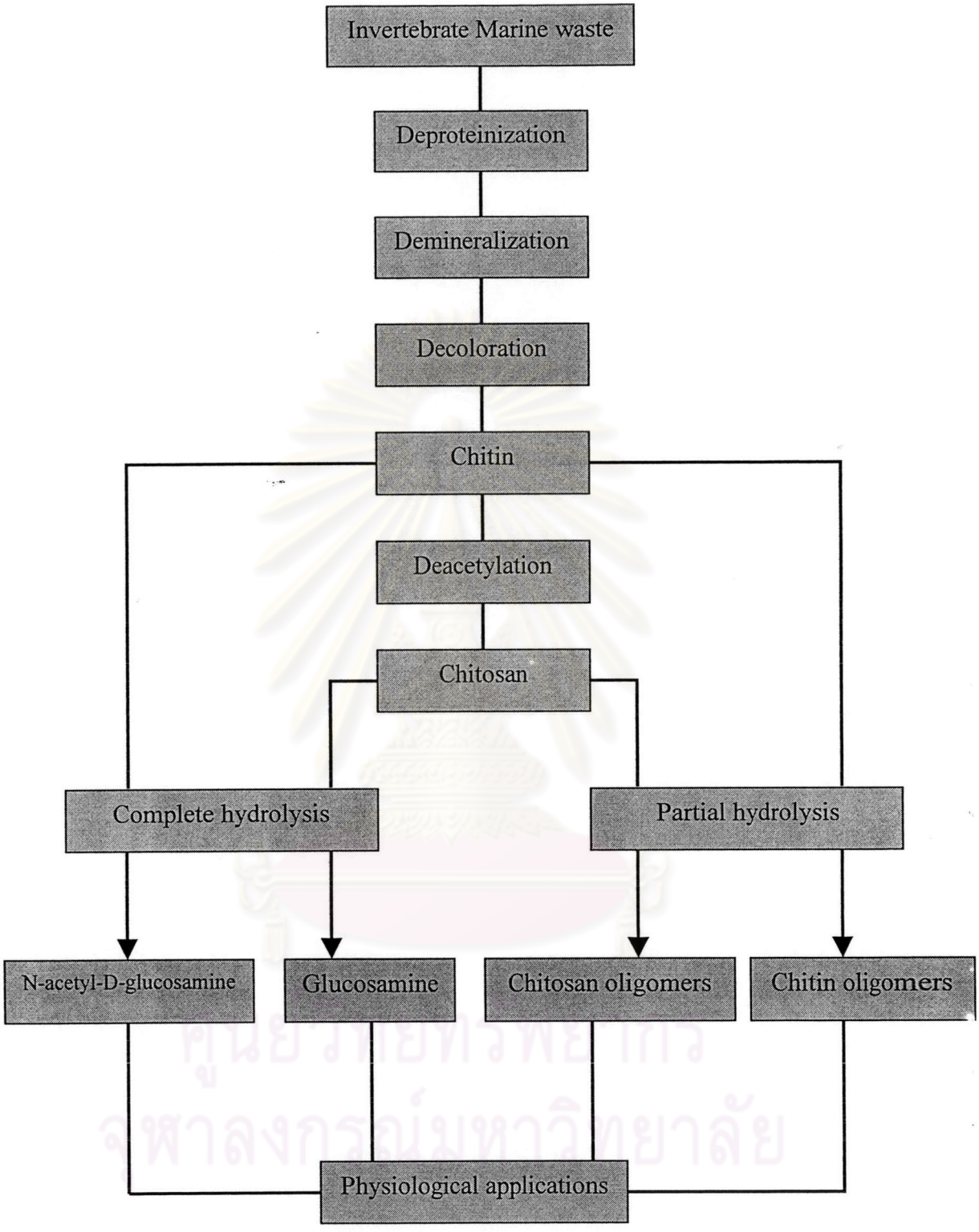



**Figure 1.4** Simplified flowsheet for preparation of chitin and chitosan, their monomers and oligomers from invertebrate marine's waste

## 2. The use of monomers and oligomers of chitin and chitosan

Unlike cellulose, chitin, chitosan and their subunits have many physiological activities. These activities have led to progressively increased utilization of these materials in food and pharmaceutical fields for human health and in chemistry as biologically important synthesis building blocks (**Table 1.1**).

Hirano and Nagao[11] studied relationships between the degree of polymerization (DP) of chitosan and the degree of pathogen inhibition. They showed that chitosan oligomers (DP 2-8) as well as partially hydrolyzed chitosan with a low molecular weight possessed stronger growth inhibition than high molecular weight chitosan against several phytopathogens including *Fusaruim oxyporum*, *Phomopsis fukushi*, and *Alternaria alternata*.

Chitin, chitosan, and their oligomers have been reported to exhibit elicitor activities toward several plants, and have been widely used as elicitors for the induction of secondary products in plant cell cultures.[12,13] Chitin oligomers were active as elicitors for defending mechanism of higher plants, whereas chitosan oligomers had almost no eliciting activity.[14,15]

Suzuki[16] demonstrated that chitin and chitosan oligomers inhibited the growth of tumor cells by immuno-enhancing effect. Suzuki *et al.*[17] also revealed the chitin oligomers from $(GlcNAc)_4$ to $(GlcNAc)_7$ displayed strong attracting response to peritoneal exudate cells in BALB/c mice, whereas chitosan oligomers from $(GlcN)_2$ to $(GlcN)_6$ did not show this effect. For the antitumor effect of chitin and chitosan oligomers with hexamer, $(GlcNAc)_6$ and $(GlcN)_6$, respectively, Tokoro *et al.*[18] showed that the two hexamers had growth-inhibitory effect against Meth-A solid tumor transplanted into BALB/c mice.

On the effects of water-soluble chitin and chitosan oligomers, Suzuki *et al.*[19] demonstrated that chitin hexamer, $(GlcNAc)_6$, possessed a strong candidacidal activity. Tokoro *et al.*[20] found that $(GlcNAc)_6$ exerted strong growth-inhibitory effect on *Listeria monocytogenes* by elevating the function of cellular immunity.

**Table 1.1** Application of chitin, chitosan, their monomers and oligomers

| Field | Chitin and chitosan | Monomer and oligomers |
|---|---|---|
| Food | Antimicrobial agents | Antimicrobial agents |
| | Preservative agents | Preservative agents |
| | Edible film | |
| Pharmaceutical | Antibacterial infection | Antibacterial infection |
| | Antitumor agents | Antitumor agents |
| | Immunopotentialting agents | Immunopotentialting agents |
| | Carrier for drug delivery system | |
| Medical | Accelerator for wound healing | Osteoarthritis and |
| | Artificial skin | inflammatory |
| | Fiber for absorbable sutures | bowel disease treatment |
| Nutritional | Dietary fiber | Hypocholesterolemic agents |
| | Hypocholesterolemic agents | Calcium absorption accelerator |
| | Antihypertensive agents | in vitro |
| Biotechnological | Carrier for immobilized | |
| | enzymes and cells | |
| | Porous beads for bioreactors | |
| | Resin for chromatography | |
| | Membrane materials | |
| Agricultural | Seed coating preparation | Activator of plant cells |
| | Activator of plant cells | Plant growth |
| Other | Coagulant for wastewater | Chemistry building blocks |
| | treatment | Cosmetics materials |
| | Protein recovery preparation in | |
| | food processing plants | |
| | Removal of heavy metal from | |
| | wastewater | |
| | Cosmetics materials | |

Tsukada *et al.*[21] reported a significant antimetastic effect for $(GlcNAc)_6$ in mice bearing Lewis lung carcinoma. Suzuki *et al.*[22] analyzed the change of the spleen cells from tumor-bearing mice administered with chitooligosaccharide such as $(GlcNAc)_6$ to unravel the tumor inhibition mechanism and cell growth by immuno-enhancing effects of the oligomers. It was demonstrated that increase of cytotoxic T lymphocytes activity by accelerating the differentiation of helper T cell was remarkable and paralleled a decrease of suppressor T cell activity.

