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PREPARATION OF GLUCOSAMINE HYDROCHLORIDE FROM α-CHITIN BY MICROWAVE ASSISTED ACID HYDROLYSIS

Miss Sulaleewan Supsvetson

A Thesis Submitted in Partial Fulfillment of the Requirements
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เกลือกลูโคซามีนไฮโดรคลอไรด์เป็นยาที่ใช้ในการบรรเทาอาการเจ็บปวดข้อกระดูกกับผู้ป่วยที่เป็นโรคออสทีโออาร์ไธรทิส (osteoarthritis) การไฮโดรไลซิสแอลฟาไคตินจากเปลือกกุ้งในกรดไฮโดรคลอริกเข้มข้นภายใต้อุณหภูมิสูงเป็นวิธีปกติที่ใช้ในการผลิตเกลือกลูโคซามีนไฮโดรคลอไรด์ เพื่อเร่งระยะเวลาในกระบวนการไฮโดรไลซิสจึงได้นำคลื่นไมโครเวฟมาช่วยเร่งปฏิกิริยาในการงานวิจัยนี้ ซึ่งพบว่าการย่อยโดยใช้คลื่นไมโครเวฟช่วยลดระยะเวลาในการเกิดปฏิกิริยาไฮโดรไลซิสเมื่อเปรียบเทียบการย่อยด้วยวิธีดั้งเดิม คือใช้เวลาในการทำปฏิกิริยาเพียง 12 นาทีเท่านั้นเพื่อให้เกิดการย่อยอย่างสมบูรณ์ในขณะที่วิธีให้ความร้อนแบบดั้งเดิมต้องใช้เวลา 90-120 นาที โดยได้เปอร์เซ็นต์ผลิตภัณฑ์ประมาณ 55 เปอร์เซ็นต์ และมีความบริสุทธิ์ 99-100 เปอร์เซ็นต์

สาขาวิชาปิโตรเคมีและวิทยาศาสตร์พอลิเมอร์ ลายมือชื่อนิสิต...

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SULALEEWAN SUPSVETSON : PREPARATION OF GLUCOSAMINE HYDROCHLORIDE FROM α-CHITIN BY MICROWAVE ASSISTED ACID HYDROLYSIS. THESIS ADVISOR : ASSOC. PROF. MONGKOL SUKWATTANASINITT, Ph.D., THESIS CO-ADVISOR ANAWAT AJAVAKOM, Ph.D. 63 pp.

Glucosamine hydrochloride (GlcNHCl) is a well known neutrapharmaceutical agent prescribed for osteoarthritis patients. Hydrolysis of shrimp shell α-chitin in concentrated hydrochloric acid under elevated temperature is a general method for production of GlcNHCl. To speed up the hydrolysis process, microwave assisted hydrolysis is studied in this work. With microwave irradiation, the hydrolysis is faster comparing to the conventional heating. Only 12 minutes of reaction time is required to complete the hydrolysis when the microwave is utilized while 90-120 minutes is generally required for conventional heating. The reaction typically gave 55% isolated yield with 99-100% pure of GlcNHCl.

Field of Study : Petrochemical and Polymer Science Student's Signature…………..
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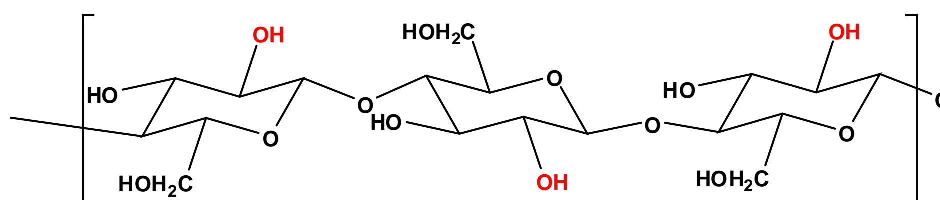
CHAPTER I

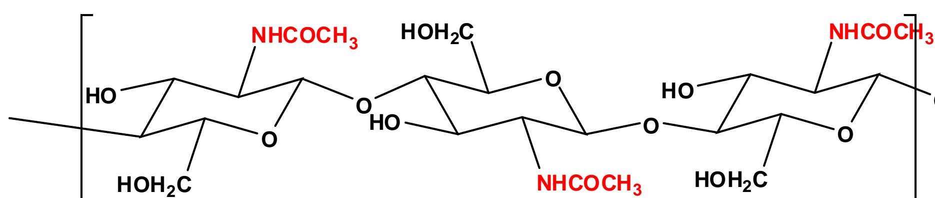
INTRODUCTION AND THEORY

1.1 Chitin and Chitosan

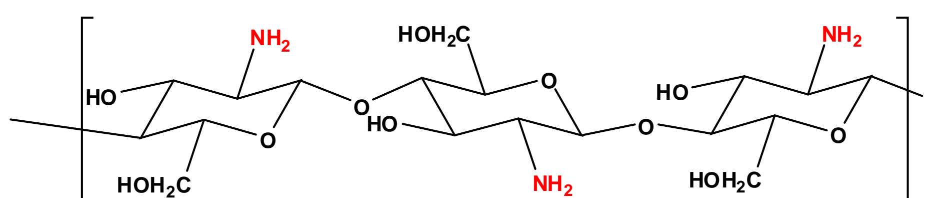
Chitin, a naturally abundant mucopolysaccharide, and the supporting material of crustaceans, insects, etc., is well known to consist of 2-acetamodo-2-deoxy-β-D-glucose through a β (1→4) linkage. Chitin can be degraded by chitinases. Its immunogenicity is exceptionally low, in spite of the presence of nitrogen. It is highly insoluble and has low chemical reactivity. It may be regarded as cellulose with hydroxyl at position C-2 replaced by an acetamido group. Chitin is a white, hard, inelastic, nitrogenous polysaccharide and the major source of surface pollution in coastal areas. Chitosan is the *N*-deacetylated derivative of chitin, although this *N*-deacetylation is almost never complete. A sharp nomenclature with respect to the degree of *N*-deacetylation has not been defined between chitin and chitosan [1, 2]. The structures of cellulose, chitin and chitosan are shown in Figure 1.1. Chitin and chitosan are of commercial interest due to their high percentage of nitrogen (6.89%) compared to synthetically substituted cellulose (1.25%). This makes chitin a useful chelating agent [1]. As most of the present-day polymers are synthetic materials, their biocompatibility and biodegradability are much more limited than those of natural polymers such as cellulose, chitin, chitosan and their derivatives. However, these naturally abundant materials also exhibit a limitation in their reactivity and processability [3, 4]. In this respect, chitin and chitosan are recommended as suitable functional materials, because these natural polymers have excellent properties such as biocompatibility, biodegradability, non-toxicity, adsorption properties, etc.



(a) Cellulose



(b) Chitin



(c) Chitosan

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

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 [5, 6]. Chitin and chitosan offer wide range of applications, including clarification and purification of water and beverages, applications in pharmaceuticals and cosmetics, as well as agricultural, food and biotechnological uses [7, 8]. 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, function 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 immune-enhancing, antibacterial activity, hypocholesterolemic activity, and antihypertensive action [7].

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 monomers and oligomers (Figure 1.2). The monomers and oligomers of chitin and chitosan have low viscosity due to their low molecular weight and short-chain lengths that allow them to be readily soluble in neutral aqueous solution and absorbed in the *in vivo* system.

