CHAPTER I

INTRODUCTION

1.1 Chitin

Chitin is the second most abundant biopolymer found in nature. It consists predominantly of unbranched homopolymer chains 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 presents in the exoskeleton of various marine invertebrates and insects, and in cell walls of fungi and yeasts. It, likes cellulose in plant, acts as supportive and protective materials for biological living systems. Chitin may be produced at approximately 10^9 metric tons annually in the world.

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.[1] X-ray diffraction was the first to show the crystalline nature of chitin.[2] Chitin chains are assembled into microfibrils form in a crystalline structure *via* inter- and intramolecular hydrogen bonds. A comparison of X-ray data for chitin from different sources revealed the existence, in nature, of three polymeric forms such as α, β, and γ forms.[3] 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.[2]

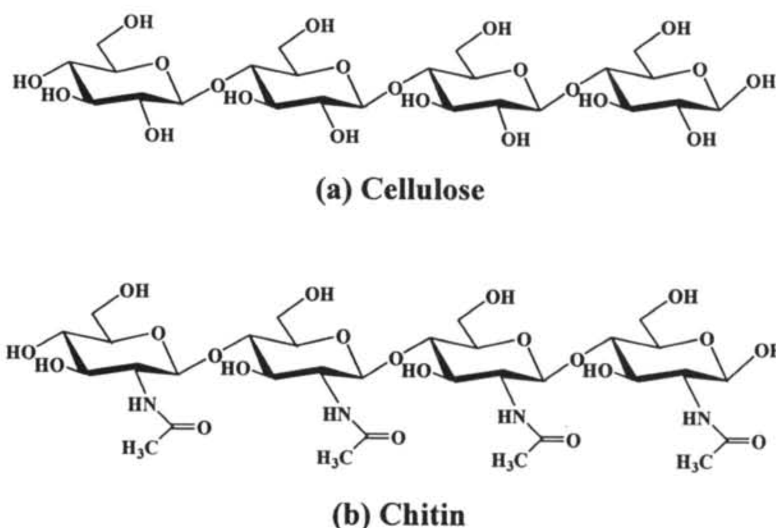

Figure 1.1 Chemical structures of (a) cellulose and (b) chitin

The structure refined for α-chitin either by X-ray diffraction[4] or linked atom least-square procedure[3] revealed an antiparallel arrangement 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 hydrogen 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 chains (**Figure 1.3**). In this arrangement, there is no hydrogen bond 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.[5] Since, 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.[2,3] The infrared spectra of α-chitin and β-chitin are essentially similar. It is probable that α-chitin and β-chitin do not differ significantly in any essential chemical manners, since both are readily hydrolyzed by chitinase from a number of sources.[2] 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.(6,7) 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.(8,9) 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.(8)

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 cellulose, chitin and chitosan. Recently, studies have attracted interest to converting 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 low molecular weight and short-chain lengths that allows them to be readily soluble in neutral aqueous solution and absorbed in the in vivo system.