Shikhman *et al.*[23] reported that glucosamine and its derivatives, including *N*-acetylglucosamine, are some of the most commonly used drugs to treat osteoarthritis. However, the mechanisms of their antiarthritic activities are still poorly understood.

Hiroshi synthesized amphiphilic chitooligosaccharide, having antitumor activity, by using $(GlcNAc)_2$ as stating material. The researcher expected those chitooligosaccharide derivative would aggregate in water and form a micelle, which improve the biological activity.[24] Takahashi *et al.*[25] have synthesized the an azapseudodisaccharide related to allosamidin and they reported that it was chitinase inhibitor as insecticides and antifungal agents by using $(GlcNAc)_2$ as starting material.

Richardson *et al.* reported the potential of low molecular weight chitosans as a DNA delivery system. The low molecular weight chitosans were neither toxic nor hemolytic, and could complex DNA and protect against nuclease degradation compared with high molecular weight chitosan.[26]

## 3. Preparation of *N*-acetyl-D-glucosamine and chitooligosaccharide

*N*-acetyl-D-glucosamine and D-glucosamine are monomers of chitin and chitosan, respectively. Chitooligosaccharides are the oligomers of β-(1→4) linked *N*-acetyl-D-glucosamine and D-glucosamine units, respectively. There are two hydrolytic methods, chemical hydrolysis and enzymatic hydrolysis, used for the preparation of monomers and chitooligosaccharides from chitin and chitosan.

### 3.1 Chemical hydrolysis

Chemical method for the preparation of GlcNAc, GlcN, and chitooligo-saccharides mostly deals with acid hydrolysis.[27-29] Recently, the series of chitooligosaccharide have become commercially available. They are usually prepared by hydrolysis of chitin and chitosan with concentrated hydrochloric acid, followed by extensive column chromatographic fractionation.[27] The conventional procedure for

their isolation is as follow: 1) acid hydrolysis, 2) neutralization, 3) demineralization, 4) charcoal-celite column fractionation, 5) HPLC fractionation, and 6) lyophilization.[28]

Rupley[27] used concentrated hydrochloric acid to digest chitin for preparation a substrate for lysozyme assaying. Moreover, Horowitz *et al.*[33] explained that acid hydrolysis of chitosan with concentrated HCl also led to the production of chitosan oligomers with low degree of polymerization (DP) (monomer to trimer) in quantitative yields. However, such a simple method, using only concentrated hydrochloric acid associates with some inherent problems such as cost for purification of the products, environmental concerns, and a low yield of product with many by-products. Acetolysis, fluorolysis, fluorohydrolysis, and hydrolysis with sonolysis have thus been studied to alleviate these problems (**Figure 1.5**).

Inaba *et al.*[30] used acetolysis of chitin to synthesize a substrate for the assay of lysozyme. In addition, Kurita *et al.*[31] suggested squid β-chitin as a starting material for simple acetolysis giving rise to the formation of *N*-acetyl chitooligosaccharide peracetates in high yields with considerable reproducibility.

Defaye *et al.*[32] noted that fluorohydrolysis of chitin in anhydrous hydrogen fluoride (HF) led to chitin oligomers in almost quantitative yield and conditions can be conveniently monitored in order to optimize the preparation of specific oligomers ranging from 2 to 9 residues. However, major products of chitin oligomers obtained are mainly dimer to tetramer and chitin oligomer isomers (β-(1→6)-linked 2-acetamino-2-deoxy-D-glucosyl oligosaccharide) exclusively formed when solutions of chitin were kept in HF for over 10 hrs at room temperature.

Takahashi *et al.*[28] reported a production of chitin oligomers by a combination method of mild acid degradation and sonolysis, which is able to degrade chitin without dependence on the temperature of the bulk solution and hydrolyze chitin by hydrochloric acid under ultrasound irradiation.

Moreover, the preparation of these small carbohydrate molecules is also achieved by a free radicle reaction. Nordtveit *et al.*[34] demonstrated that the viscosity of chitosan solution decreased rapidly in the presence of hydrogen peroxide ($H_2O_2$) and $FeCl_3$. They attributed this to a random radical depolymerization of chitosan. Tanioka *et al.*[35] showed that Cu(II), ascorbate, and UV-$H_2O_2$ system gradually reduced the molecular weight of chitosan. They postulated that the hydroxyl radicals

generated in the experimental system caused the polymer degradation and that this phenomenon may help to explain the disappearance of chitosan *in vivo* during biomedical applications.

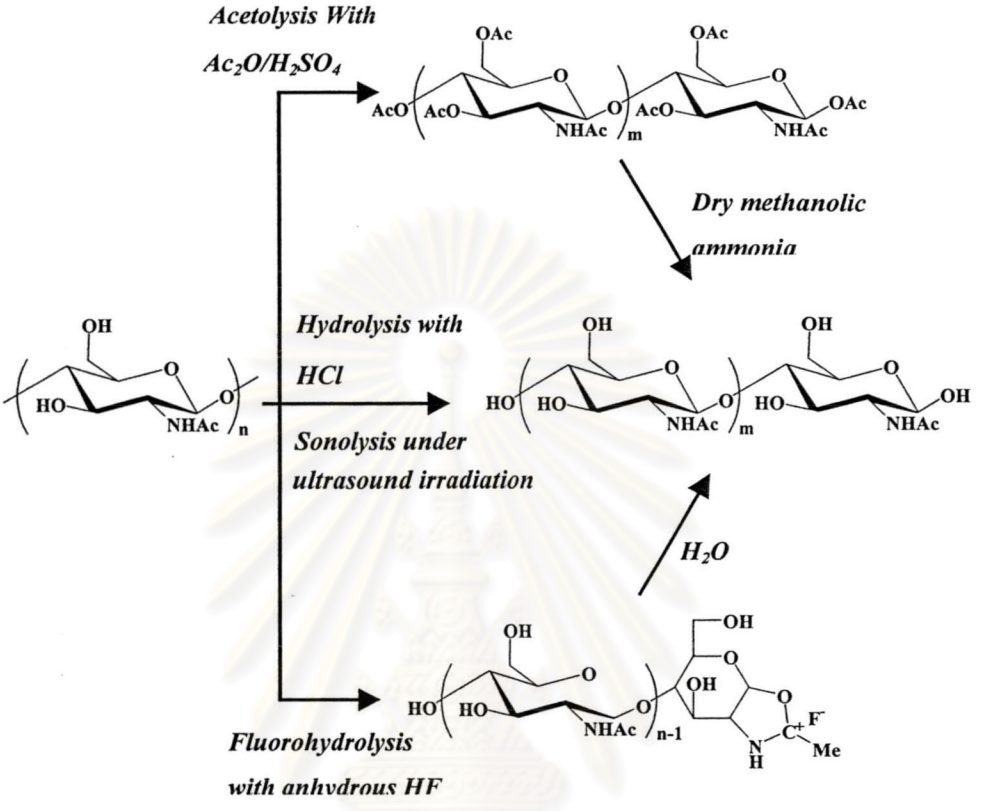



**Figure 1.5** Reactions for acid hydrolysis of chitin.

### 3.2 Enzymatic hydrolysis

In contrast to chemical hydrolysis, enzymatic hydrolysis of chitin and chitosan has several benefits to produce monomers and oligomers with milder reaction condition. Uchida *et al.*[36] explained that the enzymatic hydrolysis was a useful method for the preparation of oligomers from chitin and chitosan because the yield of specific products was usually greater in the enzymatic hydrolysis than in the acid hydrolysis.

