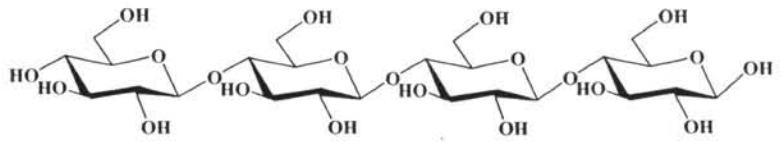
CHAPTER I

INTRODUCTION

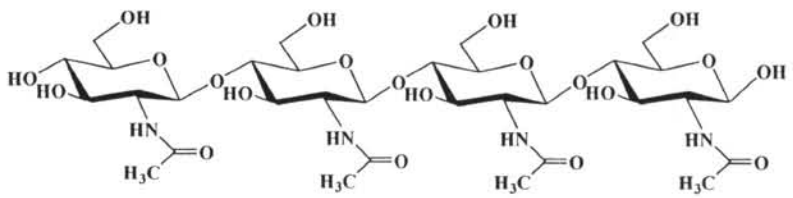
1.1 Chitin and Chitosan

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 formed 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]



(a) Cellulose



(b) Chitin

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 revealed an antiparallel arrangement of two adjacent polysaccharide chains[3] (**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 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. 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-glycosidic 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.

α-Chitin

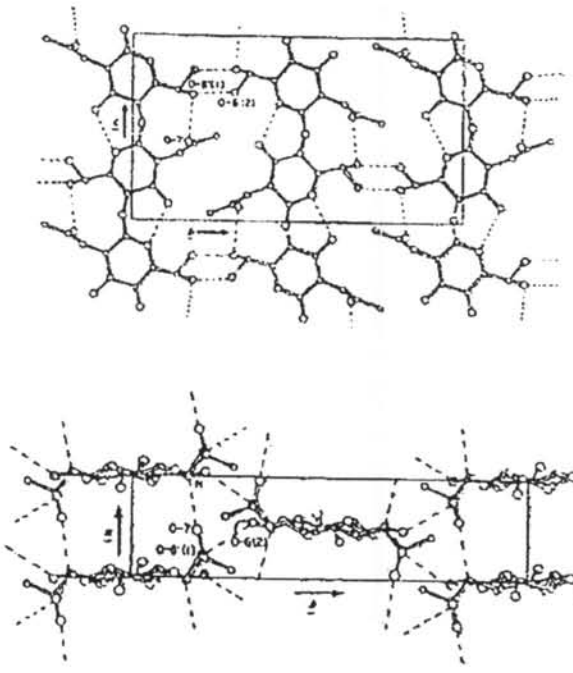


Figure 1.2 Diagrammatic illustration of antiparallel arrangement and X-ray crystal structure of hydrogen bond linkage between O=CNH groups of α-chitin.

β-Chitin

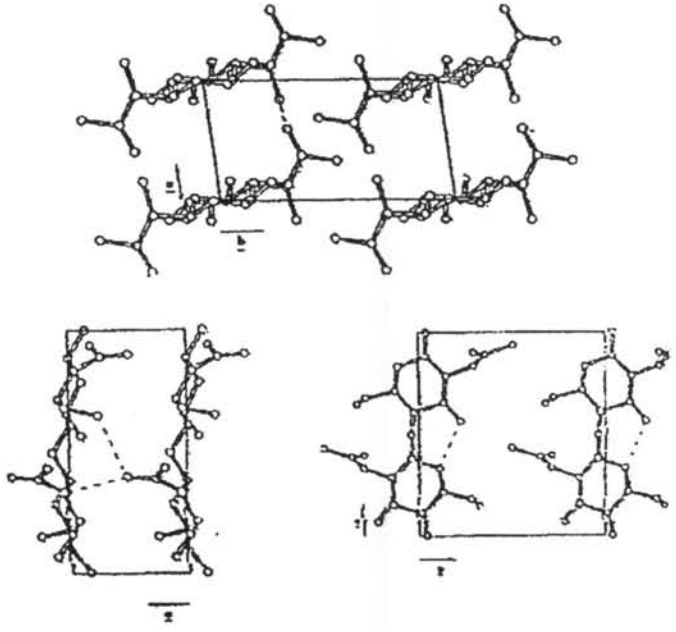


Figure 1.3 Diagrammatic illustration of parallel arrangement and X-ray crystal structure of hydrogen bond linkage between to O=CNH groups of β-chitin.