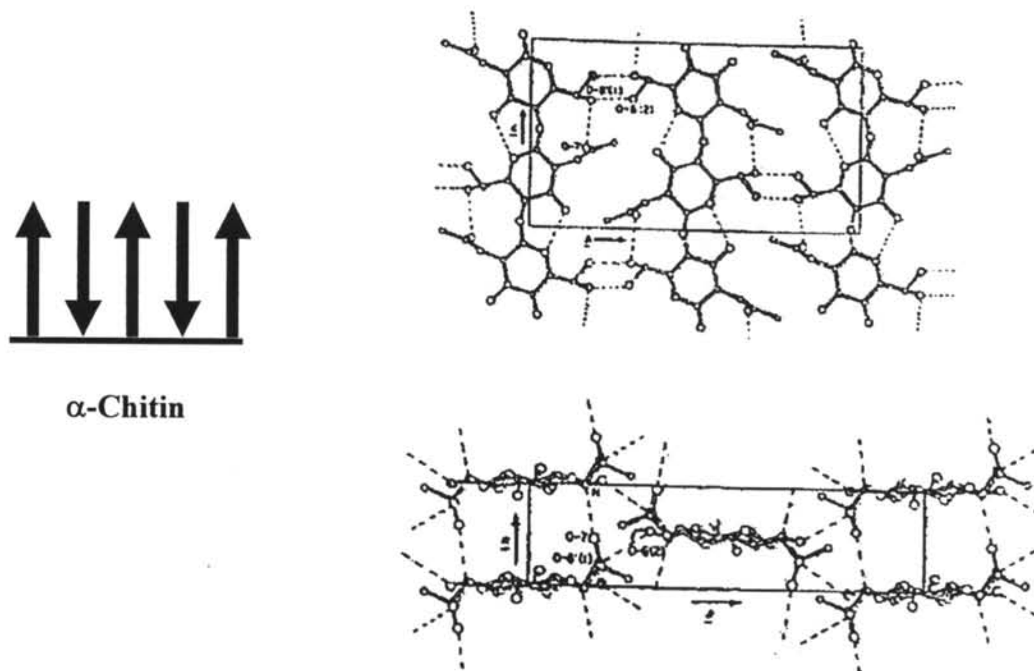

Figure 1.2 Diagrammatic illustration with arrangement as antiparallel and X-ray crystal structure of hydrogen bond linkage between to O=CNH group of α-chitin.

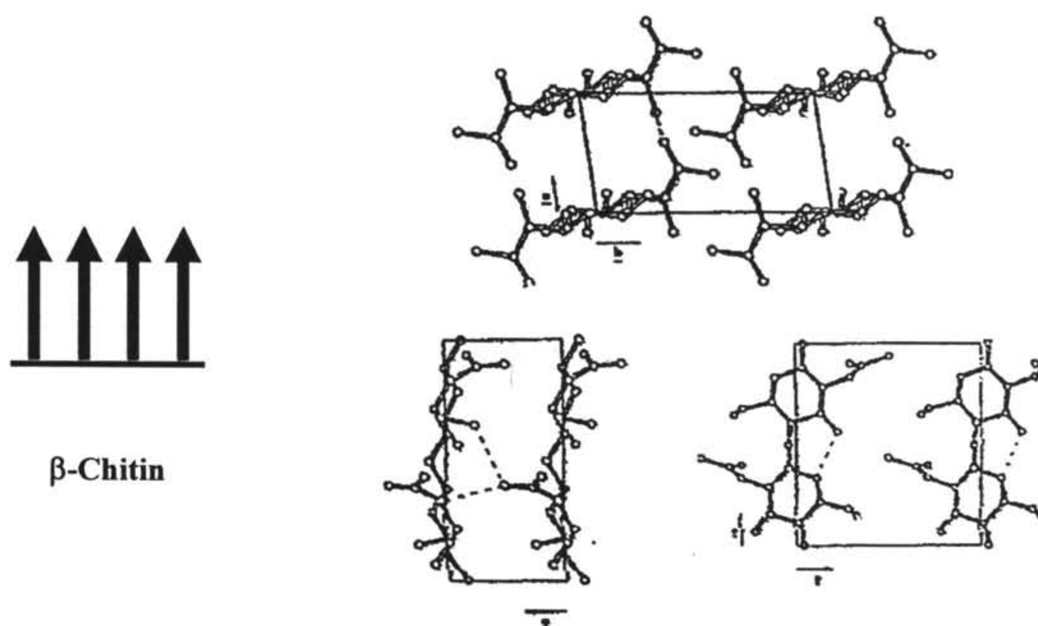

Figure 1.3 Diagrammatic illustration with arrangement as parallel and X-ray crystal structure of hydrogen bond linkage between to O=CNH group of β-chitin.