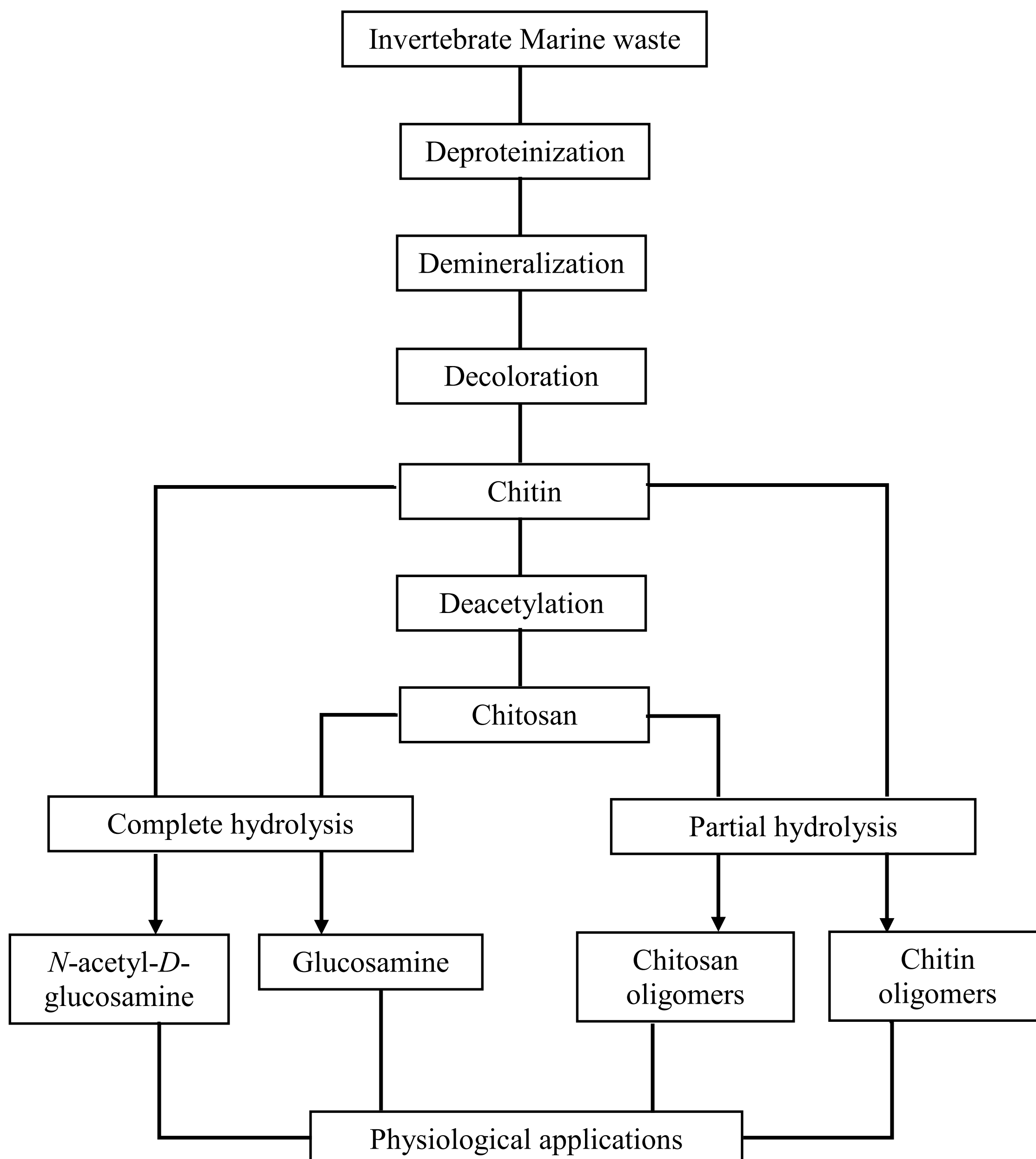


Figure 1.2 Simplified flow chart for the preparation of chitin, chitosan, their monomer and oligomers from invertebrate marine waste.

1.2 Properties of chitin and chitosan

Most of the naturally occurring polysaccharides, e.g. cellulose, dextran, pectin, alginic acid, agar, agarose and carragenans, are neutral or acidic in nature, whereas chitin and chitosan are examples of highly basic polysaccharides. Their unique properties include polyoxysalt formation, ability to form films, metal ions chelation and optical characteristic structure [9].

Like cellulose, chitin functions naturally as a structural polysaccharide, but differs from cellulose in its properties. It's high hydrophobicity makes chitin insoluble in water and almost organic solvents. However it is soluble in hexafluoroisopropanol, hexafluoroacetone, chloroalcohols in conjugation with aqueous solutions of mineral acids [10] and dimethylacetamide containing 5% lithium chloride. Chitosan, the deacetylated product of chitin, is soluble in dilute acids such as acetic acid, formic acid, etc. Recently, the gel forming ability of chitosan in *N*-methylmorpholine *N*-oxide and its application in controlled drug release formulations has been reported [11-13]. The hydrolysis of chitin with concentrated acids under drastic conditions produces relatively pure D-glucosamine.

The nitrogen content of chitin varies from 5 to 8% depending on the extent of deacetylation, whereas the nitrogen in chitosan is mostly in the form of primary aliphatic amino groups. Chitosan, therefore, undergoes reactions typical of amines, of which *N*-acylation and Schiff reaction: are the most important. Chitosan derivatives are easily obtained under mild conditions and can be considered as substituted glucans.

1.2.1 Degree of *N*-acetylation

An important parameter to examine closely is the degree of *N*-acetylation of chitin, i.e. the ratio of 2-acetamido-2-deoxy-D-glucopyranose to 2-amino-2-deoxy-D-glucopyranose structural units. This ratio has striking effect on chitin solubility and solution properties. Chitosan is the universally accepted non-toxic *N*-deacetylated derivative of chitin, where chitin is *N*-acetylated to such an extent that it becomes soluble in dilute aqueous acetic and formic acids. Chitosan is the fully or partially *N*-deacetylated derivative of chitin with a typical degree of acetylation of less than 0.35. To define this ratio, attempts have been made with many analytical tools [14-23], which include IR spectroscopy, pyrolysis gas chromatography, gel permeation chromatography and UV spectrophotometry, first derivative of UV spectrophotometry, ^{1}H-NMR spectroscopy, ^{13}C solid state NMR, thermal analysis, various titration schemes, acid hydrolysis and HPLC, separation spectrometry methods and, more recently, near-infrared spectroscopy [24].

1.2.2 Molecular weight

The weight-average molecular weight (M_w) of chitin and chitosan has been determined by light scattering [25]. Viscometry is a simple and rapid method for the determination of molecular weight; the constants α and K in Mark-Houwink equation

have been determined in 0.1 M acetic acid and 0.2 M sodium chloride solution. The intrinsic viscosity is expressed as

$$[\eta] = KM^{\alpha} = 1.81 \times 10^{-3}\, M^{0.93}$$

The charged nature of chitosan in acid solvents and chitosan's propensity to form aggregation complexes require care when applying these constants. Furthermore, converting chitin into chitosan lowers the molecular weight, changes the degree of deacetylation, and thereby alters the charge distribution, which in turn influences the agglomeration. The weight-average molecular weight of chitin is 1.03×10^6 to 2.5×10^6, but the *N*-acetylation reaction reduces this to $1 \times 10^5 \sim 5 \times 10^5$ [26].

1.2.3 Solvent and solution properties

Both cellulose and chitin are highly crystalline, intractable materials and only a limited number of solvents are known to be applicable as reaction solvents. Chitin and chitosan degrade before melting, which is typical for polysaccharides with extensive hydrogen bonding. This makes it necessary to dissolve chitin and chitosan in an appropriate solvent system to impart functionality. For each solvent system, polymer concentration, pH, counterion concentration and temperature effects on the solution viscosity must be known. Comparative data from solvent to solvent are not available. As a general rule, the maximum amount of polymer is dissolved in a given solvent towards a homogeneous solution. Subsequently, the polymer is regenerated in required form. A coagulant is required for polymer regeneration or solidification. The nature of the coagulant is also highly dependent on the solvent and solution properties as well as the polymer used [27-28].

1.3 Applications of chitin, chitosan and their monomers and oligomers

The interest in chitin originates from the study of the behavior and chemical characteristics of lysozyme, an enzyme present in human body fluids [29]. A wide variety of medical applications for chitin and chitin derivatives have been reported over the last three decades [30-32]. It has been suggested that chitosan may be used to inhibit fibroplasias in wound healing and to promote tissue growth and differentiation in tissue culture [33].

The poor solubility of chitin is the major limiting factor in its utilization. Despite this limitation, various applications of chitin and modified chitins have been reported, e.g. as raw material for man-made fibers [27]. Fibers made of chitin and

chitosan are useful as absorbable sutures and wound-dressing materials [34,27,35]. Chitin sutures resist attack in bile, urine and pancreatic juice, which are problem areas with other absorbable sutures [34]. It has been claimed that wound dressings made of chitin and chitosan fibers have applications in wastewater treatment. Here, the removal of heavy metal ions by chitosan through chelation has received much attention [28-36]. Their use in the apparel industry, with a much larger scope, could be a long-term possibility [37].

Unlike cellulose, chitin, chitosan 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 [38-39]. Chitin oligomers are active as elicitors for defending mechanism of higher plants, whereas chitosan oligomers have almost no eliciting activity [40-41].