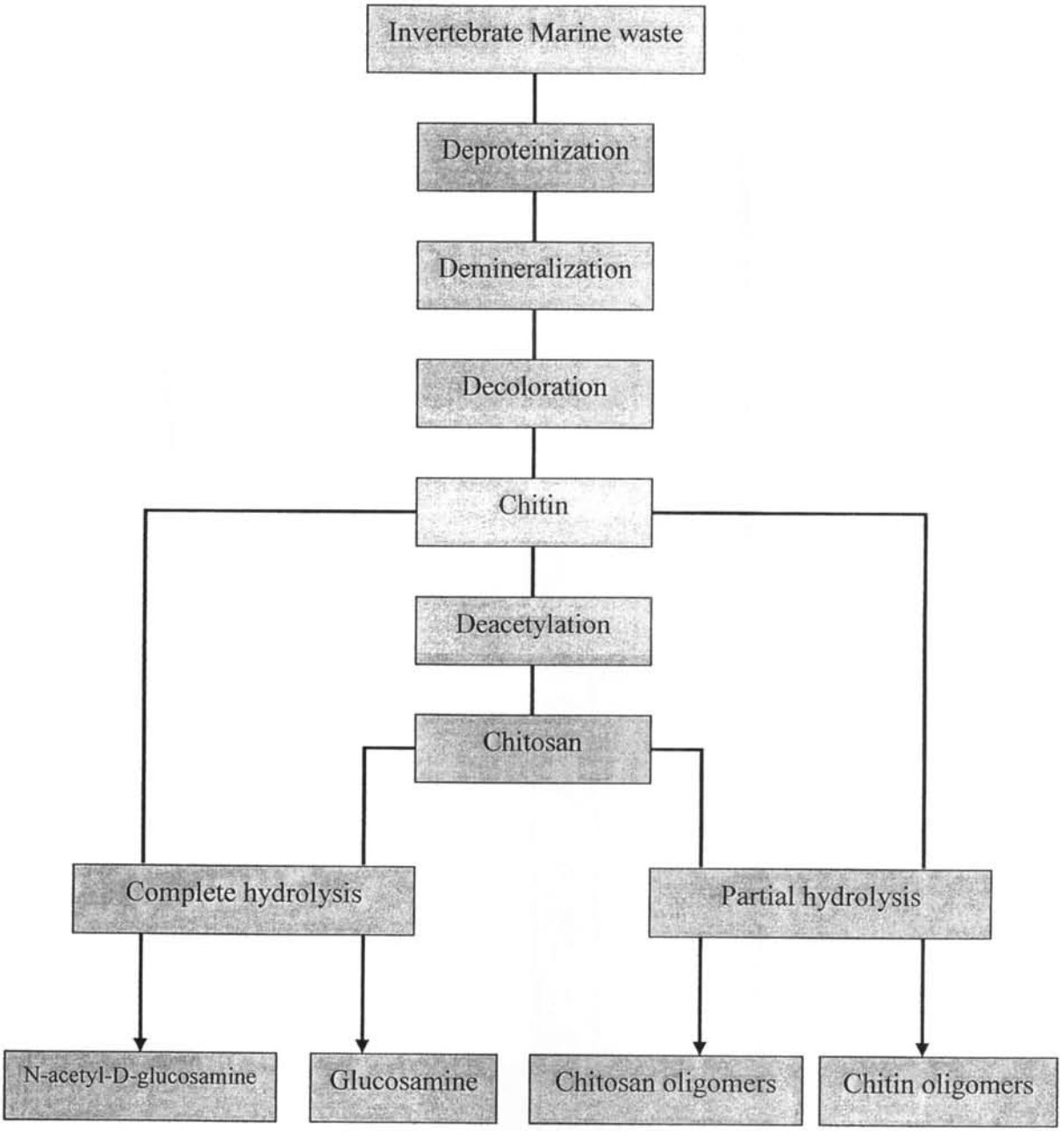


Figure 1.4 Simplified flow-chart for the preparation of chitin, chitosan, their monomers and oligomers from invertebrate marine waste

