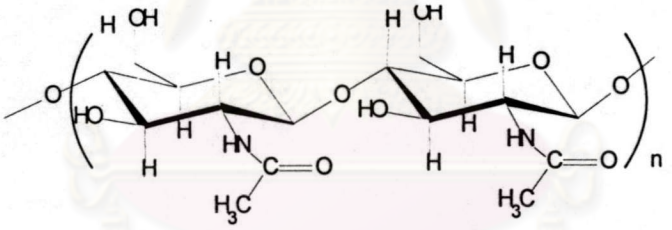
CHAPTER I

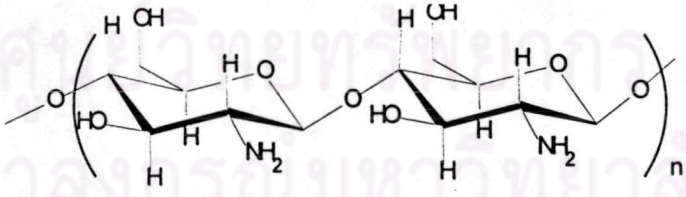
INTRODUCTION

1.1 Chitin

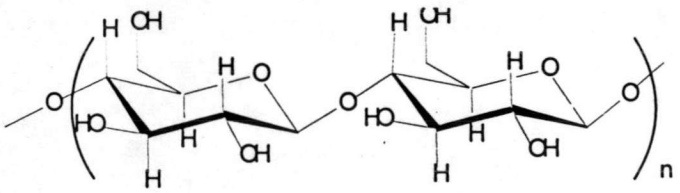
Chitin, a natural homopolymer of β-(1-4) linked *N*-acetyl-D-glucosamine (2-acetamido-2-deoxy-D-glucose), belongs to a large class of amino sugars found in the exoskeletons or cuticles of invertebrates, as well as in the cell wall of many fungi and some algae. The global yield of chitin is amounted to be 1 to 100 billion metric tons, making chitin the second most abundant polysaccharide on the earth. It may be regarded as a derivative of cellulose, the most abundant organic compound, in which the hydroxyl groups (-OH) at the second carbon position of the pyranose ring is replaced with an acetamide ($-NHCCH_3$, with O on the C) group **(Figure 1.1)**.[1]