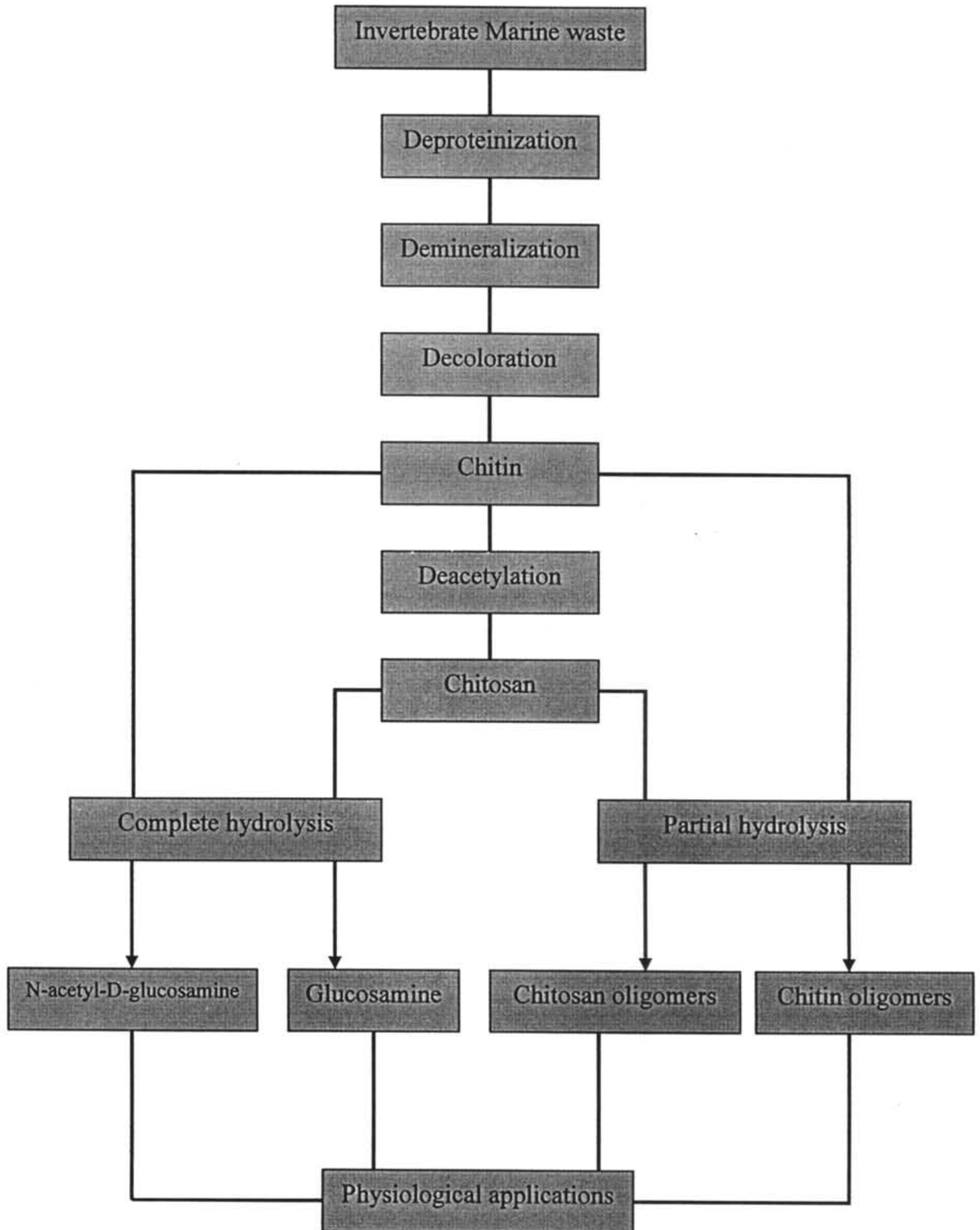

Figure 1.4 Simplified flowsheet for preparation of chitin and chitosan, their monomers and oligomers from invertebrate marine waste

1.2 Applications of monomer, dimer and oligomers of chitin

Chitin and its 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 synthetic building blocks of biologically important compoumds (**Table 1.1**).