1.2 Applications of *N*-acetyl-*D*-glucosamine and glucosamine hydrochloride

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 compounds (**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]

Shikhman *et al.*[14] reported that glucosamine and its derivatives, including *N*-acetylglucosamine, were some of the most commonly used drugs to treat osteoarthritis. However, the mechanisms of their antiarthritic activities still poorly understood. Recently, Hatano *et al.*[15] reported that the long-term intake of soymilk beverage containing GlcNAc improved the subjective symptom and range of motion in subjects with slight pain, stiffness, and/or discomfort at knee joint.

Xing *et al.*[16] reported that the multiple antioxidant activity of glucosamine hydrochloride was evident as it showed considerable reducing power, superoxide/hydroxyl-radical scavenging ability. These *in vitro* result suggest the possibility that glucosamine hydrochloride could be effectively employed as an ingredient in health or functional food, to alleviate oxidative stress.

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	

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

N-acetyl-D-glucosamine is a monomer of chitin and chitooligosaccharides are the oligomers of β-(1→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 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.*[17] explained that the enzymatic hydrolysis was a useful method for the preparation of oligomers from chitin and chitosan because it could produce (or yield) specific products.

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.[17] Chitinase is the enzyme 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**).

Amano and Ito[19] 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[20] prepared chitooligosaccharides from hydrolysis of chitin slurry by chitinase from *Vibrio parahemolyticus* and *Streptomyces griseus*. They reported that the major product of this hydrolysis reaction is *N,N'*-diacetylchitobiose. The increasing of temperature increased the yield of $(GlcNAc)_4$ and $(GlcNAc)_5$.

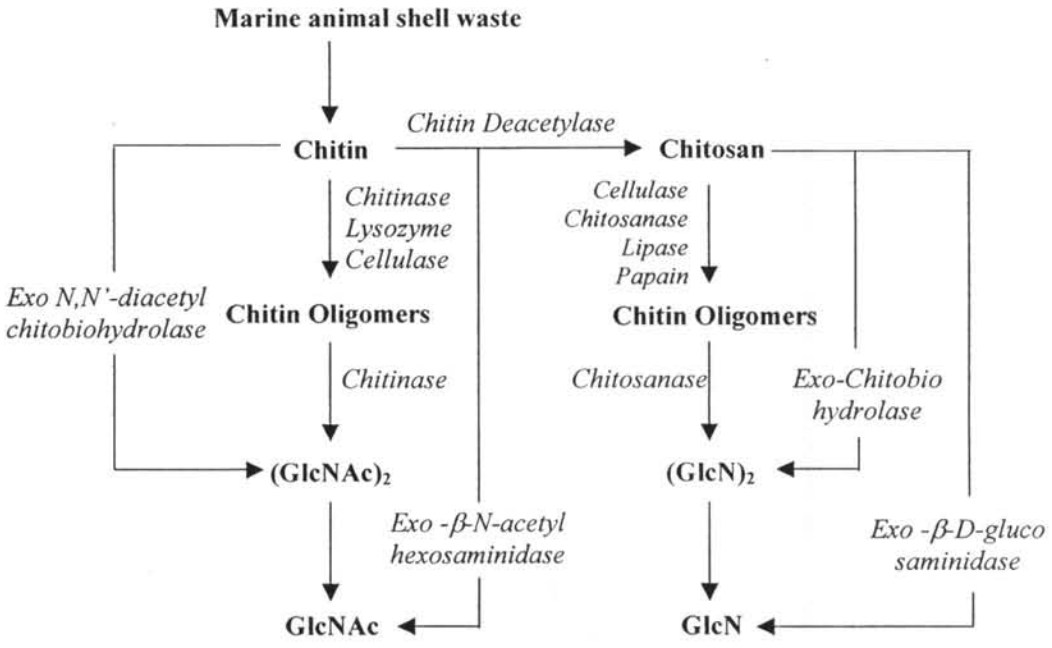


Figure 1.5 Pathway for the conversion of chitin and chitosan into their oligomers by enzymatic means.