Chitin may be degraded *via* enzymatic hydrolysis by lysozyme and chitinase. Lysozyme hydrolyzes partially *N*-acetylated chitosans (PNACs) under homogeneous condition. The lysozyme digestibility of PNACs increases with the increasing of the degree of *N*-acetylation of PNACs because lysozyme recognizes GlcNAc sequences with more than three residues.[37] Chitinase is the enzymes from bacteria that of the

endo-type and produce oligomers larger than $(GlcNAc)_2$. In contrast, β-*N*-acetylhexosaminidase is an exo-type involved in hydrolysis of *N*-acetylchito-oligosaccharide or $(GlcNAc)_2$ to release free *N*-acetyl-D-glucosamine (**Figure 1.6**).

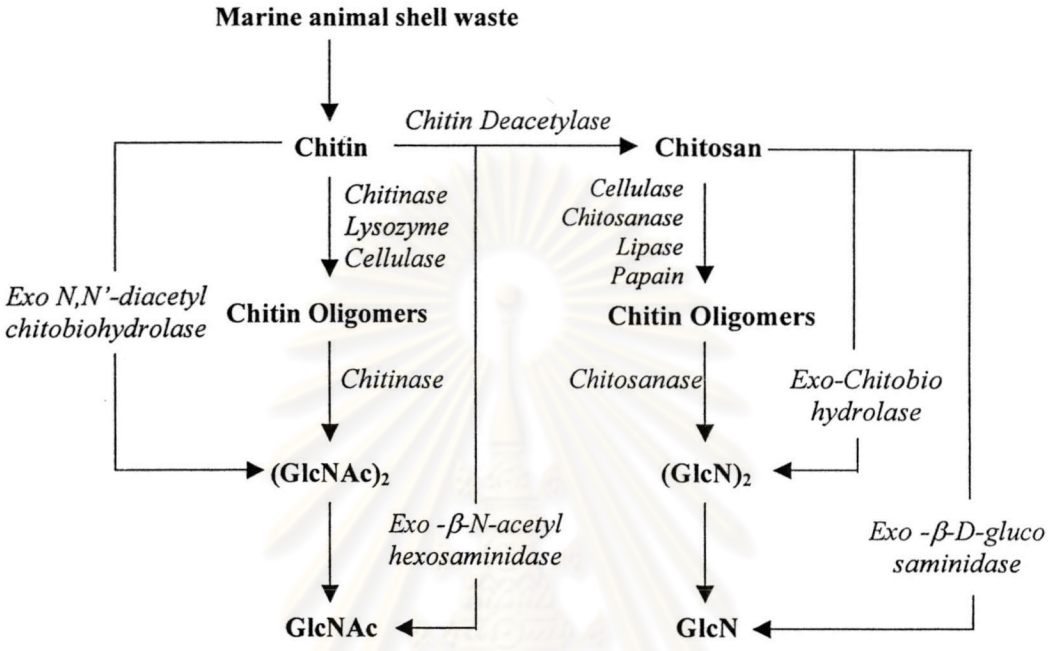



**Figure 1.6** Pathway for the conversion of chitin and chitosan into their oligomers by enzymatic means.

Takiguchi and Shimahara[38] reported a production of only $(GlcNAc)_2$ from chitin with an enzyme from thermophilic bacterium. Takayanagi *et al.*[39] reported that four kinds of thermostable chitinase isolated from the cell-free culture broth of *Bacillus licheniformis* X-7u produced $(GlcNAc)_2$ and GlcNAc. Mitsutomi *et al.*[40] revealed that the chitinase A1 and D from *Bacillus circulans* WL-12 specifically hydrolyzed the *N*-acetyl-β-D-glucosaminidic bonds in a 50% *N*-acetylated chitosan to produce heterooligosaccharide with GlcNAc at the reducing end residue and hetero-oligosaccharides with DP 2 or 3 were produced as major hydrolytic products. Ohtakara *et al.*[41] and Mitsutomi *et al.*[42] also reported that main oligosaccharides produced during the course of hydrolysis of partially-*N*-acetylated chitosans (PNACs) by chitinase from *Streptomyces griseus* and *Aeromonas hydrophila* were heterochitooligosaccharides with 2 to 4 residues.

Aiba[43] also suggested that, in the case of degradation of chitin by chitinase, hydrolyzed sites can not be regulated by the enzyme. If chitosan is used as a substrate

in a homogeneous state, hydrolyzed sites might be regulated as chitosan has partial GlcNAc residues recognized by chitinase. Preparation of *N*-acetylchitooligosaccharide with two to six residues from chitosan with chitinolytic hydrolysis followed by *N*-acetylation with acetic anhydride. When 20% acetylated chitosan was hydrolyzed by *Streptomyces griseus* chitinase for seven days, the yields of $(GlcNAc)_3$, $(GlcNAc)_4$, $(GlcNAc)_5$, and $(GlcNAc)_6$ were 23.5, 25.5, 19.6, and 12.3%, respectively.

Fenton and Eveleigh[44] reported a production of heterooligomer, GlcN-GlcN-GlcNAc and GlcN-GlcNAc, with GlcNAc at the reducing end residues in the hydrolysis of 30% and 60% acetylated chitosan, respectively, with *Penicillium islandicum* chitosanase. Izume *et al.*[45] showed that chitin oligomers from dimer to heptamer could be prepared by enzymatic hydrolysis of 10% acetylated chitosan by a chitosanolytic enzyme.

Recent studies on enzymatic transglycosylation have revealed production of higher oligomers, such as hexamer and heptamer form lower oligomers. Kobayashi *et al.*[46] prepared *N,N'*-diacetylchitobiose by combining a sugar oxazoline derivative as a glycosyl donor and *N*-acetyl-D-glucosamine as glycosyl acceptor for chitinase (from *Bacillus* sp.), a hydrolytic enzyme of chitin (**Figure 1.7**).

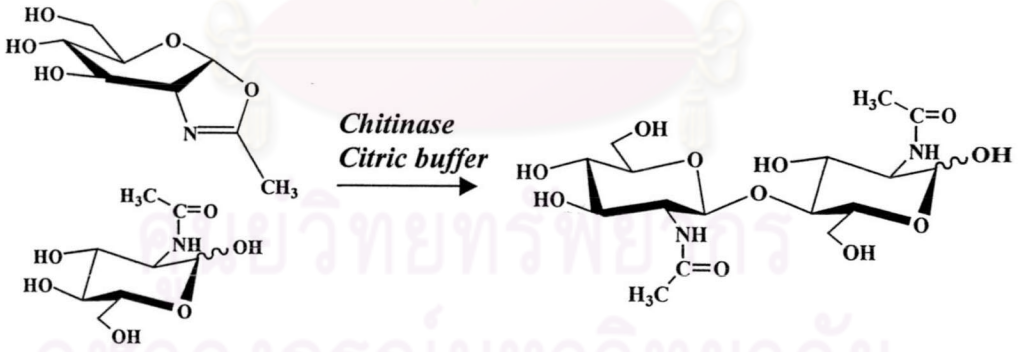



**Figure 1.7** Preparation of $(GlcNAc)_2$ by enzymatic transglycosylation.

Usui *et al.*[47] found that transferase activity of a chitinase purified from *Nocardia orientalis* IFO 12806 could be used for the preparative scale synthesis of $(GlcNAc)_6$ and $(GlcNAc)_7$ form $(GlcNAc)_4$ and $(GlcNAc)_5$.