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
	Inmunopotentialting agents	Inmunopotentialting agents
	Carrier for drug delivery system	
Medical	Accelerator foe wound healing	Osteoarthritis and inflammatory
	Artificial skin	bowel disease treatment
	Fiber for absorbable sutures	
Nutritional	Dietary fiber	Hypocholesterolemic agents
	Hypocholesterolemic agents	Calcium absorption accelerator
	Antihypertensive agents	*in vitro*
Biotechnological	Carrier for immobilized	
	enzyme and cell	
	Porous beads for bioreactors	
	Resin for chromatography	
	Membrane materials	
Agriculture	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	

1.4 Osteoarthritis

Osteoarthritis (OA) is the most common form of arthritis and is a major cause of disability in people aged over 65 years. The disease most frequently affects weight-bearing joints, such as the medial tibiofemoral compartment of the knee and the prevalence of radiological OA in people aged over 65 years is approximately 30% [42]. Although the disease affects all societies and races, the prevalence and distribution of joints involved varies. Women particularly have a greater risk of developing the disease than men [43]. The major clinical features of OA are pain and stiffness, leading to a decline in physical function which may ultimately require joint replacement surgery. While the major pathological feature of OA is articular cartilage degeneration, there is new evidence that changes in bone morphology may play a role in disease initiation [44]. With increasing disease severity, pain, swelling, loss of cartilage, bone spur formation and decreased range of motion can occur. Despite these changes in joint morphology, the etiology of this condition remains unclear. Recently, there has been a growing interest in the biomechanical factors associated with the pathogenesis of OA.

Glucosaminoglycans (mucopolysaccharides) are large complexes of negatively-charged carbohydrate chains that are incorporated into mucous secretions, connective tissue, skin, tendons, ligaments and cartilage. Glucosamine and its acetylated derivatives, *N*-acetylglucosamine, are readily synthesized in the body from glucose. Because of its high concentration in joint tissues, the hypothesis that glucosamine supplements would provide symptomatic relief for osteoarthritis was developed more than 30 years ago [45]. Many clinical trials have tested this hypothesis and glucosamine supplements are widely used to relieve arthritic complaints [46].

To meet the demand for glucosamine nutritional supplement, three forms of glucosamine are commonly available: glucosamine hydrochloride, glucosamine sulfate, and *N*-acetyl-glucosamine. These glucosamine compounds are generally derived from chitin, a biopolymer present in the exoskeleton of marine invertebrate animals. The glucosamine derived from chitin in the cell walls of many fungi appears to be chemically identical to that found in marine invertebrates.

Glucosamine is a prominent component of the hexosamine pathway, an important branch of glycolysis. Exogenous glucosamine is actively transported from extracellular tissue into cells by glucose transporters (Figure 1.3) [47]; insulin

facilitates glucosamine transport into cells [48]. Glucosamine is phosphorylated by one of the hexakinase families to glucosamine-6-phosphate (GlucN-6-P). Endogenous GlucN-6-P is formed from fructose-6-phosphate and glutamine by GlucN-6-P synthetase, commonly called glucosamine:fructose-6-P amidotransferase (GFAT) [49]. GFAT irreversibly catalyzes the first and rate-controlling step in the synthesis of uridine diphosphate-*N*-acetylglucosamine (UDP-GlucNAc), a precursor of all macromolecules containing amino sugar. GlucN-6-P is readily converted back to fructose-6-phosphate by glucosamine-6-phosphate deaminase (GNPDA) [50]. GlucN-6-P is acetylated to *N*-acetyl-glucosamine-6-P (GlucNAc-6-P) by glucosamine-phosphate-*N*-acetyltransferase and subsequently converted to UDP-GlucNAc by UDP-*N*-acetyl-glucosamine pyrophosphorylase. In some tissue, GlucNAc-6-P is converted to GlucNAc-1-P by phosphoacetylglucosaminemutase during the formation of UDP-GlucNAc [51]. UDP-GlucNAc can be converted to UDP-*N*-acetylgalactosamine (UDP-GalNAc) by UDP-*N*-acetylglucosamine-4-epimerase [49].

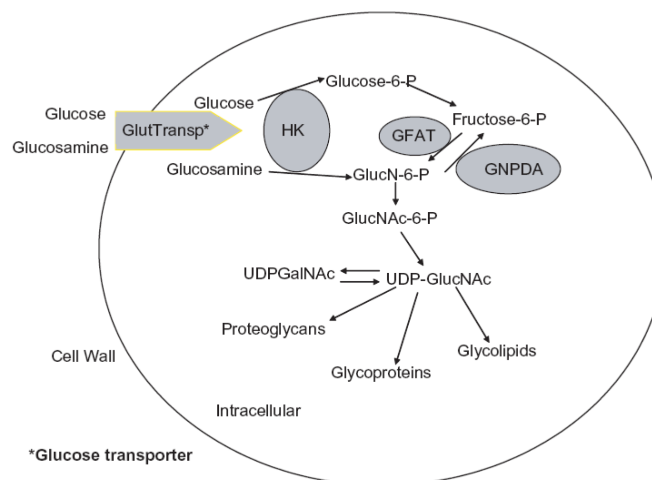


Figure 1.3 Metabolic pathways of glucosamine glucose transporters are indicated by arrow and major enzyme are included in ellipses. Abbreviations are: Glucose-6-P, glucose-6-phosphate; Fructose-6-P, fructose-6-phosphate; GlucN-6-P, glucosamine-6-phosphate; GlucNAc-6-P, *N*-acetyl-glucosamine-6-phosphate; UDPGalNAc, uridine diphosphate (UDP)-*N*-acetyl-galactosamine; UDP-GlucNAc, UDP-*N*-acetyl-glucosamine; HK, Hexokinase; GFAT, glucosamine; fructose-6-phosphate amindotransferase; and GNPDA, Glucosamine-6-phosphate deaminase.

The metabolism of glucosamine is highly regulated by rates of transport into various tissues and by effects of intermediates on key enzymatic steps. For example, in many tissues the affinity of glucosamine for glucose transporters is several-fold lower than for glucose but in some mammalian tissues, the affinity of glucosamine for GLUT2 transporters is higher than for glucose [47]. The affinity of the family of hexokinases in different tissues for glucosamine compared to glucose may also regulate utilization of glucosamine in various tissues. GFAT is unique among the subfamily of amidotransferase enzymes because it does not display any ammonia-dependent activity and requires glutamine as amino donor [51]. GFAT is strongly inhibited by the end-product of this synthetic pathway, UDP-GlucNAc [52]. Ambient testosterone or estrogen levels may affect tissue GFAT activity [51]. Between 2-5% of fructose-6-P or of the flux trough the glycolytic pathway enters the hexosamine pathway *via* glucosamine [51]. In humans the endogenous production of glucosamine is in the range of 4-20 g/day or ~12 g/day [52].

1.5 Hydrolysis of chitin

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, which be normally used for the preparation of monomers and chitooligosaccharides from chitin and chitosan.

1.5.1Enzymatic 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.* [53] 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 3 residues [54]. 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 1.4).

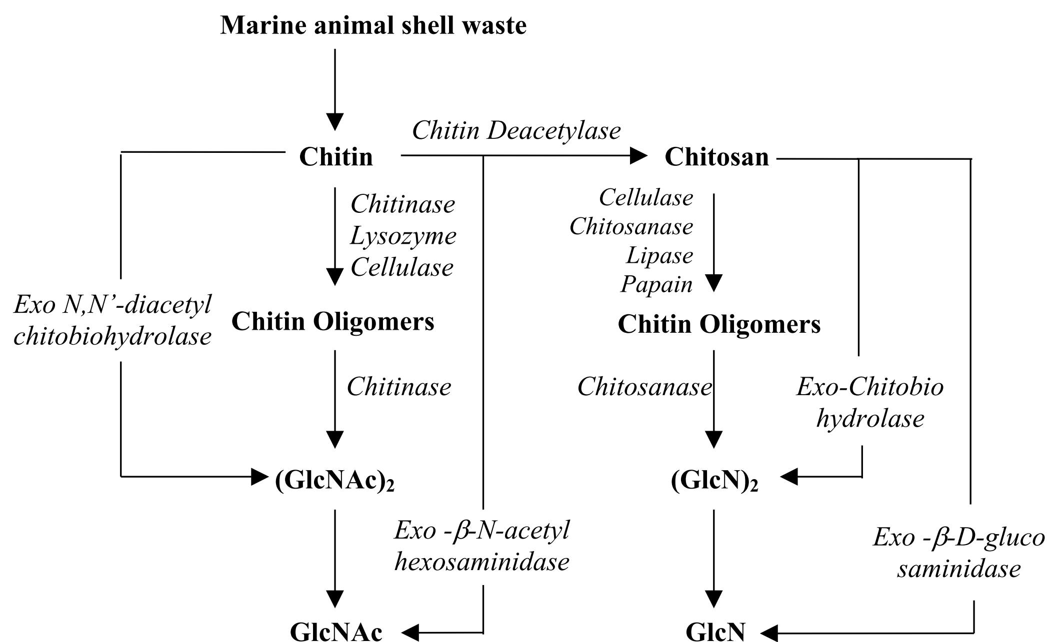


Figure 1.4 Pathway for the conversion of chitin and chitosan into their oligomers by enzymatic hydrolysis.