Aiba[21] also suggested that, in the case of degradation of chitin by chitinase, hydrolyzed sites could not be regulated by the enzyme. If chitosan was 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 was 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.

Sukwattanasinitt *et al.*[22] 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. In addition, Pichyangkura *et al.*[23] used crude chitinase from *Burkholderia cepacia* TU09 and *Bacillus lichenniformis* SK-1 to digest α- and β-chitin powder. The results from this work suggested that certain enzymes was able to hydrolyze crystalline chitin to give GlcNAc in high yield (>70%).

Although a number of chitinases and chitosanases have been isolated from microorganisms over the past two decades, their costs remain too high to be utilized in the industrial process.

1.3.2 Chemical hydrolysis

Chemical method for the preparation of GlcNAc and chitooligosaccharides mostly deals with acid hydrolysis.[24-26] Recently, the serie of chitooligosaccharides has become commercially available. They are usually prepared by hydrolysis of chitin and chitosan with concentrated hydrochloric acid, followed by extensive column chromatographic fractionation.[24] 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) lypohilization.[25]

Horowitz *et al.*[27] 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, associated 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 1.6**).

Inaba *et al.*[28] 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.*[26] noted that fluorohydrolysis of chitin in anhydrous hydrogen fluoride (HF) led to chitin oligomers in almost quantitative yield. The conditions could also be conveniently monitored in order to optimize the preparation of specific oligomers ranging from 2 to 9 residues. However, major products of chitin oligomers 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.*[29] reported a production of chitin oligomers by a combination method of mild acid degradation and sonolysis, which was able to degrade chitin not depending on the temperature of the bulk solution.

Moreover, the preparation of these small carbohydrate molecules is also achieved by a free radical reaction. Nordtveit *et al.*[30] 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.*[31] 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. This phenomenon may help to explain the disappearance of chitosan *in vivo* during biomedical applications.