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.(10,11) Chitin oligomers are active as elicitors for defending mechanism of higher plants, whereas chitosan oligomers have almost no eliciting activity.(12,13)

Suzuki(14) demonstrated that chitin and chitosan oligomers inhibited the growth of tumor cells by immuno-enhancing effect. Suzuki *et al.*(15) also revealed that 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.*(16) showed that both 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.*(17) demonstrated that chitin hexamer, $(GlcNAc)_6$, possessed a strong candidacidal activity. Tokoro *et al.*(18) found that $(GlcNAc)_6$ exerted strong growth-inhibitory effect on *Listeria monocytogenes* by elevating the function of cellular immunity.

Tsukada *et al.*(19) reported a significant antimetasatic effect for $(GlcNAc)_6$ in mice bearing Lewis lung carcinoma. Suzuki *et al.*(20) 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 to a decrease of suppressor T cell activity.

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 inflammatory bowel disease treatment
	Artificial skin	
	Fiber for absorbable sutures	
Nutritional	Dietary fiber	Hypocholesterolemic agents
	Hypocholesterolemic agents	Calcium absorption accelerator in vitro
	Antihypertensive agents	
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	

Shikhman *et al.*[21] 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. Recently, Hatano *et al.*[22] reported that the long-term intake of the soymilk beverage containing GlcNAc improves the subjective symptom and range of motion in subjects with slight pain, stiffness, and/or discomfort at knee joint.

Many researchers have synthesized $(GlcNAc)_2$ derivatives which were found to have many interesting activity. Hiroshi[23] synthesized amphiphilic chitooligosaccharide, having antitumor activity, by using $(GlcNAc)_2$ as a starting material. The researcher expected those chitooligosaccharide derivatives would aggregate in water and form micelles, which improve the biological activity. Takahashi *et al.*[24] have synthesized azapseudodisaccharide related to allosamidin by using $(GlcNAc)_2$ as a starting material. They reported that it was chitinase inhibitor which can be used as insecticides and antifungal agents. Nishimura and Kuzuhara[25] synthesized a peripheral trisaccharide sequence of lutropin, a pituitary glycoprotein hormone by using $(GlcNAc)_2$ as a key starting material. Kobayashi *et al.*[26] reported that $(GlcNAc)_2$ linked glycopeptide was found to inhibit hemagglutination activity of wheat germ agglutinin.

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

N-acetyl-D-glucosamine is a monomer of chitin and chitooligosaccharides are the oligomers of β-(1$\rightarrow$4) linked *N*-acetyl-D-glucosamine. There are two hydrolytic methods, chemical hydrolysis and enzymatic hydrolysis, used for the preparation of monomers and chitooligosaccharides from chitin.

1.3.1 Chemical hydrolysis

Chemical method for the preparation of GlcNAc 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 preparation is as follow: 1) acid hydrolysis, 2) neutralization, 3) demineralization, 4) charcoal-celite column fractionation, 5) HPLC fractionation, and 6) lyophilization.[28]

Horowitz *et al.*[30] found 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.*[31] used acetolysis of chitin to synthesize a substrate for the assay of lysozyme. In addition, Kurita *et al.*[32] 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.*[33] 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.

Moreover, the preparation of these small carbohydrate molecules is also achieved by a free radicle reaction. Nordtveit *et al.*[34] demonstrated that 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.