Takiguchi and Shimahara [55] reported a production of only $(GlcNAc)_2$ from chitin with an enzyme from thermophilic bacterium. Takayanagi *et al.* [56] 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.* [58] 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.* [58] and Mitsutomi *et al.* [59] 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 2-4 residues.

Aiba [60] 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 has partial GlcNAc residues recognized by chitinase. *N*-acetylchitooligo-saccharide with 2-6 residues was successfully prepared from chitosan with chitinolytic hydrolysis followed by *N*-acetylation with acetic anhydride. When 20% acetylated chitosan was hydrolyzed by *Streptomyces griseus* chitinase for 7 days, the yields of $(GlcNAc)_3$, $(GlcNAc)_4$, $(GlcNAc)_5$, and $(GlcNAc)_6$ were 23.5, 25.5, 19.6, and 12.3%, respectively.

According to Fenton and Eveleigh [61], 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.* [62] 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.* [63] 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 1.5).

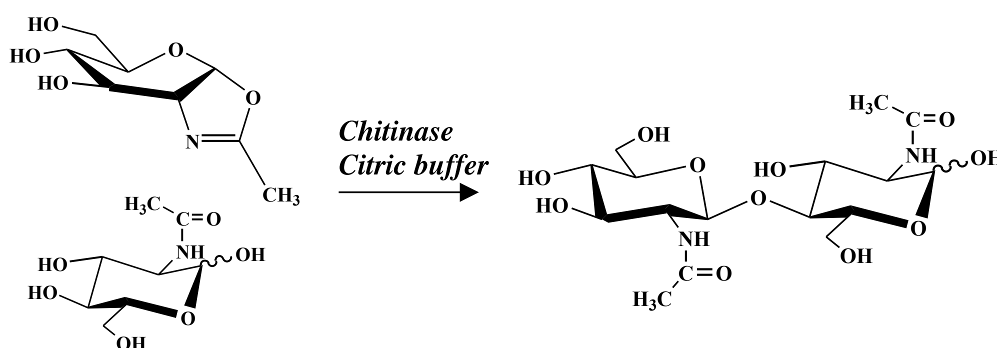


Figure 1.5 Preparation of $(GlcNAc)_2$ by enzymatic transglycosylation.

Usui *et al.* [64] 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 [65] found low-cost enzymes hemicellulase that could yield of hexamer more than 20% when chitosans with 9-22% deacetylated were used. Muzzarelli *et al.* [66] 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*. [67] 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 to produce the GlcNAc with high yield (76%) for 8 days by using crude enzyme from Cellulase *Tricoderma viride*. Sukwattanasinitt *et al*. [68] studied the utilization of commercial non-chitinase enzyme 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, which had high chitinase and β-*N*-acetylhexosaminidase activity. Sashiwa *et al.* [69] 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% without by-product. In addition, Pichyangkura *et al*. [70] used crude chitinase form *Brukholderia cepacia* TU09 and *Bacillus lichenniformis* SK-1 to digest the α- and β-chitin powder. The results 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 [71] also applied an ultrafiltration membrane in enzymatic reactor system for continuous preparation of chitosan oligomers. Matsuoka *et al.* [72] used a dialysis technique in a preparation of *N*,*N′*-diacetylchitobiose by

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

1.5.2 Chemical hydrolysis

Chemical method for the preparation of GlcNAc, GlcN, and chitooligosaccharides mostly deals with acid hydrolysis [73-75]. 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 [73]. 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 [75].

Rupley [73] used concentrated hydrochloric acid to digest chitin for preparation a lysozyme assaying substrate. Moreover, Horowitz *et al.* [76] 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 1.6).

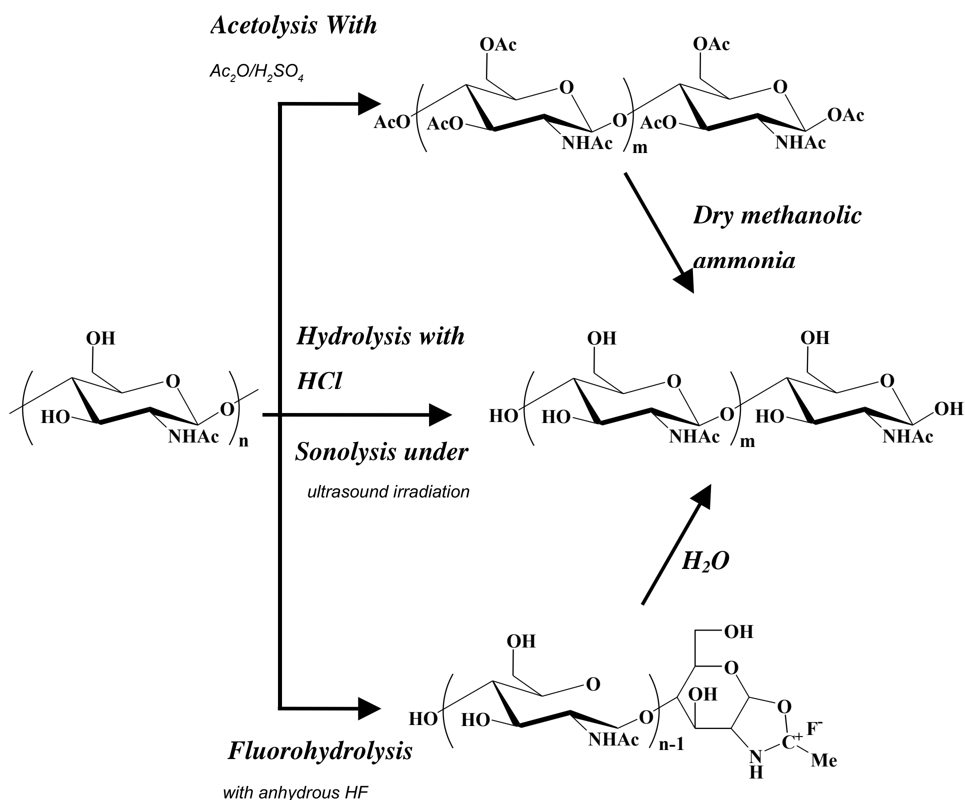


Figure 1.6 Reactions for acid hydrolysis of chitin.

Inaba *et al.* [77] used acetolysis of chitin to synthesize a substrate for the assay of lysozyme. In addition, Kurita *et al.* [78] 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.* [79] noted that fluorohydrolysis of chitin in anhydrous hydrogen fluoride (HF) led to specific chitin oligomers2-9 residues in almost quantitative yield and conditions can be conveniently monitored. 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 hours at room temperature.

Takahashi *et al.* [74] 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 radical reaction. Nordtveit *et al.* [79] 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.* [80] 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.

Purchase and Braun [81] reported the chitin (40g) was hydrolyzed with concentrated hydrochloric acid (12M, 200mL) by heating on a boiling water bath for 2.5 hours with mechanical stirrer. The hydrolysis temperature was obtain about 60°C for an hour and stirred continuously during the process of decolorization. The white crystals obtain from filtration are washed with 95% ethanol and dried to yield 67% glucosamine hydrochloride 95% purity.

Rupley [73] studied over a range of acid concentration and temperature in acid hydrolysis of chitin. The chitin concentration 20 mg/mL was hydrolyzed by acid. Number 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 [82] prepared glucosamine hydrochloride 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 glucosamine hydrochloride crystal salts. The reaction mixture was filtered and washed with 95% ethanol (194g). The white glucosamine hydrochloride crystal was obtained in 70% yield and 100% purity (by pH titration).

N. Gandhi and J.K. Laidlhi [83] reported the preparation of glucosamine hydrochloride salts from hydrolysis of chitin, 20 mesh, by concentrated hydrochloric acid. The ratio of chitin/concentrated hydrochloric acid is 1:2 w/w. The concentrated hydrochloric acid was pre-heated until 65°C followed by addition chitin. The reaction was heated to temperature of 95°C for 75 min. After purification, glucosamine hydrochloride was obtained in 70% yield and 100% purity.

Varum *et. al*. [84] studied the hydrolysis of the glycosidic linkages (depolymerization) and the *N*-acetyl linkage (deacetylation) of chitosan in dilute and concentrated hydrochloric acid. The hydrolysis rate of glycosidic linkage was found to be equal to the rate of deacetylation 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 hydrochloric at temperature of 30°C.

Shao *et. al*. [85] studied on preparation of oligoglucosamine by oxidative degradation of chitosan with neutral hydrogen peroxide under microwave irradiation. In this reaction, hydrogen peroxide acts on C-O-C glycosidic bond and leading to the chain of chitosan scission. The optimum reaction condition was as follows: volume of H_2O_2 (ml): 50 ml; irradiation time: 4min; concentration of H_2O_2:15%; amount of chitosan: 2 g. The structure of the product was confirmed by FT-IR spectrum. The average molecular weight of oligoglucosamine obtained by this method is about 900–1000. The changes in the yield of oligoglucosamine are strongly dependent on the reaction time and the concentration of H_2O_2.