(a) Chitin



(b) Chitosan



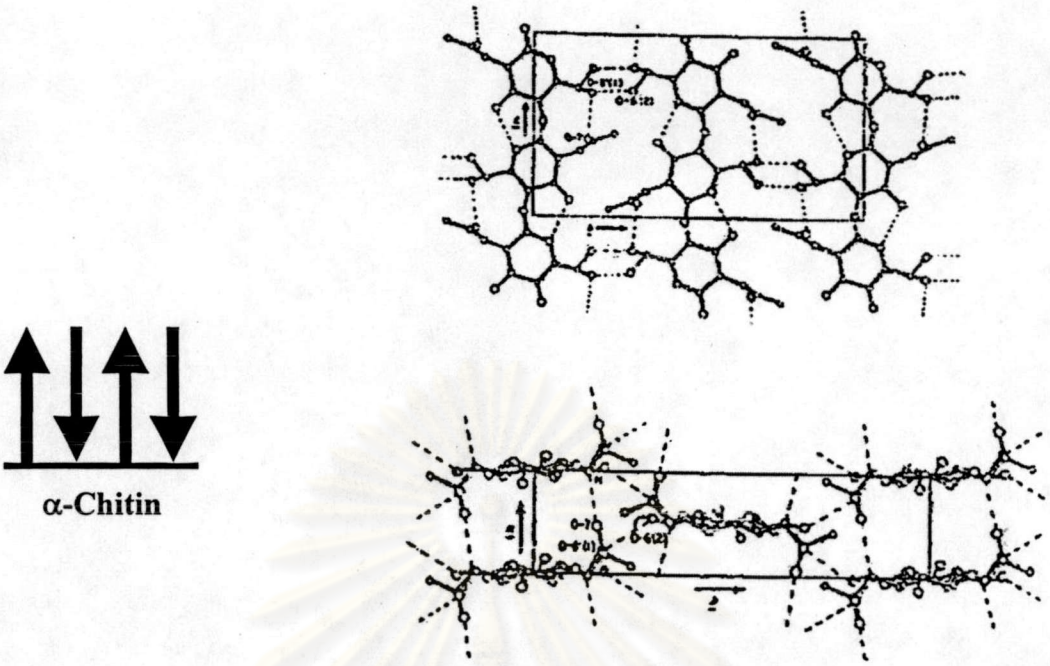
(c) Cellulose

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

The structure refined for α-chitin either by X-ray diffraction[2] or linked atom least-square procedure revealed an antipararell 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 chains and the other 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.

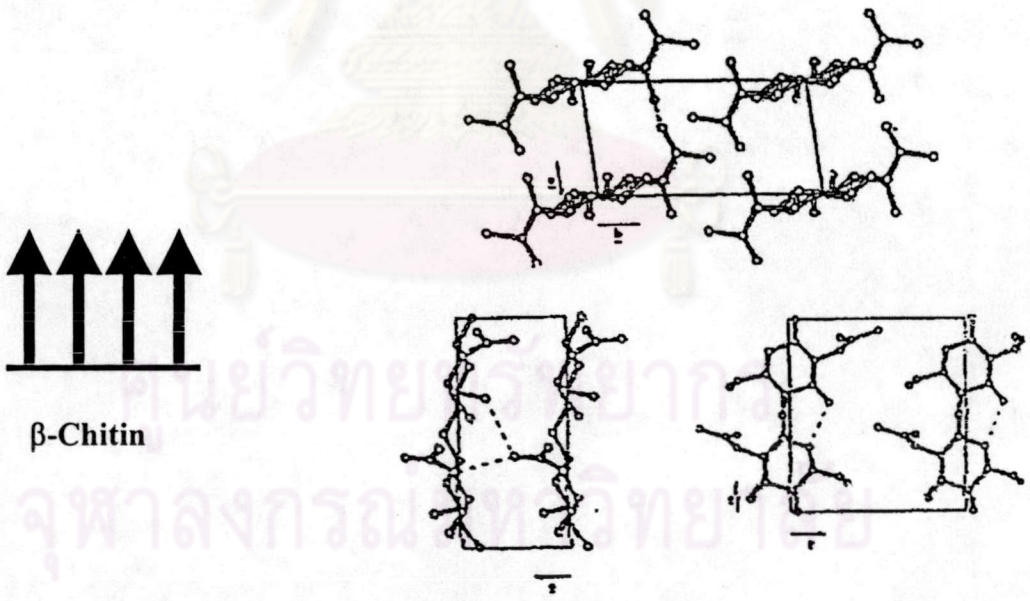
The β-chitin is characterized by a pararell arrangement of the polysaccharide chains (**Figure 1.3**). In this arrangement, there is no hydrogen bond between the adjacent chitin stacks. Thus, β-chitin is easily swollen by intercalation of water molecules between the stacks of the chitin chains. In this regard, it is interesting that β-chitin is found exclusively in aquatic organisms.[3] The differences between two forms are slight, however the α-form is more stable. The 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.[4,5] 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.[5] The third form, γ-chitin, is a mixture of anitipararell and pararell arrangements of chitin chains.

Although chitin and chitosan are known to have very interesting physiological properties, but there is a 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 convert chitin and chitosan to their monomer and oligomers (**Figure 1.4**). The monomers and oligomers of chitin and chitosan have low viscosity due to their small molecular weights 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 representing the antipararell and the X-ray crystal structure showing the hydrogen bond linkage between to C=O···NH group of α-chitin.



β-Chitin

Figure 1.3 Diagrammatic illustration representing the antipararell and the X-ray crystal structure showing the hydrogen bond linkage between to C=O···NH group of β-chitin.