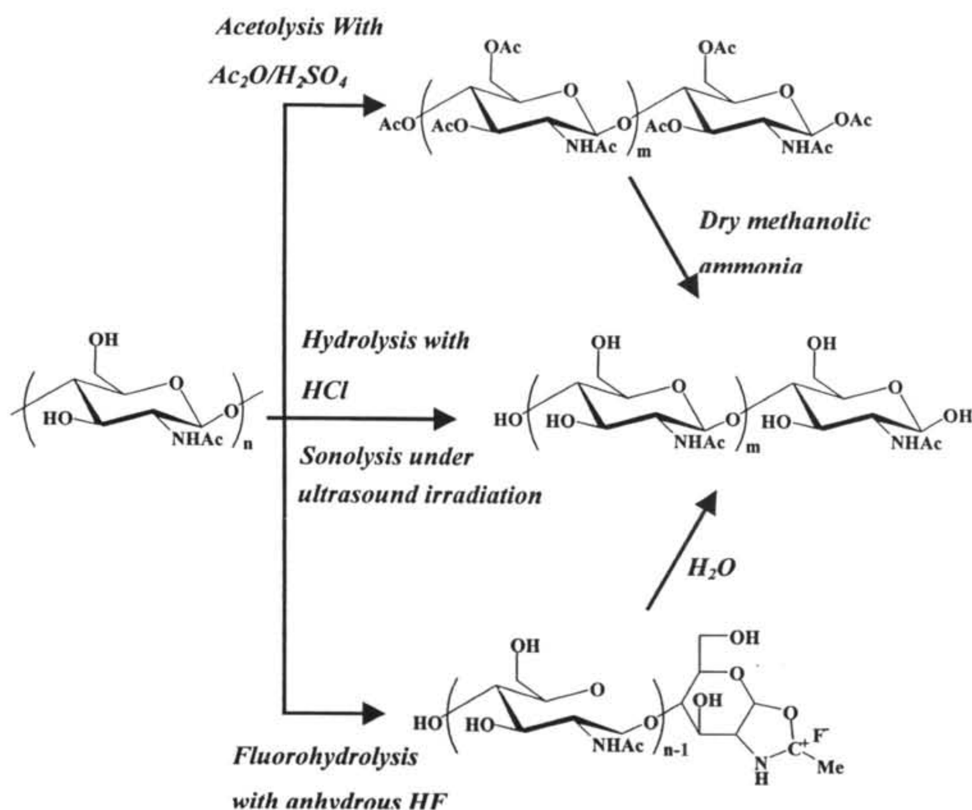

Figure 1.5 Reactions for acid hydrolysis of chitin.

1.3.2 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**).

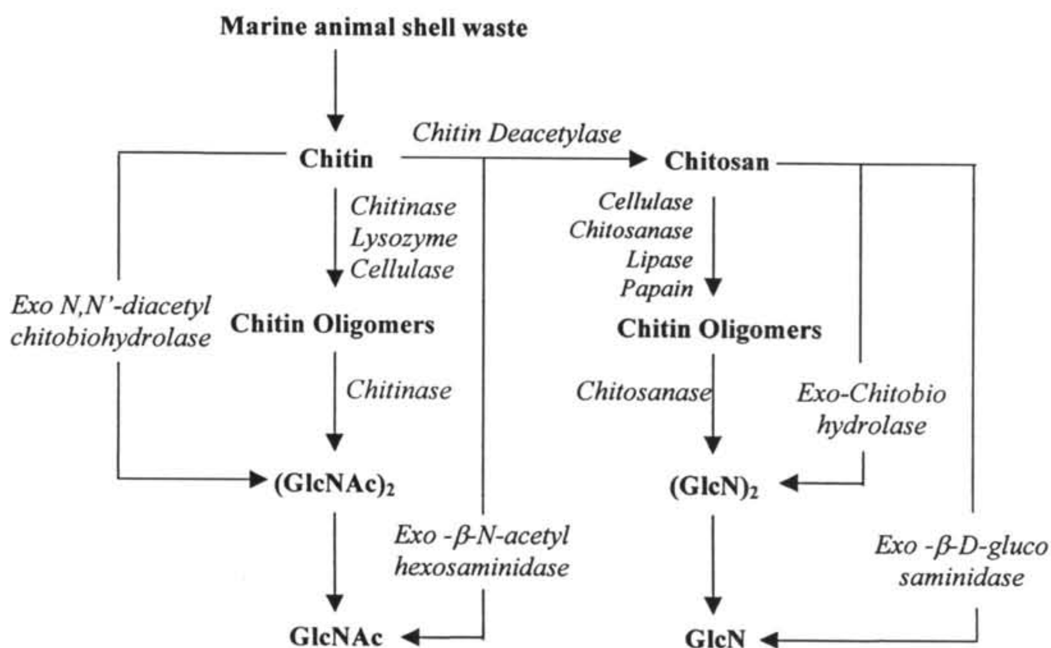

Figure 1.6 Pathway for the conversion of chitin and chitosan into their oligomers by enzymatic means.