Warrand and Jenssen [86] prepared the malto-oligosaccharides from pure amylase under dilute acidic conditions (0.45 M HCl, 90°C) with 2 sorts of heating: microwave irradiation and conventional heating. The microwave irradiation seems to act only on the speed of heat transfer without any specific effect on the amylose. With microwave treatment, the temperature is then much quickly reached and heat transfer in the medium is more efficient than the conventional heating process. A similar range of oligosaccharides as seen in the conventional heating procedure was observed, but without any appearance of degradation compounds (brown products) and a 10 times faster reaction rate leading to very short production times (maximum 15 min).

Kunlan *et. al*. [87] studied the effect of inorganic salts on the hydrolysis of starch in a microwave field, revealing that some inorganic salts can effectively accelerate the acid hydrolysis of starch. The results suggested that the metal halide's ability to promote the hydrolysis of starch is due to the salt's ability to cause superheating of the solution.

Xing et. al. [88] also investigated the effect of inorganic salts but on the hydrolysis of chitosan in a microwave field. The molecular weight of degraded chitosan obtained by microwave assisted hydrolysis under the conditions of added salt was considerably lower than that obtained by microwave irradiation without added

salt. It was also found that microwave heating assisted inorganic salt is a convenient way to obtain a wide range of products of different molecular weight only by changing reaction time or/and radiation power.

Goncalves and Schuchardt [89] converted hydrolytic eucalyptus lignin to oils by hydrogenolysis using microwave and ultrasound irradiations. It was found that the polymeric chains in lignin brokedown due to the action of water near the boiling point under microwave irradiation. By using ultrasound, the formation of radicals that probably caused the reticulation of lignins, decreased the conversion and yield.

1.6 Glucosamine hydrochloride vs. glucosamine sulfate

Glucosamine hydrochloride was chosen instead of glucosamine sulfate for a number of reasons. The hydrochloride form is more concentrated than the sulphate form contains substantially less sodium per effective dose than the sulfate form. Glucosamine sulfate is stabilized with sodium chloride (table salt) and can contain as much as 30% sodium. This is a consideration for individuals who want to reduce their dietary intake of sodium.

Glucosamine hydrochloride offers the promise of the same efficacy as glucosamine sulfate, since glucosamine is not absorbed intact with its carrier. The body doesn't care how it gets glucosamine as long as it is bioavailable. Nonetheless, they embarked on clinical research to prove the efficacy of the hydrochloride form.
As mentioned above, detailed human studies on the absorption, distribution, and elimination of orally administered glucosamine sulfate have shown an absorption rate of as high as 98% and that once absorbed it is then distributed primarily to joint tissues where it is incorporated into the connective tissue matrix of cartilage, ligaments, and tendons, In addition, there are the impressive clinical studies on thousands of patients.

L. Setnikar *et. al*. [90] states that after oral administration, glucosamine sulfate is rapidly split into glucosamine and sulfate ions and absorbed. After absorption, the sulfate ions enter the blood stream where a steady level already exists. None of the clinical studies performed with glucosamine sulfate indicate that sulfate contributed to the benefits shown in the study. As a matter of clarification, while this study references glucosamine sulfate, it was actually glucosamine hydrochloride that was radiolabeled and used to prove the bioavailability of glucosamine.

J.R. Schleck *et. al.* [91] reported the preparation of glucosamine sulfate sodium chloride using isopropanol as a precipitant. The ratio of glucosamine hydrochloride aqueous solution/sodium sulfate is 2:1 w/w was added in the water and the reaction mixture was stirred for 1 hour at room temperature. Thereafter, isopropanol were added to precipitate the glucosamine sulfate sodium chloride. The product recovered by filtrate was washed with isopropanol and dried under vacuum to yield yellow production product (85.4%).

However, some researchers believe that the sulfate part of glucosamine sulfate might actually be the active ingredient, not the glucosamine, for various reasons. Early studies (which used glucosamine sulfate) showed positive results. Later studies, which used either other forms or combinations of different forms, often showed little or no benefit. In addition, taking glucosamine does not actually increase the level of glucosamine in the blood, leading researchers to suspect that it might be the sulfate part of the molecule that is contributing to the effects.

It appears the sulfur component of glucosamine sulfate may be critical to the beneficial effects noted. Sulfur is an essential nutrient for joint tissue where it functions in the stabilization of the connective tissue matrix of cartilage, tendons, and ligaments. As far back as the 1930’s, researchers demonstrated that individuals with arthritis are commonly deficient in this essential nutrient [92]. Restoring sulfur levels brought about significant benefit to these patients [93]. Therefore, it appears the sulfur portion of glucosamine sulfate is extremely important and is another reason why glucosamine sulfate is the preferred form of glucosamine.

The standard dose for glucosamine sulfate is 500 mg three times per day. Obese individuals may need higher dosages based on their body weight (20 mg/kg body weight/day). Glucosamine sulfate is extremely well-tolerated. In addition, there are no contra-indications or adverse interactions with drugs. Individuals taking diuretics may need to take higher dosages. Glucosamine sulfate may cause some gastrointestinal upset (nausea, heartburn, etc.) in rare instances. If this occurs, have the patient try taking it with meals.

1.7 Microwave irradiation

Microwaves are electromagnetic waves; contain electric and magnetic field components. The microwave radiation region is located between infrared radiation and radio waves. The wavelength of microwaves ranges from 1 cm to 1m, corresponding to the frequencies from 30GHz to 300MHz. However, some wavelengths in this region are employed for radar and telecommunication. In order to avoid disruption, only limited wavelengths have been allocated for industrial and domestic microwave ovens intended for heating and drying by international convention. The most commonly used frequency for microwave heating is 2.45GHz, corresponding to a wavelength of 12.2 cm. It has been known for a long time that microwaves can be used to heat materials. In fact, the development of microwave ovens for the food heating has more than a 50 year history [94]. In the 1970s, the construction of the microwave generator, magnetron, was both improved and simplified. Consequently, the prices of domestic microwave ovens fell considerably, leading them to become a mass product. The design of the oven chamber or cavity, however, which is crucial for the heating characteristics, was not significantly improved until the end of the 1980s.

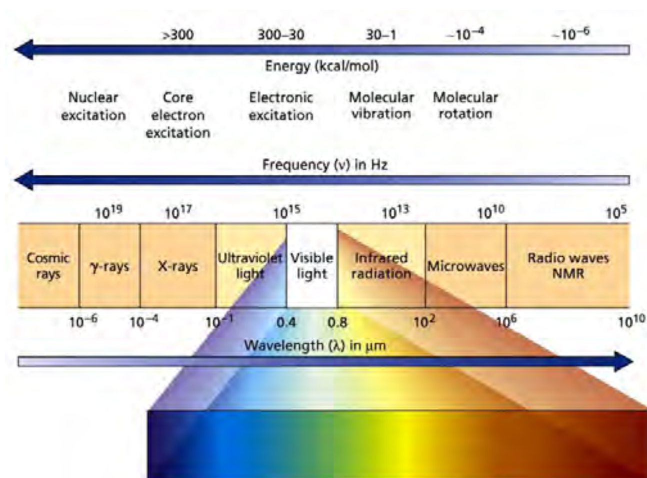


Figure 1.7 The electromagnetic spectrum.

In the past few decades, many significant advances in organic chemistry, such as the novel synthetic reagents and methods, as well as the advent of an array of analytical apparatus and techniques, have made the organic synthesis more dynamic and effective than ever before. However, the practical aspects for carrying out laboratory-

scale reactions have changed little during this period. Especially when heating is necessary, oil baths and heating jackets are the main equipment used. These traditional heating techniques are slow and time-consuming, and sometimes can lead to overheating and decomposition of the substrate and product. To this end, microwaves have been employed in organic chemistry to reduce the reaction times from hours to minutes, and also to increase yield and selectivity.

In organic chemistry, microwave technology has been used since the late 1970s, while it has only been implemented in organic chemistry since the mid-1980s. The development of the technology for organic chemistry has been rather slow compared, to for example, combinatorial chemistry and computational chemistry. This slow uptake of the technology has been principally attributed to its lack of controllability and reproducibility, safety aspects and a generally low degree of understanding of the basics of microwave dielectric heating. Since the mid-1990s, however, the number of publications has increased significantly (Figure 1.7). The main reasons for this increase include the availability of commercial microwave equipment intended for organic chemistry and the development of the solvent-free technique, which has improved the safety aspects, but are mostly due to an increased interest in shorter reaction times.