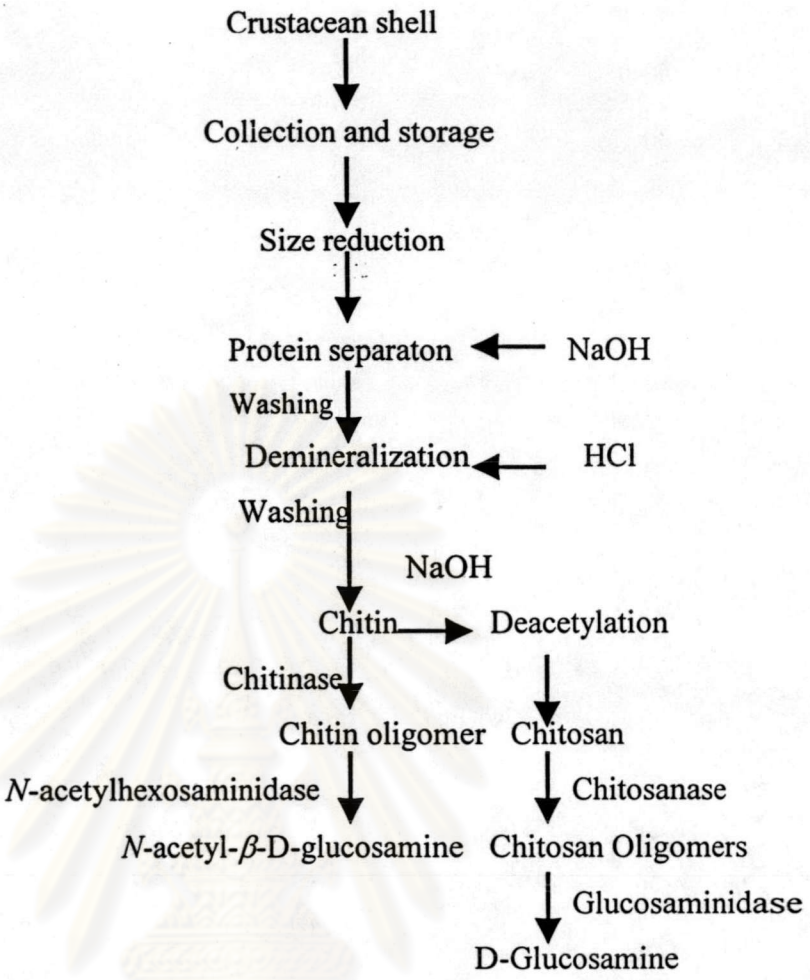


Figure 1.4 Simplified flowsheet for preparation of chitin, chitosan and their oligomers.

1.2 The applications of chitin and their derivatives.

Monomers and oligomers of chitin and chitosan have potential applications in the pharmaceutical industry, medical, agriculture, cosmetics and in chemistry as biologically important synthesis building blocks (**Table 1.1**). GlcNAc was a part of the makeup of the body tissues and blood vessels.[6]

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 waste water and heavy metal adsorption in industry, immobilization of enzyme and cells, resin for chromatography, 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. Recently, 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.[6]

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*.

On the effects of water-soluble chitin and chitosan oligomers, Suzuki *et al.*[12] demonstrated that chitin hexamer, $(GlcNAc)_6$, possessed a strong candiacidal activity. Tokoro *et al.*[13] found that $(GlcNAc)_6$ exerted strong growth-inhibitory effect on *Listeria monocytogenes* by elevating the function of cellular immunity.

Kobayashi and Kiyosoda[14] studied chitin, a mucopolysaccharide from invertebrates, which has recently been of great interest in numerous scientific and application fields as a multifunctional substance, activator for immune system, inhibitor of metastases of tumor cells, antibacterial substance, wound-healing materials, additives for cosmetics, drug carrier, health foods, biodegradable polymers, chelating polymer, *etc.*

Khor[15] studied chitin, extracted primarily from shellfish sources is a unique biopolymer based on *N*-acetyl-glucosamine monomer, shown to be useful as a wound dressing material, drug delivery vehicle an increasingly a candidate for tissue engineering.