Amano and Ito[37] reported that lysozyme acts like a chitinase which can hydrolyze copolymer of chitin and chitosan. Lysozyme hydrolyzed selectively at *N*-acetyl-D-glucosamine unit. Zhu and Laine[38] prepared chitooligosaccharides from hydrolysis of chitin slurry by chitinase from *Vibrio parahemolyticus* and *Streptomyces griseus*. They found that the major product of this hydrolysis reaction is *N,N'*-diacetylchitobiose. The increasing of temperature increased the yield of $(GlcNAc)_4$ and $(GlcNAc)_5$.

Terayama *et al.*[39] studied the hydrolysis of chitin by chitinase from *Streptomyces griseus*. They found that this enzyme is an endo-chitinase enzyme which can hydrolyze colloidal chitin to give *N,N'diacetylchitobiose* as a major product. Matsuoka *et al.*[40] also found that *Streptomyces griseus* produced chitinase enzyme to hydrolyze colloidal chitin at 40 °C and pH 6.3 to give *N,N'*-diacetylchitobiose.

Takiguchi and Shimahara[41] reported a production of only $(GlcNAc)_2$ from chitin with an enzyme from thermophilic bacterium. Takayanagi *et al.*[42] 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.*[43] 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.*(44) and Mitsutomi *et al.*(45) 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(46) 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*-acetylchitooligo-saccharide with two to six residues from chitosan could be done by 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(47) 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.*(48) 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 from lower oligomers. Kobayashi *et al.*(49) 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. (**Figure 1.7**).

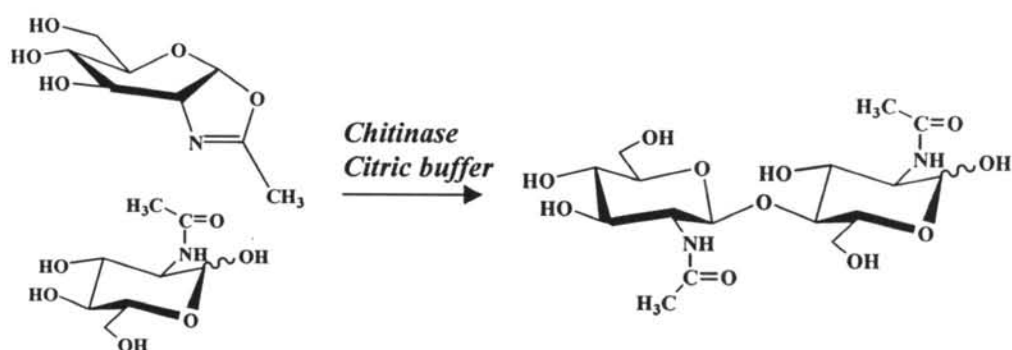

Figure 1.7 Preparation of $(GlcNAc)_2$ by enzymatic transglycosylation.

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(50) 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 chitosan with 9-22% deacetylated was used. Muzzarelli *et al.*(51) 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 commercial crude enzymes without purification for preparation the monomer and oligomers of chitin and chitosan. Naranong(52) studied the possibility of crude enzyme production from some species of fungi. They found that *Mucor sp.* and *Actinomycetes* produced both chitinase and chitobiase depending on the enzyme preparation condition. But *Paecilomyces lilacinus* produced both chitinase and chitobiase at the same condition. The major hydrolytic product of enzyme from *Actinomycetes* was GlcNAc while enzyme from *Mucor sp.* and *Paecilomyces* produced both GlcNAc and $(GlcNAc)_2$. Sashiwa *et al.*(53) 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 hydrolyzed β-chitin with crude enzyme from Cellulase *Tricoderma viride* to produce GlcNAc in high yield (76%) after 8 days. They also