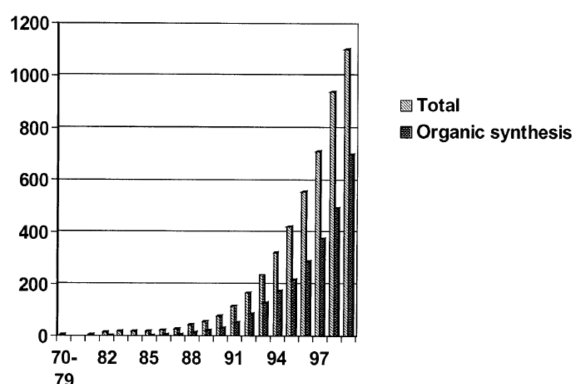


Figure 1.8 The accumulated number of published articles involving organic and inorganic microwave assisted synthesis 1970-1999.

In general, most organic reactions have been heated using traditional heat transfer equipment such as oil baths, sand baths and heating jackets. These heating techniques are, however, rather slow and a temperature gradient can develop within

the sample. In addition, local overheating can lead to product, substrate and reagent decomposition.

In contrast, in microwave dielectric heating, the microwave energy is introduced into the chemical reactor remotely and direct access by the energy source to the reaction vessel is possible. The microwave irradiation passes through the walls of the vessel and heats only the reactants and solvents, not the reaction vessel itself. If the apparatus is properly designed, the temperature increase will be uniform throughout the sample, which can lead to less by-products and/or decomposition products. In pressurized systems, it is possible to rapidly increase the temperature far above the conventional boiling point of the solvent used.

Mingos *et. al*. [95] have given a through explanation of the underlying theory of microwave dielectric heating. Gedye [96] and Langa [97] have discussed the suggested "specific microwave effect", Loupy *et. al*. [98] have published a number of reviews on solvent-free reactions and Strauss [99] has reported on organic synthesis in high temperature aqueous systems. The last microwave organic chemistry review was published by Caddick [100] in 1995.

1.7.1 Two mechanisms of microwave heating

As with all electromagnetic radiation, microwave radiation can be divided into an electric field component and a magnetic field component. The former component is responsible for the dielectric heating, which is effected *via* 2 major mechanisms.

Dipolar polarization mechanism is one of the interactions of the electric field component with the matrix called. For a substance to generate heat when irradiated with microwaves it must possess a dipole moment, such as a water molecule. A dipole is sensitive to external electric fields and will attempt to align itself with the field by rotation (Figure 1.9).

Figure 1.9 Dipolar molecules which try to align with and oscillating electric field.

The second mechanism, ionic conduction, also contributes to microwave heating effect, if ions are involved in the sample. When the ions move through the

solution under the applied field, heat is generated by frictional losses, which depend on the size, charge and conductivity of the ions, converting the kinetic energy to heat (Figure 1.10). The conductivity mechanism is a much stronger interaction than the dipolar mechanism with regard to the heat generating capacity. The heat generated by the conduction mechanism due to the presence of ions adds to the heat produced through the dipolar mechanism.

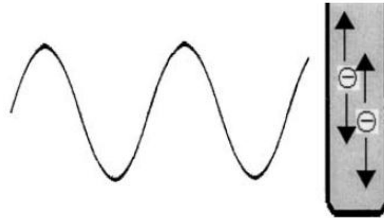


Figure 1.10 Charged particles in a solution will follow the applied electric field.

1.7.2 Loss angle

The properties ε′ and ε″ are associated with the extent of heating which the material can undergo in a dielectric field. The exact dependence of the heating rate upon the presence of a dielectric field is given by eqn. (1).

$$\tan\delta = \varepsilon'/\varepsilon'' \qquad (1)$$

ε′ is the relative permittivity, which is a measure of the ability of a molecule (or assembly of molecules) to be polarized by an electric field.

ε″ is the dielectric loss, which is indicative of the ability of a medium to convert dielectric energy into heat.

tanδ is the dielectric loss tangent and defines the ability of a material to convert electromagnetic energy into heat energy at a given frequency and temperature.

The value of tanδ of an assembly of molecules depends on several factors: on the frequency of the electromagnetic waves, the temperature and the physical state and composition of the mixture.

For water, the relative permittivity ε′ decreases when the microwave frequency increases, but the dielectric loss factor ε″ increases in the frequency range of 3×10^8 to 1×10^{10} Hz. The presence of electrolyte (*e.g.* NaCl) does not seem to influence the relative permittivity significantly, but has a marked effect on the dielectric loss factor, specifically at frequencies of 3×10^8 and 3×10^9 Hz [101].

The relaxation time, τ, defines the time it takes for one molecule to return to 36.8% of its original situation when the electric field is switched off [94]. The relaxation time is temperature dependent and decreases as the temperature is increased. Since both ε' and ε'' are dependent on τ, the ability of a solvent to convert microwave energy into heat will be dependent not only the frequency, but also on the temperature. Consequently, an organic solvent with a relaxation time >65 ps irradiated at 2.45 GHz will have a loss tangent that increases with temperature. The heating rate for these solvents will increase during microwave dielectric heating, most probably by limiting the formation of "boiling nuclei" [102]. This phenomenon is described as superheating and may result in boiling points of solvents being raised by up to 26 °C above their conventional values [97, 102]. In a pure solvent, the higher boiling point can be maintained as long as the microwave irradiation is applied. Substrates or ions present in the solvent will, however, aid the formation of "boiling nucleuses" and the temperature will eventually return to that of the normal boiling point of the solvent. The superheating phenomenon is widely believed to be responsible for many of the rate increases which often accompany solution phase microwave assisted organic reactions at atmospheric pressure [96].

1.7.3. Effect of microwave dielectric heating

Effect of microwave dielectric heating can be divided into two kinds: thermal effects and non-thermal effects. Thermal effects are those which are caused by the different temperature regime which can be created due to microwave dielectric heating. Non-thermal effects are effects specifically inherent to the microwaves and are not caused by different temperature regimes.

a) Temperature effects

Mingos *et. al.* [95] has described which effects can be expected when reactions are being carried out in a microwave dielectric field. He described the factors which play a role in microwave heating: (1) superheating in the presence of a large number of ions; (2) more rapid achievement of the reaction temperature and (3) efficient mixing and boundary effects.

I. Effects of rapid heating

In the case of preparation of intercalation compounds a better crystallinity can be obtained, and in the case of zeolite and ceramic processed there is also an advantage to be gained. The rate acceleration effect is increased if microwave

energy is absorbed by the reactants themselves and not by an absorbent, for example by the solvent.

In the case of polymer curing, the rapid heating is also thought by most researchers to be the cause of the better curing yields.

II. Hot spots, surface effects

Specifically in the case of solids being heated in the microwave oven, there are some dramatic effects with respect to heating rates, whereas in organic solvents there is not really any thermal effect. The synthesis of many organometallic compounds under microwave radiation reflux conditions is accompanied by a decrease in reaction time. These effects can be exploited in the efficient synthesis of complexes of second and third row transition metal ions which are considered difficult to prepare under standard conditions, but can be readily produced in a microwave oven.

Problems with field and energy distribution have been identified [103], but then again these can also be used as an advantage: local "hot spots" can be used to synthesis germanium derivatives.

III. Pressure cooker effect

The reaction media used in the experiments of Gedye *et. al.* [104] and Majetich *et. al.* [105] was generally heated to quite high temperatures and (sometime) also high pressure. The high temperatures could have been the cause of the rate accelerations observed. In some cases it was claimed that the temperature effects observed during microwave heating could be caused by local "hot spots" which would occur while the bulk temperature remained low. When conventionally heated reactions were carried out in a sealed tube the yield of reaction became comparable to those carried out in the microwave oven (studied for Diels-Alder reactions in DMF). This finding illustrates that the temperature effect due to the buildup of pressure in the sealed tubes accounts for the effects observed during microwave heating.

b) Non-thermal microwave effects

Non-thermal effects were claimed initially by Gedye *et.al*. [104] and Majetich *et. al*. [105] when they observed significant rate enhancements for hydrolysis and esterification reactions. However, reevaluation of reaction rates under conventional conditions revealed that sometimes reaction times suggested in the literature were erroneously long.

Although there was a general agreement that microwaves contain only 1 J /mol of photons, there were still claims of special effects [106] such as lowering of Gibbs energy of activation of reactions. This was envisaged to happen through either (1) storage of microwave energy in the vibrational energy of a molecule by *e.g.* an antenna group (enthalpy effect) or (2) by alignment of molecules (entropy effect).

The discussion which involved this issue is interesting. Of course it would be quite exciting if it had been established that there is a non-thermal microwaves effect. This could have far reaching consequences for reaction chemistry. In the food industry it would be of great concern if there could be reactions taking place during microwave heating which would not take place during conventional cooking or were not thought viable by normal thermal conditions.