Although a number of chitinases and chitosanases have been isolated from microorganisms over the past two decades, they are still very expensive to be utilized in the industrial process. Several commercial enzymes have been examined for their potential usage in the preparation of GlcNAc and chitooligosaccharides by enzymatic hydrolysis of chitin and chitosan with a low production cost. Aiba and Muraki[48] used low-cost enzymes such as lipase, cellulase and hemicellulase and found that, in the case of hemicellulase, the yield of hexamer was more than 20% when chitosans with 9-22% deacetylated were used. Muzzarelli *et al.*[49] also reported that wheat germ lipase, which is widely used as an additive in laundry detergents for removal of fatty stains, was very active in depolymerization of chitosan and modified chitosans in slightly acidic aqueous solutions. These results suggested the possibility of using a number of commercial enzymes in place of lysozymes and high cost chitinases.

Recently, there are approaches of using the commercially crude enzymes without purification for preparation the monomer and oligomers of chitin and chitosan. Sashiwa *et al.*[50] reported that crude enzymes had some advantage to produce the GlcNAc owing to their low cost and their inclusion of both endo- and exo-type chitinases. These researchers can hydrolyzed β-chitin and produced the GlcNAc with high yield (76%) for 8 days when used crude enzyme from Cellulase *Tricoderma viride*. Sukwattanasinitt *et al.*[51] studied the utilization of commercial non-chitinase enzyms form fungi to prepare GlcNAc. They found that 64% of GlcNAc was obtained within only 4 days with fewer enzymes used by combination of two enzymes, that had high chitinase and β-*N*-acetylhexosaminidase activity. Sashiwa *et al.*[52] also attempted to digest the α-chitin with crude enzyme from *Aeromonas hydrophila* H-2330. The selective and efficiency production of GlcNAc was achieved by obtaining of 77% yield and clean reaction without by-product. In addition, Pichyangkura *et al.*[53] used crude chitinase form *Brukholderia cepacia* TU09 and *Bacillus lichenniformis* SK-1 to digesting the α- and β-chitin powder. The results from these suggested that certain enzymes could hydrolyze crystalline chitin to give GlcNAc in high yield (>70%).

In the development process for efficient enzymatic hydrolysis of chitin and chitosan, ran immobilized enzyme was employed for a continuous production of oligosaccharides. Jeon and Kim[54] also applied an ultrafiltration membrane in enzymatic reactor system for continuous preparation of chitosan oligomers. In addition, Matsuoka *et al.*[55] used a dialysis technique in a preparation of *N,N'*-

diacetylchitobiose by continuous enzymatic degradation of colliodal chitin with chitinase from *Streptomyces griseus* and the method had potential to be used for large-scale industrial production.

**4. Chitinolytic enzymes**

In the nature, the enzymes which can degrade chitin and chitosan have been found in many living animals, plants and pathogens. In higher plants, chitinases are used for defending themselves against plant pathogens and pests. The seaweed chitinases also play a role in self-defense similar to plant chitinases. In insects and crustaceans, chitinases are required for degradation of the exoskeletal chitin in the cuticle or shell in ecdysis process. Microorganisms produce chitinases to digest the chitinous nutrient or to partially hydrolyze the chitinous cell wall for cell proliferation. Fish and mammals also use chitinases for defense. Furthermore, chitinases are found in other organisms. Thus, these living organisms produce and use chitinase for their own specific biological purposes.[56,57]

Lysozyme (EC 3.2.1.17) is one of the early known non-chitinase enzymes that have chitinolytic activity. Hen egg white lysozyme hydrolyzes the β-(1,4) linkage between C-1 of *N*-acetylmuramic acid (MurNAc) and C-4 of *N*-acetyl-D-glucosamine (GlcNAc) in cell wall of gram positive bacteria such as *Micrococcus lysodeikticus* (*leteus*). Certain plant chitinases such as hevamine also have lysozyme activity in addition to chitinase activity. However, this chitinase hydrolyze the β-(1,4) linkage between the C-1 of *N*-acetyl-D-glucosamine and C-4 of *N*-acetylmuramic acid, which is different from the lysozyme at the hydrolytic site of the substrate.[58] Such chitinases with lysozyme activity were also found in microorganism such as *Pseudomonas aeruginosa*, *Choanephora cucurbitanum*, and *Phascolomyces articulosus* and in plants such as bean, pea, sweet orange and wasabi. Chitinases (EC 3.2.1.14) is classified as a class of hydrolytic enzyme which hydrolyze chitinous materials at GlcNAc-GlcNAc or GlcNAc-GlcN or GlcN-GlcNAc glycosidic bond.[59] Chitosanase (EC 3.2.1.132) shows that glucosamine is necessarily observed at reducing end or at the non-reducing end in its hydrolytic products.[60] Furthermore, *N*-acetylhexosaminidase or chitobiase (EC. 3.2.1.52) is an exo-type chitinolytic enzyme and hydrolyzes the chitin oligosaccharides from the non-reducing end to release a

monomeric *N*-acetyl-D-glucosamine.[61] These chitinolytic enzymes are different from chitinase in antigenicity, amino acid sequences and three-dimentional structures.

### 4.1 Chitinase (EC 3.2.1.14, glycosylhydrolase family 18 and 19)

Chitinases hydrolyze β-(1,4)-glycosidic linkage bond randomly within the polymeric chitin chain giving the mixture of *N*-acetylchitooligosaccharide including *N,N'*-diacetylchitobiose as a major product and may be with *N*-acetyl-D-glucosamine. Chitinases are found primarily in two of the families of glycosylhydrolases, family 18 and family 19 chitinase. The differences of these types of chitinases are the specificity of glycosidic linkage recognition to degrade chitin including the different mechanism of catalytic hydrolysis. The family 18 and 19 chitinase also have no similarity in their structures (**Figure 1.9** and **1.10**).

The specificity of linkage recognition for various chitinolytic enzymes was usually investigated using 20-30% *N*-acetylated chitosan as substrate (**Figure 1.8**).

**Bacterial chitinase (family 18)**
***Bacillus circulans* WL-12**[62]

**Bacterial chitinase (family 19)**
***Streptomyces griseus* HUT 6037**[63]

**Figure 1.8** The specificity of linkage recognition for chitinolytic enzymes family 18 and 19 chitinase when ● GlcNAc and ◯ GlcN.