Sashiwa *et al.*[16] remarked for D-glucosamine and *N*-acetyl-D-glucosamine (GlcNAc) have attracted much attention owing to their therapeutic activity in osteoarthritis. They have also been evaluated as a food supplement and GlcNAc is more suitable than D-glucosamine for oral administration because of its sweet taste non toxic and dissolve easily in water.

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
	Immunopotential agents	Immunopotential 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 treatment	Chemistry building blocks
		Cosmetics materials
	Protein recovery preparation in food processing plants	
	Removal of heavy metal from wastewater	
	Cosmetics materials	

1.3 Preparation of N-acetyl-D-glucosamine and chitooligosaccharides

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 preparation of the monomers and chitooligosaccharides from chitin and chitosan.

1.3.1 Chemical Hydrolysis

Chemical method for the preparation of GlcNAc, GlcN, and chitooligosaccharides mostly deals with acid hydrolysis **(Figure 1.5)**.[17-19] Recently, the series of chitooligosaccharide have become commercially available.

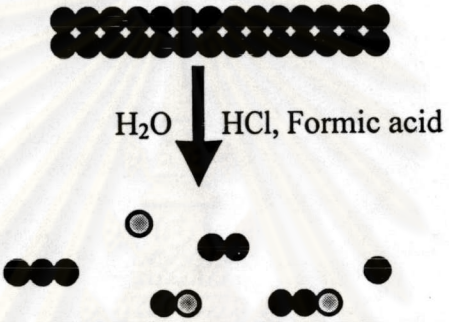


Figure 1.5 Acid hydrolysis of chitin

They are usually prepared by hydrolysis of chitin and chitosan with concentrated hydrochloric acid, followed by extensive column chromatographic fractionation.[17] The conventional procedure for their preparation is as follow: 1) acid hydrolysis, 2) neutralization, 3) demineralization, 4) charcoal-celite column fractionation, 5) HPLC fractionation, and 6) lyophilization.[18]

Rupley[17] used concentrated hydrochloric acid to digest chitin for preparation a substrate for lysozyme assaying. Moreover, Horowitz *et al.*[19] 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). 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 the specific product with many by-products. Acetolysis, fluorolysis, fluorohydrolysis, and hydrolysis with sonolysis have thus been studied to alleviate these problems.

Defaye *et al.*[20] 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 chitinoligomer 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.

In addition, Kurita *et al.*[21] 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 good reproducibility.

1.3.2 Enzymatic hydrolysis of chitin and chitosan

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.*[22] explained that the enzymatic hydrolysis was a useful method for the preparation of oligomers from chitin and chitosan because the yield of the specific products was usually greater in the enzymatic hydrolysis than in the acid hydrolysis.