Finot and Merabet [106] have reviewed thoroughly the work carried out in food research and came to the conclusion that no non-thermal microwave effects have been observed when food was dielectrically heated. They observed that any effect observed during microwave heating could be reproduced when food mixtures were heated under conventional conditions.

In the field of reaction chemistry, however, initially it was believed that there was a non-thermal microwave effect when Diels-Alder reactions were carried out under homogeneous reaction conditions (both in apolar as well as slightly polar reaction mixtures [106]) since reaction halflives seemed to depend on the heating mode, despite comparable bulk temperatures.

However, when careful temperature control was guaranteed, no special rate effects were observed. This was confirmed for reactions [106] as well as for isomerisation reactions and Diels-Alder reactions [107]. Neither of the researchers found a reproducible microwave effect. K.D. Raner *et. al.* [107] provided the first example of a systematic study to determine and evaluate activation parameters for reaction both heated dielectrically and conventionally. No differences depending on heating mode were observed. Reactions studied were the isomerisation of carvone and the Diels-Alder reaction between diethyl maleate and anthracene and the acid catalyzed esterification of 2,4,6-trimethylbenzoic acid in isopropyl alcohol. It was also argued by Raner *et. al.* [107] that the notion that microwaves can excite rotational of the microwave oven was needed to guarantee a constant temperature during microwave heating with a constant presence of a dielectric field.

Thus, it was concluded that most rate enhancement effects were observed during microwave heating because there was inadequate temperature monitoring and control.

1.8 Aims of the thesis

The aim of this work is to depolymerize chitin into glucosamine hydrochloride (GlcNHCl) by microwave assisted acid hydrolysis. It was hypothesized that the energy provided by microwave irradiation 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 increases the efficiency of the acid hydrolysis. The effect of hydrolysis parameter such as the reaction time, the chitin/acid ratio, microwave irradiation power, mechanical stirrer and metal halide on the yields of glucosamine hydrochloride was investigated.

CHAPTER II

EXPERIMENTAL

2.1 Instruments and apparatus

1. LC/MS/MS (Quattomicro, MicromassAPI, UK)
2. Hot-plated magnetic stirrer (Corning, USA)
3. Syringe filter (0.45 μm PTFE, Minisart SRP4, Satorious, Germany)
4. Pipette man (P200, Gilson, France)
5. Solvent membrane filters (0.45 μm cellulose, Millipore, USA)
6. Centrifuge (Centuar 2, Sanyo, UK)
7. Vial-capped 1.5 mL (MCT-150-C, Axygen Scientific, Inc., USA)
8. Filter papers No.1 (125 mm ∅ X 100 circles, Whatman, England)
9. Rotary evaporator (Buchi rotavapor R-200, Switzerland)
10. Vacuum oil pump (RV3 rotary vane pump, Edwardsvacuum, England)
11. Electrical food blender 500 watt (Cucina HR 1791/6, Philips, Netherlands)
12. Microwave oven (M183GN, 850 watts, Samsung, Korea)
13. Weight scale (XT220A, Precisa, UK)
14. Ultrasonic bath (S30H, 50/60 Hz, 275 watts, Elmasonic, England)
15. Nuclear magnetic resonance spectrometer (NMR) (Varian Mercury 400 NMR spectrometer)
16. Mechanical stirrer (IKA RW20 digital dual-range mixer, Cole-Parmer, USA)
17. pH meter (pHScan3+, Eutech Instruments)

2.2 Materials and Chemicals

1. Shrimp α-chitin (Ta-ming Enterprises, Thailand)
2. Glucosamine hydrochloride ≥ 99% HPLC Grade (Fluka Chemicals, Ltd., Switzerland)

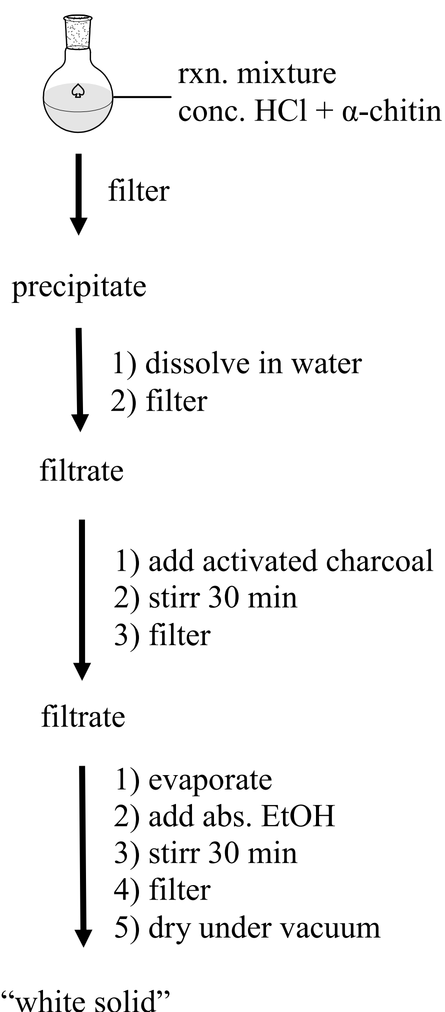
3. Sodium hydroxide, analar grade (Merck, Germany)
4. Concentrated hydrochloric acid (Merck, Germany)
5. Activated charcoal (Fluka Chemicals, Ltd., Switzerland)
6. Ethanol commercial grade (Carlo Erba Reagents, France)
7. Absolute ethanol (Merck, Germany)
8. Potassium hydrogen phthalate (Fluka Chemicals, Ltd., Switzerland)
9. Deuterium oxide (Merck, Germany)

2.3 Shrimp α-chitin (Starting materials)

Shrimp chitin flakes were purchased from Ta-ming Enterprises, Thailand and it is pulverized to fine particle by a 500 watt food blender (Philip HR 1791/6). The moisture and ash content of powder chitin were measured at the Metallurgy and Materials Science Research Institute, Chulalongkorn University.

2.4 General procedure for acid hydrolysis of chitin

Concentrated hydrochloric acid (8.5 mL) was pre-warmed to 60 °C in a controlled temperature bath. Shrimp chitin (5 g) was added portion wise into pre-warmed acid with stirring to prevent excess foaming. The reaction should be maintained at about 95 °C for about 75 minutes to produce slurry. After stopping the heater, the slurry was allowed to cool to room temperature and then filtered through a filter paper (No. 1). The precipitate containing glucosamine hydrochloride (GlcNHCl) was collected and then dissolved in distilled water (15 mL), stirred for 30 minutes with activated charcoal (0.1 g) for decolorization. The solution was filtered through a filter paper (No. 1) to remove any insoluble residue and activated charcoal. The clear filtrate was evaporated to recover crude GlcNHCl as light yellow solid. The solid was then dispersed in absolute ethanol (5 mL), stirred for 30 minutes at room temperature and filtered through a filter paper (No. 1) followed by washing with cold absolute ethanol (5 mL) to provide a white solid of GlcNHCl. The solid was dried under vacuum for 24 hours and weighed to determine the yield (Scheme 2.1).



Scheme 2.1 General procedure for acid hydrolysis of chitin

2.5 Microwave oven set-up

Microwave oven (M183GN, 850 watts, Samsung, Korea) was modified by drilling 2 holes on the top of the oven to accommodate a condenser, a thermometer, a mechanical stirrer and an adapter as the apparatus set-up shown in Figure 2.1.