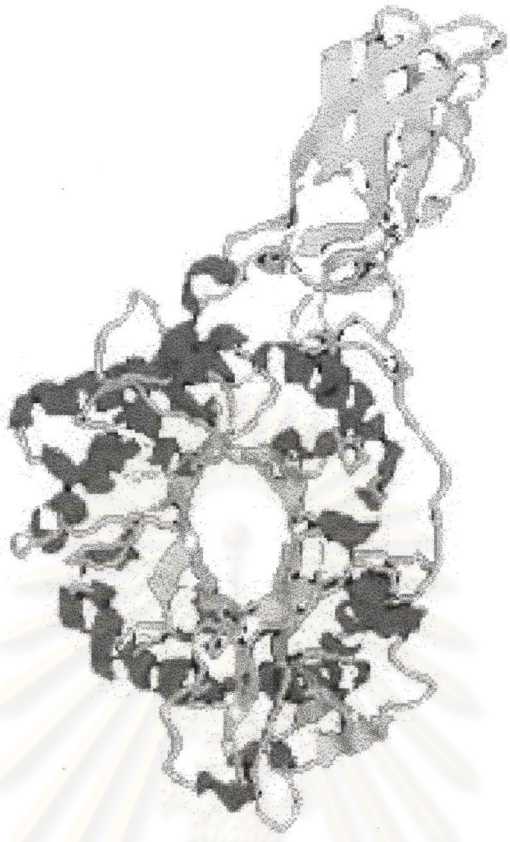


**Figure 1.9** Three dimensional structure of *S. marcescence* chitinase A, Family 18 chitinase

**Figure 1.10** Three dimensional structure of *barley* chitinase , Family 19 chitinase

### 4.1.1 Chitinase Family 18

The family 18 chitinases have a catalytic (α/β)8-barrel domain (**Figure 1.9**). The catalytic residues of this enzyme family were first reported by Watanabe *et al.* for chitinase A1 from *Bacillus circulans* WL-12.[64] Site-directed mutagenesis of Glu204 completely eliminated its activity, and the residue was considered to be a proton donor in its catalysis. From the sequence comparison, the glutamic acid residue was found to be conserved in all chitinases in family 18. In *Serratia marcescence* chitinase A, the catalytic carboxylate corresponding to Glu204 of *B. circulans* chitinase A1 is Glu315. *B. circulans* chitinase A1 produce β-anomer product,[65] 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. marcescence*. Site-directed mutagenesis of Asp200 and Asp202 in *B. circulans* chitinase A1 impaired the enzymatic activity, but did not completely eliminate the activity. The family 18 chitinases 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 (**Figure 1.12**), 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. 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*. Allosamidin was found to strongly inhibit family 18 chitinase[66], It consists of two β-1,4-linked *N*-acetylallosamine residues and an oxazoline derivative, allosamizoline (**Figure 1.11**). The strong inhibition by allosamidin suggests that the structure of allosamizoline residue is complementary to that of the catalytic center. Thus, the allosamizoline structure is most likely to reflect the transition state structure. In fact, Glu127 which

acts as a proton donor in its catalysis was found to be very close to the oxazoline group in the structure of *H. brasiliensis* chitinase complexed with allosamidin.[67]

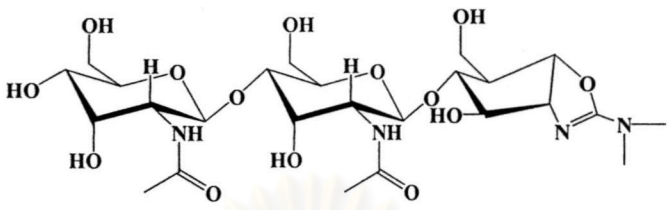



**Figure 1.11** Structure of a strong inhibitor, allosamidin, for family 18 chitinases.

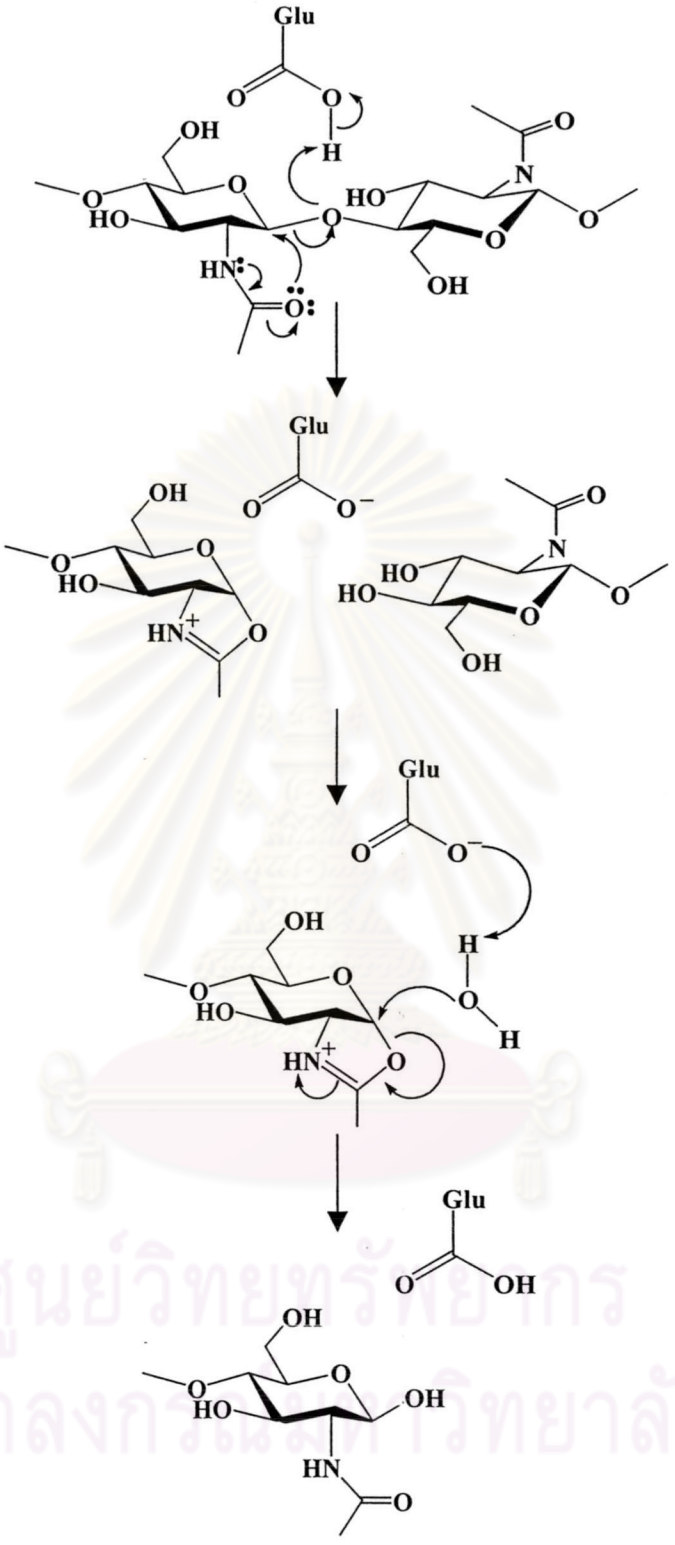



**Figure 1.12** Glycosyl hydrolysis catalyzed by family 18 chitinases following substrate-assisted catalysis. The oxazoline ion intermediate is stabilized by an anchimeric assistance of the sugar *N*-acetyl group after donating a proton from the catalytic carboxylate.