found that GlcNAc yield can be improved by mixing crude enzymes *Tricoderma viride* and *Acremonium cellulolyticus*. Sukwattanasinitt *et al.*[54] studied the utilization of commercial non-chitinase enzymes form fungi to prepare GlcNAc. They found that 64% of GlcNAc was obtained within only 4 days with less enzymes used by combination of two enzymes, which have high chitinase and β-*N*-acetylhexosaminidase activity. Sashiwa *et al.*[55] also attempted to digest α-chitin with crude enzyme from *Aeromonas hydrophila* H-2330. The selectivity and efficiency in production of GlcNAc was achieved by obtaining of 77% yield and clean reaction without by-product. In addition, Pichyangkura *et al.*[56] used crude chitinase form *Burkholderia cepacia* TU09 and *Bacillus lichenniformis* SK-1 to digest α- and β-chitin powder. The results from this work suggested that certain enzymes could hydrolyze crystalline chitin to give GlcNAc in high yield (>70%). Ilankovan *et al.*[57] reported that amorphous chitin and phosphoric decrystallized chitin were found to be the most suitable substrates in enzymatic hydrolysis. Pepsin showed the highest chitinolytic activity among other sources of enzymes. It produced 71.5% $(GlcNAc)_2$, 19% GlcNAc and 9.5% of $(GlcNAc)_3$ after incubation for 24 hours.

Recently, our group found effective enzymes which can be used for chitin hydrolysis to produce GlcNAc or $(GlcNAc)_2$ selectively. Klaikherd *et al.*[58] studied the hydrolysis of chitin by using chitinolytic enzyme from *para* rubber tree. They found that this enzyme can hydrolyze 300 mg of chitin to give mixture between 39 mg of GlcNAc and 108 mg of $(GlcNAc)_2$. They also suggested that chitin can be hydrolyzed by mixing enzyme from rubber serum with enzyme from *Aspergillus niger* to give only GlcNAc in 50% yield. Auynirundornkul[59] found that crude enzyme from fungi *Aspergillus fumigatus* showed the highest activity in chitin hydrolysis. The major product of this enzyme was GlcNAc (74% yield) at the optimum condition. Prakobkij[60] found that chitin hydrolyzed with enzyme Chi60 from cloned *Serratia sp.* can produce $(GlcNAc)_2$ as a major product in 42% yield with GlcNAc as a minor-product. By decreasing enzyme/chitin ratio, $(GlcNAc)_2$/GlcNAc ratio could be increased.

1.4 Purification of chitin enzymatic hydrolysis product

Although the enzymatic hydrolysis of chitin has been studied for many years, the purification procedure of these products in large scale remained expensive and complicated. The known process used for purification of oligosaccharides is gel permeation chromatography (GPC)[38] which was used for the separation of compound having different molecular weight. Since this method can generally separates only small amount of mixture, this technique is not suitable for manufacture.

Aiba and Muraki[61] investigated the precipitation of chitooligosacchatide from the hydrolysis of chitosan (22% degree of acetylation) in milligram scale with hemicellulase by using methanol and found that higher oligosaccharide precipitated at lower amount of methanol than lower oligosaccharide. This separation process is simple and does not include a chromatographic step.

In the development process for efficient enzymatic hydrolysis of chitin and chitosan, immobilized enzyme was employed for a continuous production of oligosaccharides. Jeon and Kim[62] also applied an ultrafiltration membrane in enzymatic reactor system for continuous preparation of chitosan oligomers. In addition, Matsuoka *et al.*[63] 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. Haynes *et al.*[64] developed the continuous process for producing GlcNAc by packing chitin in the column and flow chitinase enzyme through the column. The ultrafiltration was used to separate the high molecular weight protein from low molecular weight product. GlcNAc with 98% purity was obtained from the filtrate stream. The major contaminant was $(GlcNAc)_2$.