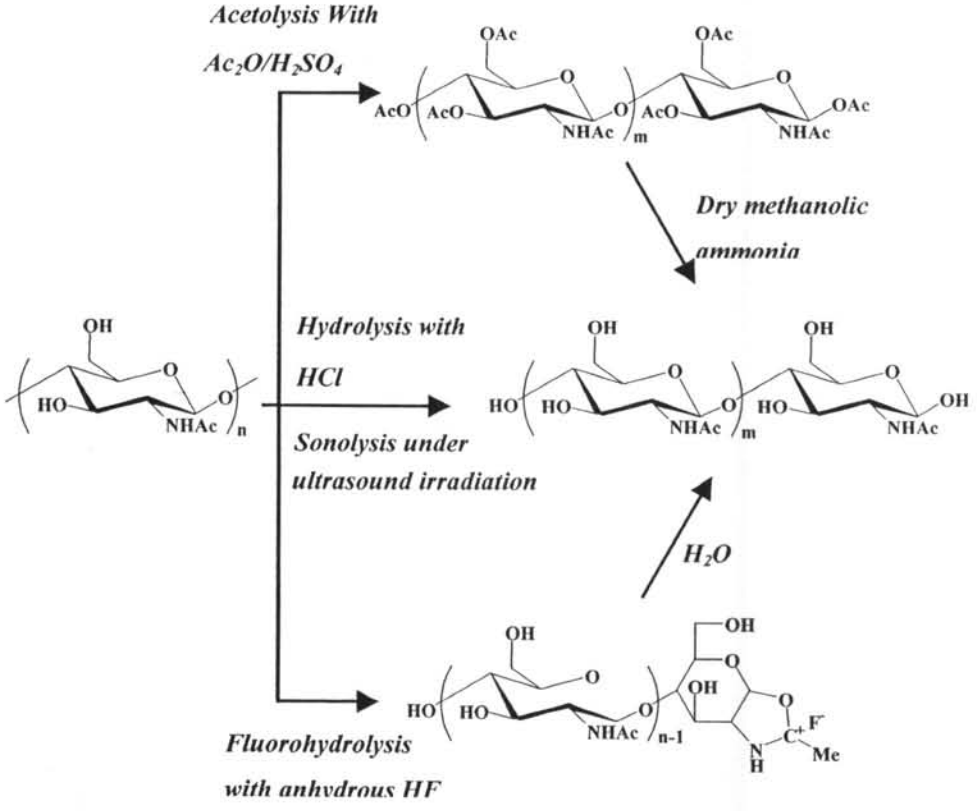


Figure 1.6 Reactions for acid hydrolysis of chitin.

Purchase and Braun[32] reported the chitin (40g) was hydrolyzed by concentrated hydrochloric acid (12 M, 200 mL) and the mixture was heated on a boiling water bath for 2.5 hours with mechanical stirrer. Water and Norit were added into the solution. The hydrolysis temperature of about 60°C for an hour and was stirred continuously during the process of decolorization. After an hour the solution was filtered and the filtrate is concentrated under diminished pressure. The white crystals of glucosamine hydrochloride are washed with 95%ethanol and dried, 67 % yield and 95% purity.

Pupley[33] studied over a range of acid concentration and temperature in acid hydrolysis of chitin. The chitin concentration 20 mg/mL was hydrolyzed by acid. No free reducing groups could be detected after completion of reaction, which were performed at 0°C. The reaction mixture was transferred to 40°C. The samples were analyzed for sugar content of reducing end by ferro-ferric cyanide method and measured the amount of deacetylated amino sugar by ninhydrin method. They found that the rate of hydrolysis upon acid concentration and temperature.

Novikov and Ivanov[34] prepared GlcNHCl from hydrolysis of chitin (100g) by concentrated hydrochloric acid (200g) at temperature of 95°C for 2 hours. After that the reaction mixture was cooled to room temperature for 24 hours to form GlcNHCl crystal salts. The reaction mixture was filtered and washed with ethanol (194 g). The white GlcNHCl crystal was obtained in 70%yield and 100%purity (by pH-titration).

Gandhi and Laidlhi[35] reported the preparation of GlcNHCl salt from hydrolysis of chitin, 20 mesh, by concentrated HCl. The ratio of chitin/concentrated HCl is 1:2 (w:w). The concentrated HCl was pre-heated until 65°C follow of the addition of chitin. The reaction was heated to temperature of 95°C for 75 min. The reaction mixture was cooled to room temperature and filtered. The precipitate was added water and activated char coal, stirred for an hour and filtered. The filtrate was dried by rotary evaporator and washed by 95%ethanol. The GlcNHCl was obtained in 70%yield and 100%purity.

Varum.*et al.*[36] studied the hydrolysis of the glycosidic linkages (depolymerization) and the *N*-acetyl linkage (de-acetylation) of the chitosan in dilute and concentrated HCl acid. The hydrolysis rate of glycosidic linkage was found to be equal the rate of de-acetylation in dilute acid at temperature of 83°C, while the glycosidic linkage was hydrolyzed more than 10 times faster than the *N*-acetyl linkage in concentrated HCl at temperature of 30°C.

The hydrolysis of chitin and chitosan by conc. HCl at temperature of 70°C were studied by Novikov [37]. They found the amount of GlcNAc, analzed by UV-vis spectrophotometer, increase in initial period of 50 min because the glycosidic linkage was hydrolyzed and then decrease because of the hydrolysis of amide bond to produced GlcNHCl salt.

Gizatulina. *et al.* [38] reported the kinetic acid hydrolysis of GlcNAc. They found the rate constant of acid hydrolysis of amide bond in chitin is similar to that in its oligomer and GlcNAc monomer, which synthesitzed by the method of Roseman and Ludowieg. The dependence of the rate of GlcNAc deacetylation on the hydrochloric acid concentration passed through a maximum at 8 molar.