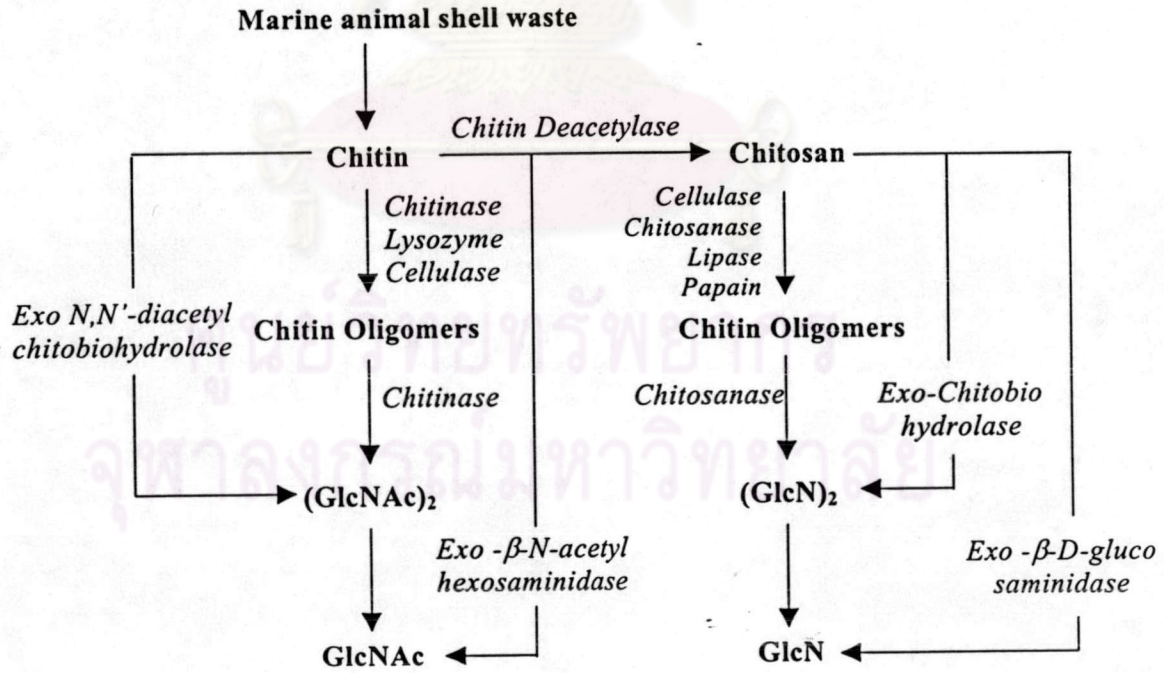
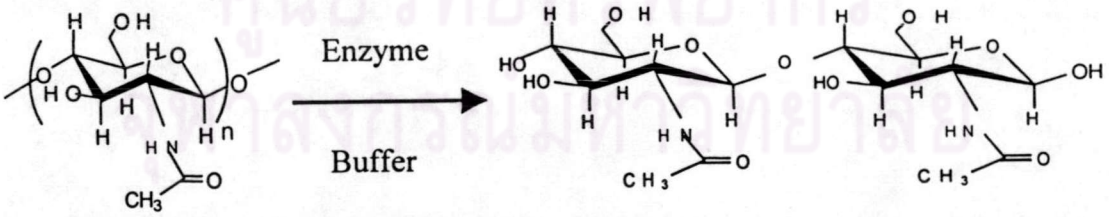


Figure 1.6 Pathway for the conversion of chitin and chitosan into their oligomers by enzymatic means

Recently, Rattanakit[23] study the optimum condition for chitinase production from *Aspergillus sp.* by isolating 3 mL of basal medium containing ammonium sulphate (0.1%). The highest yield of enzyme was obtained after 11 days of incubation at 37 °C. The chitinase activity was 110.9 mU/mL and the analysis by ion-exchange column chromatography suggested the presence of at least two chitinolytic enzymes and one *p*-nitrophenyl *β*-D-*N*-acetylglucosaminide-hydrolyzing enzyme in an extract of the solid-state culture.

Aiba[24] also suggested that, in the case of degradation of chitin by chitinase, hydrolyzed sites cannot be regulated by the enzyme. If chitosan is used as a substrate in a homogeneous state, hydrolyzed sites might be regulated as chitosan. It has a partial GlcNAc residues recognized by chitinase. Preparation of *N*-acetylchitooligo-saccharide is 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.