1.5 Column chromatography by activated charcoal

Activated charcoal is one of the stationary phases used in the adsorption mode of chromatography. Activated charcoal, charcoal prepared by activation at 1000 °C or higher, is non polar charcoal and exhibits a predominantly carbon or graphitic surface. On the other hand, polar charcoal, prepared by low temperature oxidation, is covered with various oxygenated group such as hydroxyl, carbonyl, etc. Polar charcoal shows preferential adsorption of polar and unsaturated molecules. While activated charcoal, nonpolar charcoal, shows selectively adsorption of larger molecules. Therefore, the

separation of sugars on charcoal, monosaccharides are eluted first and followed, in order, by disaccharides and higher oligosaccharides.[65]

1.6 Liquid Chromatography/Mass spectrometry (LC/MS/MS)

In this work, LC/MS/MS was used to detect *N*-acetyl-D-glucosamine, GlcNAc, and *N,N'*-diacetylchitobiose, $(GlcNAc)_2$, separated from activated charcoal column. In general, MS/MS was connected to HPLC which was used for separation of the mixture of sample. But in this thesis, the sample was separated by activated charcoal column. The LC part was thus used only as an injector to MS/MS.

Mass spectrometry composed of 3 major parts; ionization, mass analyzers and ion detector. Electrospray ionization (ESI) is an ionization technique used for ionizing the analyzed molecules. Sample solution was injected through spray needle tip with high voltage to generate ion on the surface of sample droplets. Nitrogen gas was blown to evaporate the solvent. When the droplets are getting smaller the repulsion between ions was occurred. The shrinking droplet was then separated into smaller droplet called "coulombic explosion". Coulombic explosion occurred until analyte ions were obtained and flowed into mass analyzers.

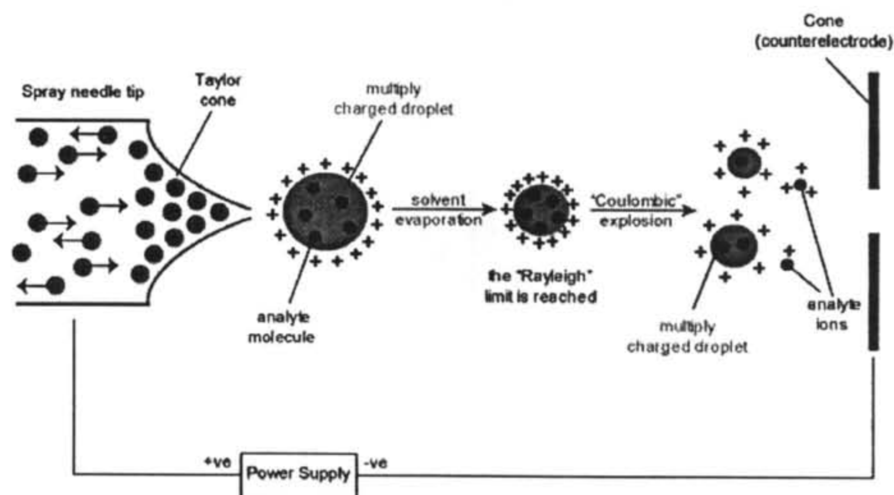

Figure 1.8 Electrospray ionization

Two quadrupoles were used in mass analyzer units. The collision cell was presented between these quadrupoles. These two quadrupoles can scan mass in range from 50 – 2000 m/z. At the collision cell, parent ion was broken into daughter ions.

In multiple reaction monitoring (MRM) mode, sample was injected through injection loop and detected by MS/MS. First MS is quadrupole which is used for

selecting the interesting parent ion. Second MS is another quadrupole which is used for selecting the interested daughter ion for the confirmation that the parent ion from MS1 is actually the interested species. The collision cell is presented between MS1 and MS2 to break parent ion into daughter ions.

1.7 Aims of thesis

This thesis focuses on the preparation of pure *N*-acetyl-D-glucosamine and *N,N'*-diacetylchitobiose by enzymatic hydrolysis of chitin using two difference enzymes; fungal enzyme from *Aspergillus fumigatus* and bacterial enzyme from cloned *Serratua sp*. Fungal enzyme, containing endochitinase and exochitinase, produced mainly *N*-acetyl-D-glucosamine, while bacterial enzyme, containing endochitinase, produced *N,N'*-diacetylchitobiose as a major product. The attempt to purify the sugar products in the hydrolysate obtained from enzymatic hydrolysis was performed by precipitation and activated charcoal column.