### 4.1.2 Family 19 Chitinases

Family 19 chitinase from barley seeds has a three-dimensional structure in the substrate binding and catalytic core composed of a three stranded β-sheet and two α-helices.[68] The hydrolytic products from barley chitinase reaction were found to be in α-form as determined by $^1$H-NMR spectroscopy, indicating that the chitinase inverts the anomeric form through its catalytic reaction. Wang *et al.*reported that distance between the two catalytic residues is closely related to the catalytic mechanism.[69] 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 the inverting enzymes. Glu67 and Glu89 are most likely to be a proton donor and a second catalytic residue. The reaction of inverting glycosyl hydrolases, which have two largely separated catalytic residues, is often explained by a single displacement mechanism[70] (**Figure 1.13**). 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, 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. Chitinases from other plants were reported to be an inverter as well. All of these inverting chitinases from plants should have a similar catalytic mechanism.[71]

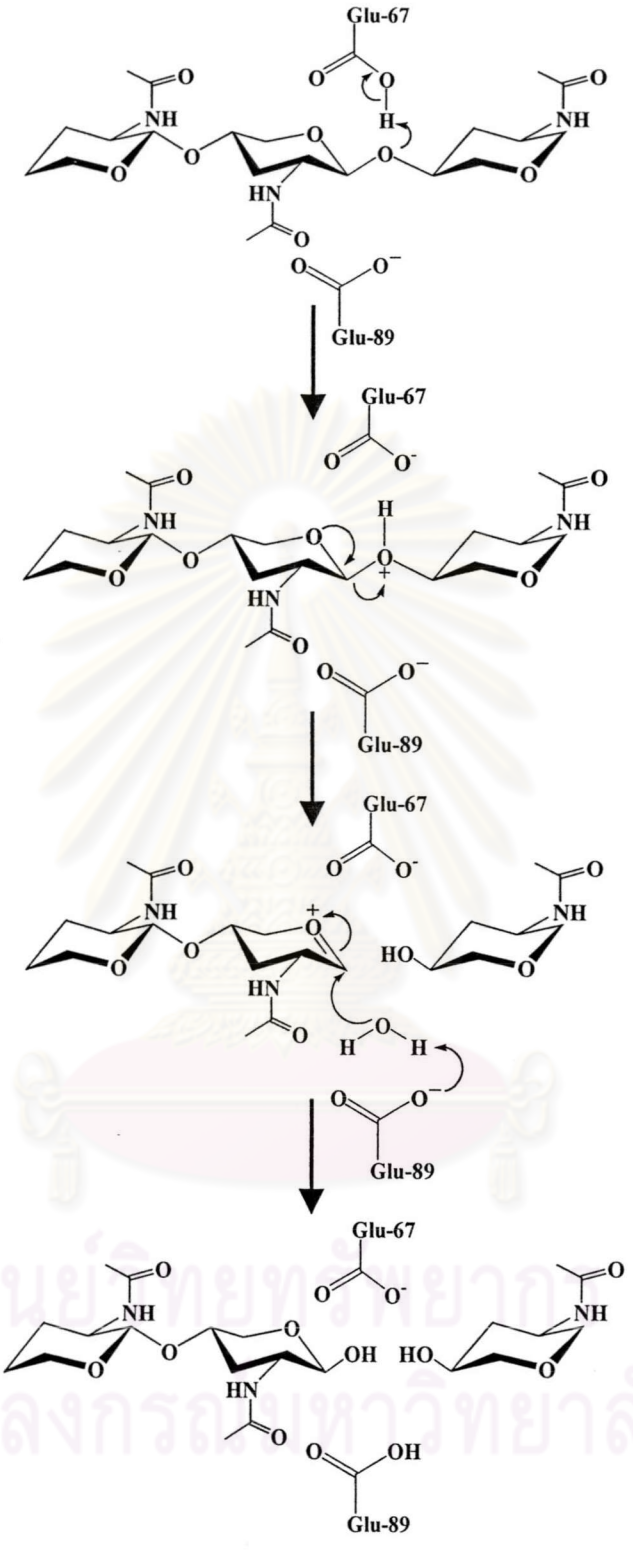



**Figure 1.13** Glycosyl hydrolysis catalyzed by barley chitinase following single displacement mechanism. Glu67 as the general acid acts as the proton donor, and Glu89 as the general base activates the water molecule which then attacks the C1 atom of the intermediate sugar residue at site (-1).

### 4.2 β-*N*-acetylhexosaminidase (EC 3.2.1.52, Glycosylhydrolase family 3 and 20)

The β-*N*-acetylhexosaminidase (Chitobiase or *N*-acetylglucosaminohydrolase) is the enzyme which hydrolyses terminal, non-reducing GlcNAc residues in chitobiose and higher chitooligosaccharides (**Figure1.14** and **1.15**).[72]

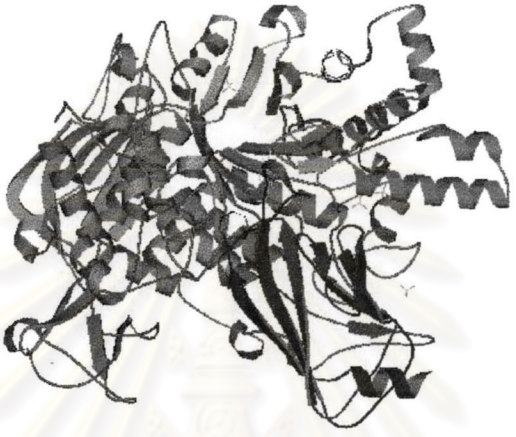


**Figure 1.14** Three dimensional structure of *S. marcescence* chitobiase, Family 20 chitinase

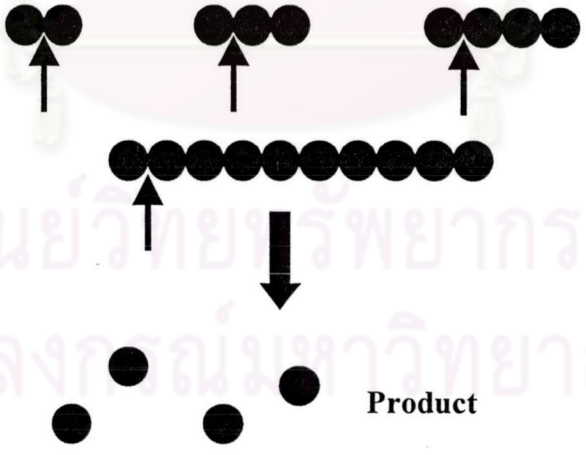



**Figure 1.15** The action of chitobiase on chitin and its product

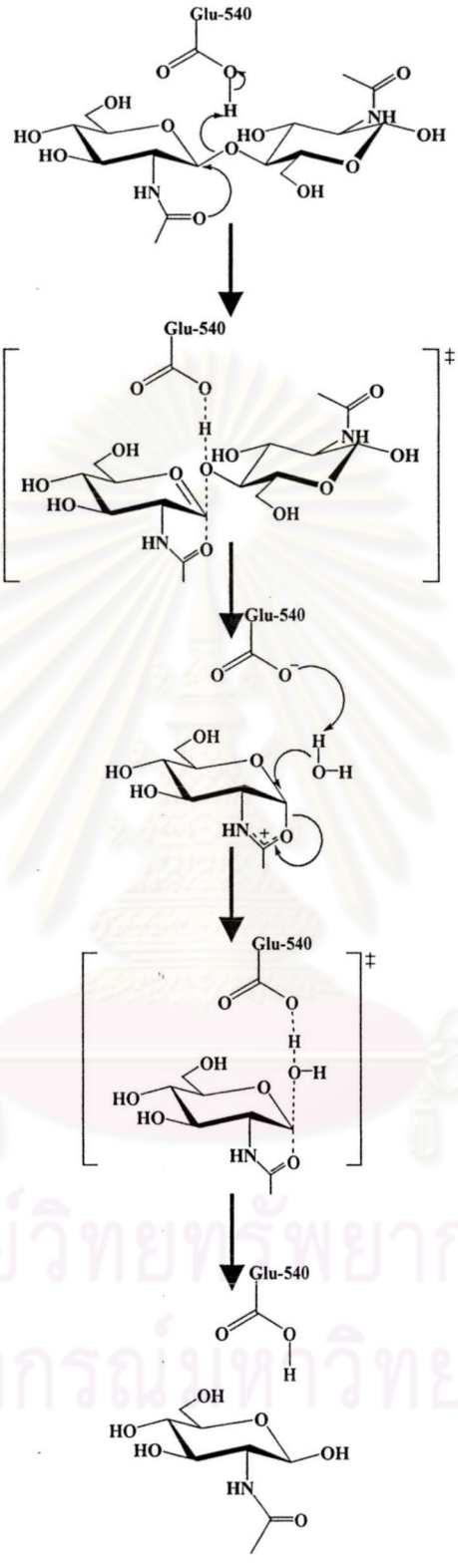



**Figure 1.16** Glycosyl hydrolysis catalyzed by *S. marcescence* chitobiase following double displacement mechanism. A covalent cyclic oxazolinium ion intermediate is formed and subsequently hydrolyzed via transition states having oxocarbenium ion character.[73]