Recent studies on enzymatic transglycosylation have revealed the production of higher oligomers, such as hexamer and heptamer from lower oligomers. Kobayashi *et al.*[25] prepared *N*, *N'*-diacetylchitobiose by combining a sugar oxazoline derivative as a glycosyl donor and *N*-acetyl-D-glucosamine as a glycosyl acceptor for chitinase (from *Bacillus* sp.), a hydrolytic enzyme of chitin **(Figure 1.7).**



Chitin

N, *N'*-diacetylchitobiose

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

Recently, there are approaches of using the commercially crude enzymes without purification for preparation of the monomer and oligomers of chitin and chitosan. Sashiwa *et al.*[26] reported that crude enzyme has some advantage to produce the GlcNAc owing to their low cost and their inclusion of both endo- and exo-type chitinases. These researchers can hydrolyze β-chitin to produce the GlcNAc with high yield (76%) in 8 days when used crude enzyme from Cellulase *Tricoderma viride*. Sukwattanasinitt *et al.*[27] studied the utilization of commercial non-chitinase enzyms form fungi to prepare GlcNAc. They found that by using enzyme combination technique, 64% of GlcNAc was obtained within only 4 days with less enzyme used. Sashiwa *et al.*[28] also reported a digestion of α-chitin with crude enzyme from *Aeromonas hydrophila* H-2330 to give GlcNAc in 77% yield. In addition, Pichyangkura *et al.*[29] used crude chitinase form *Burkholderia cepacia* TU09 and *Bacillus lichenniformis* SK-1 to digest the α- and β-chitin powder 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[30] also applied an ultrafiltration membrane in enzymatic reactor system for continuous preparation of chitosan oligomers. In addition, Matsuoka *et al.*[31] used a dialysis technique in the 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.

Tsujibo *et al.*[32] found that *Streptomyces thermoviolaceus* OPC-520 produced a thermostable chitinase when it was grown in medium containing colloidal chitin at 50 °C. The purified chitinase had optimum temperature between 70 °C and 80 °C. The optimum pH was 8.0-9.0. Moreover, it was found to be stable in a wide range of pH and more than 80 % of activity still remained in the pH range between 4.0 to 12.0.

Wang *et al.*[33] reported that *Pseudomonas aeruginosa* was the most potential strain for alkali-tolerant chitinolytic bateria. They showed that maximum chitinase activity could be obtained when the strain was grown aerobically in a medium containing chitinous compound from shrimp and crab shell. The optimum pH and temperature of the enzyme reaction were 7 and 40 °C and it was stable at pH from 5 to 10 under 60 °C. *Pseudomonas stutzeri* YPL-1 was reported to produce extracellular

chitinase as well as beta-1,3-glucanase which were key enzymes in the decomposition of fungal hyphal cell wall.

Monreal and Reese[34] reported that *Aspergillus fumigatus* QM 45 could produce chitinase in a medium containing chitin. The maximum chitinase activity was obtained when the fungus was grown in a medium containing 2.0 % chitin at an initial pH 4.5 and ambient temperature of 30 °C.

Haynes *et al.*[35] reported the process for the production of *N*-acetyl-D-glucosamine and chitobiose by hydrolysis of chitin in a two stage reactor. The two–stage reactor comprised two sequential reactors in which the first reactor contained a packed-bed of chitin-containing solid through which an enzyme-containing mobile phase passed and wherein the second reactor was a stirred tank containing a solid-free aqueous solution which was catalyzed by one or more chitobiose degrading enzymes.

1.3.2.1 Chitinase (EC 3.2.1.14, glycosylhydrolase)

Chitinases hydrolyze β-(1,4)-glycosidic linkage bond randomly within the polymeric chitin chain giving a mixture of *N*-acetylchitooligosaccharide including *N,N'*-diacetylchitobiose as a major product and may be with *N*-acetyl-D-glucosamine **(Figure 1.8).**[36]

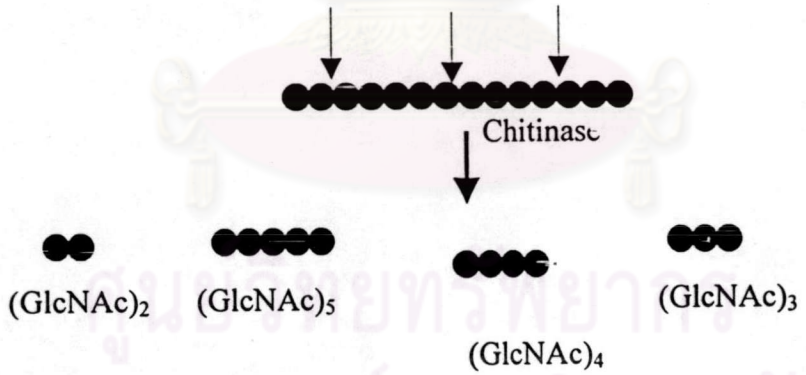


Figure 1.8 The action of chitinase on chitin and its product
when ●GlcNAc (*N*-acetyl-D-glucosamine) and ◍ GlcN (D- Glucosamine)