Chen. *et al.* [39] studied the effect of ultrasonic conditions and storage in acetic acid on change in molecular weight and polydispersity (molecular weight distribution) of treated chitosan at room temperature. They found the degradation of chitosan increased with prolonged ultrasonic time. Chitosan was degraded during storage in an acidic solution. The polydispersity decreased with ultrasonic treatment for all ultrasonic conditions studied and during storage in acidic solution. The chitosan was hydrolyzed faster in dilute solution and faster in lower temperature solution.

The effect of sonication during chitin extraction from freshwater prawn shell were studied by Kjantansson. *et al.*[40] They found that the application of ultrasonic to remove protein enhanced the amount of chitin. The glucosamine content slightly decreased with sonication probably because of losses due to depolymerization. The degree of acetylation of chitins was unaffected by sonication.

1.4 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. 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 droplets called "coulombic explosion". Coulombic explosion occurred until analyte ions were obtained and flowed into mass analyzers.

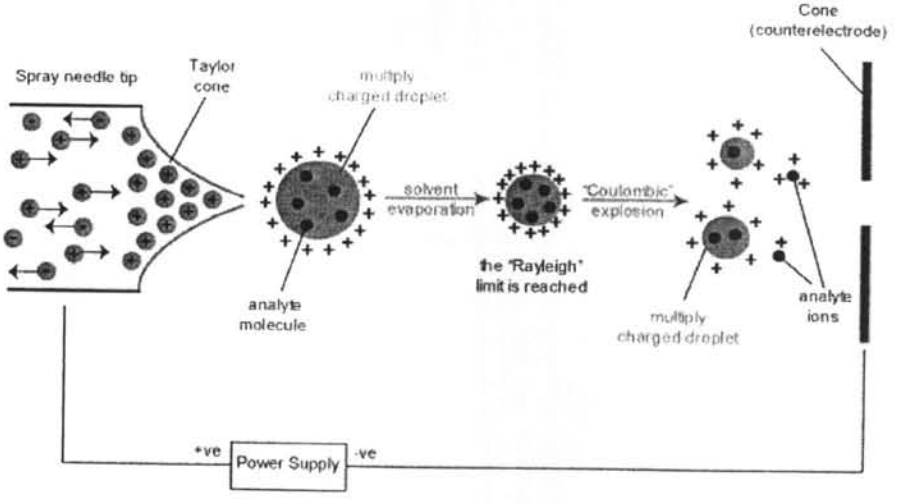
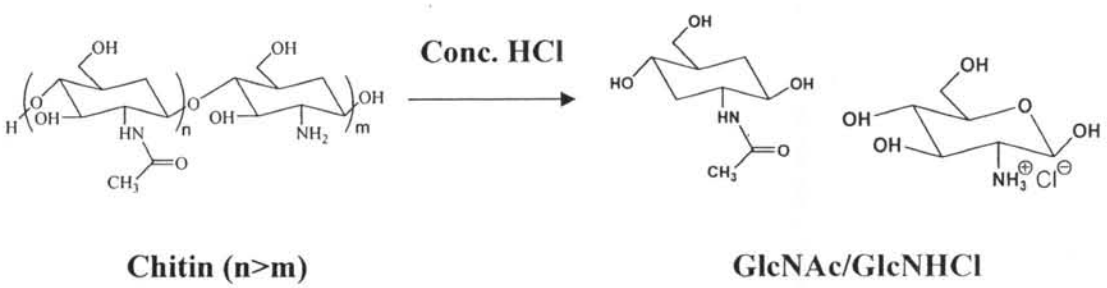


Figure 1.7 Electrospray ionization

1.5 Aims of thesis

The aim of this work is to depolymerize chitin into GlcNAc by using low temperature acid hydrolysis assisted by ultrasonic wave. It was hypothesized that the energy provided by ultrasonic wave could reduce the hydrogen bonding between chitin chains resulting in greater accessibility to oxygen protonation. This leads to the solubilization in concentrated hydrochloric acid and thus increase the efficiency of the acid hydrolysis without the need to elevate the temperature. The effects of hydrolysis parameter such as the reaction temperature, hydrolysis time and the chitin/acid ratio on the yields of GlcNHCl and GlcNAc and the product ratio were investigated (Scheme 1).



Scheme 1.1 Acid hydrolysis of chitin