Sequence data and especially the crystallographic data on the *S. marcescens*, chitobiase, enzyme in the complex with its natural substrate *N,N'*-diacetylchitobiose, give an invaluable insight into the mechanism of the substrate hydrolysis. The glycosyl transfer reaction is formally a nucleophilic substitution at the saturated carbon of the anomeric center which, for glycoside hydrolase with retention of anomeric configuration, proceeds via a double-displacement mechanism. The enzyme uses an acid-base reaction mechanism with Glu-540 as the catalytic acid (**Figure 1.16**).[72]

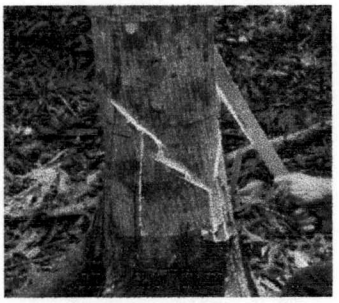
### 4.3 Substrate binding mechanism of chitinolytic enzyme

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.*, 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 liquid chromatography (HPLC)[74]

While the binding mode of $(GlcNAc)_6$ to family 19 chitinase from barley seeds was estimated by experimental time-course of $(GlcNAc)_n$ (n = 4, 5, and 6) degradation by the enzyme was first obtained by HPLC.[75] The chitinase hydrolyzed $(GlcNAc)_6$ producing $(GlcNAc)_3$ + $(GlcNAc)_3$ and $(GlcNAc)_2$ + $(GlcNAc)_4$. The amounts of (GlcNAc)3 produced was twice of those of $(GlcNAc)_2$ and $(GlcNAc)_4$, indicating that the splitting frequency into $(GlcNAc)_3$ + $(GlcNAc)_3$. They reported that the biding subsite model was (-3)(-2)(-1)(+1)(+2)(+3) for family 19 chitinase.

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. marcescence* chitinase A complexed with $(GlcNAc)_2$.[76] Brameld and Goddard have done the molecular dynamics simulations of $(GlcNAc)_6$ binding to *S. marcescence* chitinase A. Both works indicated that the binding clefts are represented by (-4)(-3)(-2)(-1)(+1)(+2) in family 18 chitinases.[77]

**5. Rubber latex tree "*Hevea brasiliensis*"**



Most of the natural rubber in the world is obtained from the latex of the rubber tree, *Hevea brasiliensis*. Latex is the cytoplasm of specialized tube-like cells, known as lactifers. The major components of latex are rubber particles. The others are of vacuolar origin and contain several hydrolase and PR(pathogenesis-related)-like proteins. Upon centrifugation (at 59,000 g for 40 min), rubber latex is divided into three layer of rubber particles, the cytosol, and the lutoids-body fraction. In the first layer, there is a white creamy layer at the top which contains virtually all the rubber particle. The translucent fluid called C-serum, which corresponds to latex cytosol, is in the second layer. The bottom fraction is called B-serum composed mainly of lutoids, but may also contain other organelles, such as some mitochondria, ribosome, and rubber particles with higher density (**Figure 1.17**).[78]

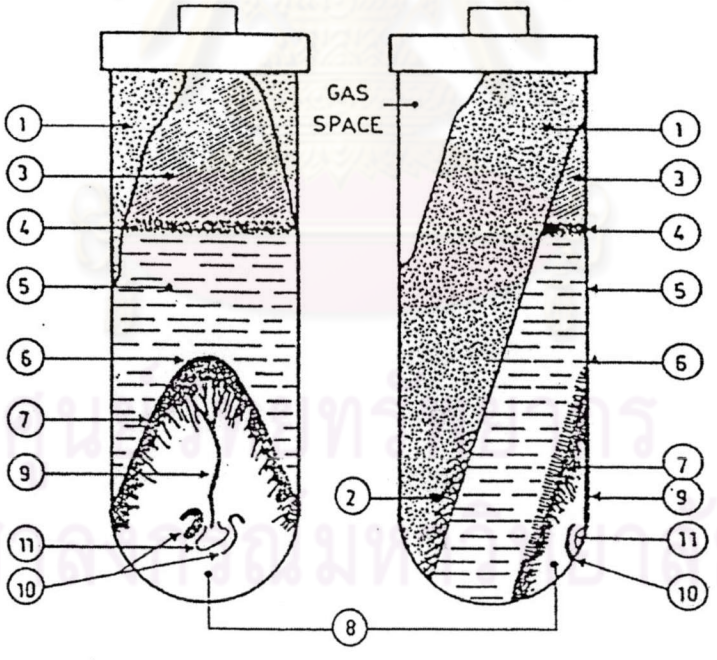



**Figure 1.17** Separation of fresh latex by ultracentrifugation at 59000 g for 40 min. Fraction 1-3 corresponds to white rubber phase. Fraction 4 is a yellow-orange layer constituted by Frey-Wyssling particle. Fractions 5 is and almost clear serum (C-serum) corresponding to the latex cytosol. Fraction 6 to 11 constituted the bottom fraction in the lutoids fraction.

Rubber latex is the cytoplasm of specialized cells known as laticifers. About 15% of its volume consist of lutiods, which are organelles of vacuolar origin containing a limited number of major proteins. Several of them having antifungal properties, hydrolase activities, and therefore are classified as pathogenesis-related protein.[79] About half of the protein content of the lutiod-body fraction can be accounted by hevein a small protein with antifungal properties, which is formed by proteolytic processing of precursor protein. Hevein is formed from the processed precursor (19 kDa) by cleavage between this domain (5 kDa) and a C-terminally located one (14 kDa), followed by further removal of residues at the C-terminus of hevein, resulting in ragged structure.[80] Another major protein in the lutoid-body fraction of rubber latex is hevamine, a bifunctional family 18 lysozyme/chitinase activity with molecular mass of 29 kDa.[81] The third major lutiod-body protein is a β-1,3-glucanase. Churngchow *et al.*[82] presented evidence that two isozymes with molecular masses of about 32 and 35 kDa are non-glycosylated and a glycoprotein, respectively. Other lutoid-body fraction proteins identified are an osmotin-like protein of 25 kDa, which may be important antifungal protein, and citrate biding protein playing a central role in citrate accumulation in lutoids also found in the this layer.