1.3.2.2 β-*N*-acetylhexosaminidase (EC 3. 2.1.52, Glycosylhydrolase)

The β-*N*-acetylhexosaminidase (Chitobiase or *N*-acetylglucosaminohydrolase) is the enzyme which hydrolyses terminal, non-reducing GlcNAc residues in chitobiose and higher chitooligosaccharides (**Figure 1.9**).[37-38]

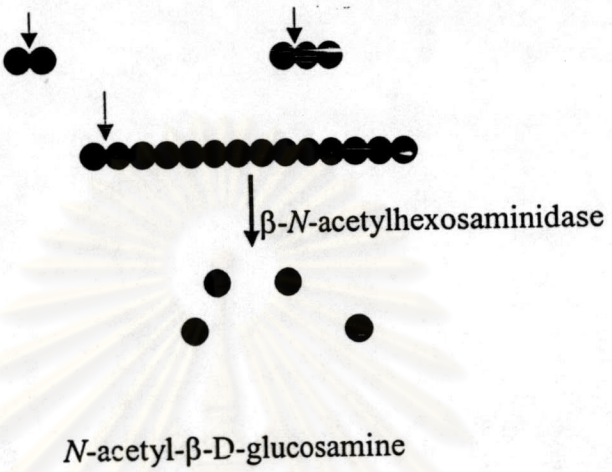


Figure 1.9 The action of chitobiase on chitin and its product

1.3.2.3 Chitosanase (EC 3.2.1.132, Glycosylhydrolase)

Chitosanases hydrolyzes β-(1,4)-glycosidic linkage bond randomly within the polymeric chitin giving a mixture of chitooligosaccharides as products and may be with *D*-glucosamine. Chitosanases are found primarily in the families of glycosylhydrolases. Chitosanase is the enzyme which hydrolyses polymer chain of chitin at the GlcNAc-GlcN or GlcN-GlcNAc (**Figure 1.10**)

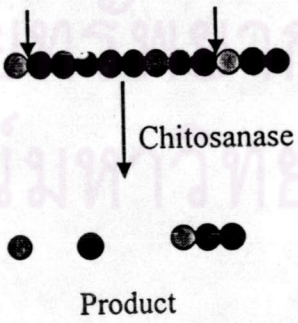


Figure 1.10 The reaction of chitosanase on chitin and its product

1.4 Enzyme from fungi

Fungal chitinolytic enzymes could be involved in the growth of the fungus itself. All types of chitinolytic enzyme activities are found in fungi, chitinase, β-*N*-acetyl-D-glucosaminidase and chitosanase. Chitinase can be localized within the cell as soluble cytoplasmic protein, sequestered in microsomes or lyosomal vacuoles or bound to the membrane or cell wall. It can also be found secreted extracellularly.[39]

1.5 Aim of thesis

N-acetyl-D-glucosamine (GlcNAc) has attracted much attention owing to their therapeutic activity in osteoarthritis.[35] They have also been evaluated as a food supplement. Comparing to glucosamine salts, GlcNAc is more suitable for oral administration because of its sweet taste. GlcNAc is mainly produced by acid hydrolysis of chitin in concentrated hydrochloric acid (HCl), which post technical and environmental concerns for acidic waste and low yield (below 65 %). This thesis focused on the suitable condition for effective production of crude chitinolytic enzyme from fungi and its potential use in the preparation of *N*-acetyl-D-glucosamine.