**5.1 Chitinase in the serum from para rubber tree**

Hevamine is a 29 kDa endochitinase that has been isolated from the vacuoles in the latex of the rubber tree (*Hevea brasiliensis*).[83] In addition, hevamine exhibits lysozyme activity, as has been observed for several other chitinases. The optimum pH of hevamine is 4.0. Its amino acid sequence contains the two conserved family 18 consensus regions (**Figure 1.18**).[84] Site-directed mutagenesis studies performed with the homologous chitinase A1 of *Bacillus circulans* and chitinase from *Alteromonas* have shown that the glutamate residue in the consensus regions is essential for catalytic activity. This glutamic acid corresponds to Glu127 in hevamine. Mutation of the residue of the chitinase from *Alteromonas* which is equivalent to Asp125 in hevamine also results in a complete loss of catalytic activity. However, this amino acid residue is not fully conserved in the chitinase family: the corresponding residue in the chitinase from *Arabidopsis thaliana* is an asparagine. After mutation of the residues of chitinase A1 of *B. circulans* corresponding to Asp120 and Asp123 of hevamine, the bacterial chitinase still showed a residual activity. These findings

indicate that the only conserved carboxylic group of the consensus regions that is essential for catalytic activity is the glutamic acid.[85]

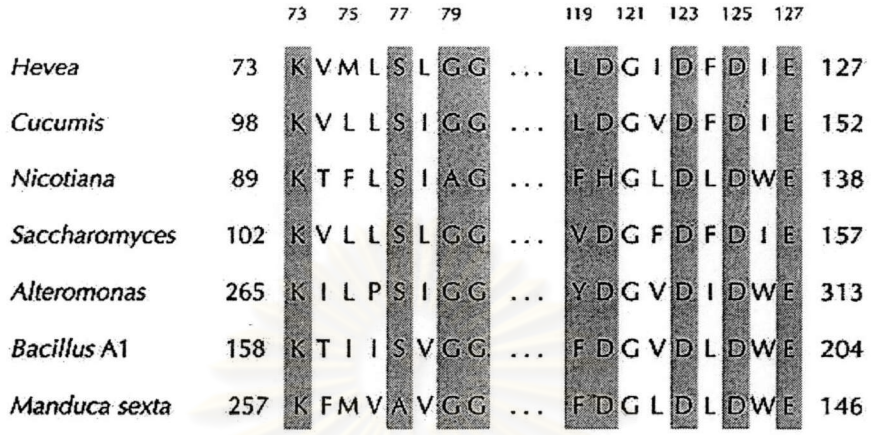



**Figure 1.18** Comparison of the conserved regions in the amino acid sequences of selected family 18 chitinases. Sequence segments shown here are from *Hevea brasiliensis* hevamine, *Cucumis sativus* acidic chitinase, *Nicotiana tabaccum* class V chitinase, *Saccharomyces cerevisiae* chitinase, *Alteromonas* sp. chitinase 85, *Bacillus circulans* chitinase A1 and insect (*Manduca sexta*) chitinase. Highly conserved amino acid residues are highlighted with shading.

Hevamine is a fairly stable enzyme, and as such it offers a potential means to confer disease resistance upon plants. Moreover, family 18 chitinases are of very diverse origin (plants, fungi, bacteria, insects) and have widely differing properties with respect to pH optimum and reaction/substrate preference (endo- versus exo-chitinase activity; chitinase versus lysozyme activity).[83]

### 5.2 Protein structure of hevamine

As no homology was found with any protein with known structure[84], the X-ray structure of hevamine was determined by the method of multiple isomorphous replacement, and subsequently refined at 2.2 Å resolution to an R-factor of 16.9%. The enzyme comprises a single domain, which has a flattened ellipsoid shape, with approximate dimensions of 50Å × 40Å × 30 Å It has a $(\beta\alpha)_8$-barrel (TIM- barrel) folding motif (**Figure 1.19**), which consists of an eight-stranded parallel β-barrel (β1–β8), surrounded by eight -helices that are antiparallel to the barrel (α1– α8). Most

connections between the carboxyl terminus of a helix and the amino terminus of a strand are 1–5 residues long; only one loop comprises 14 residues. The connections between the carboxyl terminus of a strand and the amino terminus of a helix are more variable in length, comprising 6–22 residues. Apart from the eight β-strands and eight α-helices of the $(\beta\alpha)_8$-barrel, the hevamine structure contains one extra helix after strand β8, and one extra strand located after strand β2, which form a two-stranded antiparallel β-sheet with the carboxy-terminal residues of β2. Three disulphide bridges are present, in agreement with the chemically determined connectivities[84]. The putative catalytic residue, Glu127, is located at the end of β4, with no other conserved aspartic or glutamic acid residues in its vicinity.

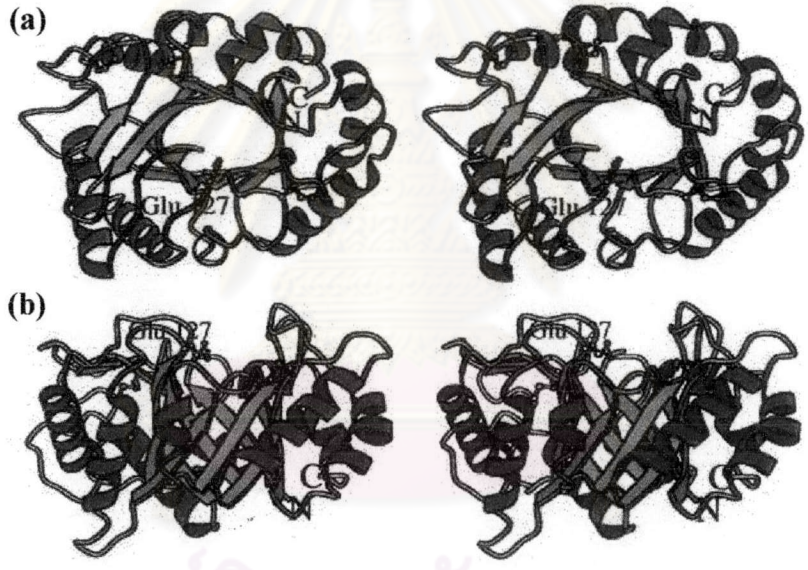



**Figure 1.19** A ribbon diagram of the structure of hevamine, drawn with the program MOLSCRIPT. Strands are shown in cyan, helices in blue and loops in yellow. Cystine residues and the conserved Glu127 are shown in ball-and-stick representation. The amino and carboxyl termini are indicated **(a)** Top view of the barrel **(b)** Side view of the barrel, showing the cleft formed by the conserved loops at the carboxyl termini of the barrel strands.

**5. Aims of thesis**

*N*-acetyl-D-glucosamine and chitooligosaccharides of chitin and chitosan possess many functional properties such as therapeutic potential in the osteoarthristis, inflammatory bowel diseases and gastritis. Furthermore, they also have the antitumor activity, antifungal activity and antimicrobial activity. The preparations of these carbohydrates are currently carried out by acid hydrolysis or enzymatic hydrolysis of chitin and chitosan. However, acid hydrolysis is usually performed by heating chitin in concentrated hydrochloric acid, which pose technical and environmental concerns. It is also virtually impossible to avoid decomposition of the desired product without having an uncompleted hydrolysis under such a harsh resulting in poor yields of the desired products. Enzymatic hydrolysis usually proceeds under much milder condition with less toxic reagents. However, the use of purified chitinases was not practical for industrial production due to its high cost.

This thesis focuses on potential use of crude enzyme from serum para rubber, waste from concentrated latex processing industry in the preparation of *N*-acetyl-D-glucosamine and chitooligosaccharide. The optimum condition of chitinolytic enzyme in serum para rubber was studied. A suitable protocol for preparation of *N*-acetyl-D-glucosamine and *N,N'*-diacetylchitobiose from chitin will be described.