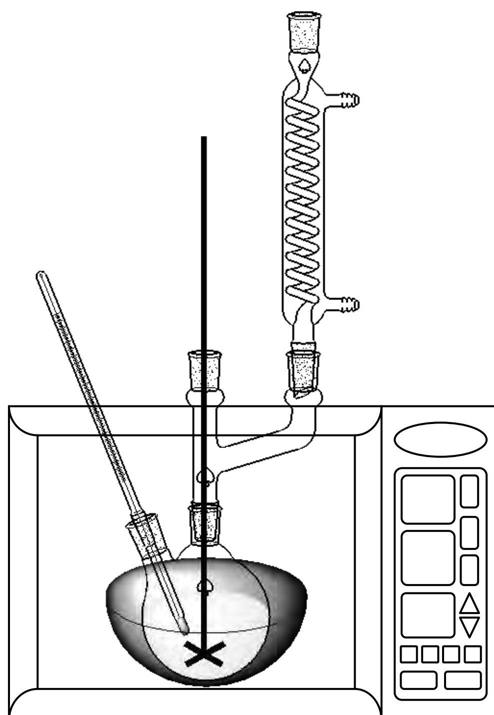
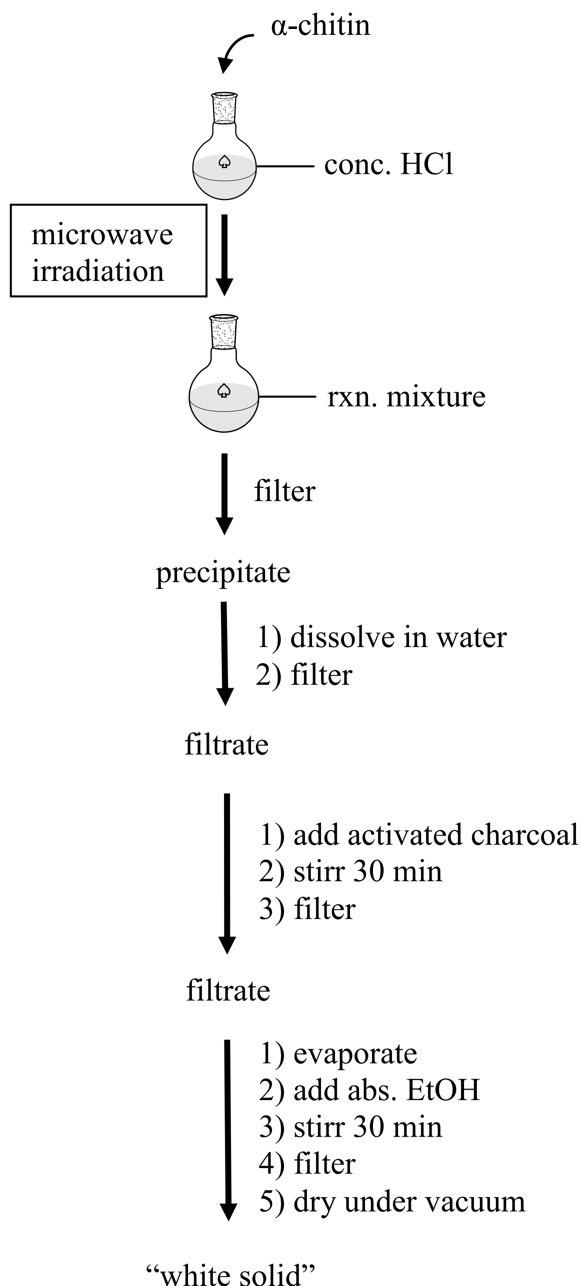


Figure 2.1 Microwave oven

2.6 Preparation of glucosamine hydrochloride (GlcNHCl) using microwave irradiation

Concentrated hydrochloric acid (50 mL) was pre-warmed by modified microwave oven at 850 watts for 30 seconds. Shrimp chitin (30g; chitin/acid ratio = 1:2 w/w) was added into the pre-warmed acid. The microwave irradiation was continued at the specified power for the designated period of time. After stopping the irradiation, the resulting slurry was allowed to cool to room temperature and filtered through a filter paper (No. 1). The precipitate containing glucosamine hydrochloride (GlcNHCl) was collected and then dissolved in distilled water (90 mL), stirred for 30 minutes with activated charcoal (0.6 g) for decolorization. The solution was filtered through a filter paper (No. 1) to remove any insoluble residue and activated charcoal. The clear filtrate was evaporated to recover crude GlcNHCl as light yellow solid. The solid obtained was then dispersed in absolute ethanol (30 mL), stirred for 30 minutes at room temperature and filtered through a filter paper (No. 1) and washed with absolute ethanol (5 mL) to provide a white solid of GlcNHCl. The solid was dried under vacuum for 24 hours and weighed to determine the yield.



Scheme 2.2 Preparation of glucosamine hydrochloride (GlcNHCl)

2.7 Preparation of glucosamine sulfate using microwave irradiation

Glucosamine hydrochloride (30 g) and sodium sulfate (10 g), at glucosamine hydrochloride/sodium sulfate ratio of 2:1 (w/w), were taken in a flask and dissolved in water (50 mL). The flask was heated by microwave oven at 850 watts for 10 minutes. After stopping the irradiation, the mixer was stirred and added dropwise to vigorously stirred 95% ethanol (30 mL) at room temperature over a period of 3 hour. The precipitation occurs too quickly, with formation of crystalline agglomerates which may encapsulate some solvent and impurity, while temperature below 30 °C, the

precipitation is completed. After overnight, stirred at 5 °C with the cool in an ice-water bath 1 hour and the crystalline mass obtained is filtered through a Buchner funnel. Glucosamine sulfate was obtained as creamy white crystal and was further dried at 25 °C under vacuum.

2.8 Product analysis

2.8.1 Purity analysis of GlcNHCl by acid-base titration

A NaOH solution (~0.5 g in 500 mL of deionized water, 0.01 M) was standardized with potassium hydrogen phthalate (KHP) solution (~0.5 g in 250 mL of deionized water, 0.01 M) using a couple drops of phenolphthalein. The NaOH solution was filled into a burette and slowly added into the KHP solution (10 mL) in the presence of a couple drops of phenolphthalein in a 100 mL Erlenmeyer flask until the perpetual pink color of phenolphthalein was observed. The titration was repeated two more times to obtain the average volume. A GlcNHCl solution was prepared by dissolving GlcNHCl salt (~0.1 g in 250 mL of deionized water, 0.01 M). The GlcNHCl solution (10 mL) was pipetted into a 100 mL volumetric flask and a couple drops of phenolphthalein were added. The NaOH solution was slowly added from the burette into the GlcNHCl solution until the perpetual pink color of phenolphthalein was observed. The titration was repeated two more times to obtain the average volume.

2.8.2 ^{1}H NMR spectroscopy

In a standard NMR tube, a solid sample (10 mg) was dissolved in deuterium oxide (D_2O, 1.5 mL). The spectra of both standard GlcNHCl (Fluka Chemicals, Ltd., Switzerland) and GlcNHCl obtained from the hydrolysis process were acquired to compare the signals and purity.

H^1 NMR data of glucosamine hydrochloride $C_6H_{14}ClNO_5$ (400 MHz, D_2O) δ 5.36 (d, 0.6H, J = 3.5 Hz, H-a$^{\alpha}$), δ 4.85 (d, 0.4, J = 8.3 Hz, H-a$^{\beta}$), δ 3.36-3.84 (m, 5H, H-b,c,d,f), δ 3.21 (dd, 0.6H, J = 3.5, 10 Hz, H-e$^{\alpha}$), δ 2.92 (dd, 0.4H, J = 8.3, 10.6Hz, H-e$^{\beta}$)

2.8.3 ESI mass spectrometry

To characterize GlcNHCl, the solution sample was prepared from white powder (1 mg) dissolved in DI-water adjusted to 1000 mL. Pipet the solution 10 μL,

filtered though a 0.45 µm PTFE filter and adjusted to 10 mL by DI-water in a 10 mL volumetric flask and the diluted sample was analyzed by ESI-MS

An ESI mass spectrometer (Quattomicro, MicromassAPI, UK) was used for the reaction monitoring. The solution sample (~1 ppm, each 1.5 mL) was injected into the mass spectrometer using the optimum injection and ionization parameters; *i.e.* the voltage at capillary, extractor and RF lens were 3.93 kV, 3 V and 0 V, respectively. The cone voltage was set at 30 V. The source and desolvation temperature were adjusted to 120 and 350 °C, respectively. The desolvation N_2 gas flow was 550 L/hr and the cone N_2 gas flow was 50 L/hr. Under MS scan mode, all other parameters were adjusted to give the highest signals corresponding to GlcNHCl *i.e.* $[GlcN+H]^+$ at m/z = 180, $[GlcN\text{-}H_3O]^+$ at m/z = 162.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Shrimp chitin

Commercial shrimp chitin purchased from Ta-ming Enterprises, Thailand in a form of thick fibrous sheets (Figure 3.1 a). The chitin sheets were ground by a 500 watt food blender to provide 10-60 mesh chitin powder (Figure 3.1 b). The moisture content and ash content of powder chitin were measured at the Metallurgy and Materials Science Research Institute, Chulalongkorn University. The moisture content is 8.18 ± 0.04% and Ash content is 0.87 ± 0.01%. In terms of purity confirmation, the resulting data demonstrated the consistency to the chemical and microbiological parameter guaranteed by Ta-ming Enterprises.

(a)

(b)

Figure 3.1 Photographs of shrimp chitin (a) as purchased and (b) after grinding.

3.2 Preparation of glucosamine hydrochloride (GlcNHCl)

To choose the power of microwave irradiation of hydrolysis α-chitin, temperature program was studied. As shown in figure 3.1, the microwave irradiation power 850 watts gives higher temperature and faster time than others. Therefore, the

850 watts was chosen to hydrolyze α-chitin based on the hypothesis that it can give the highest percent yield.

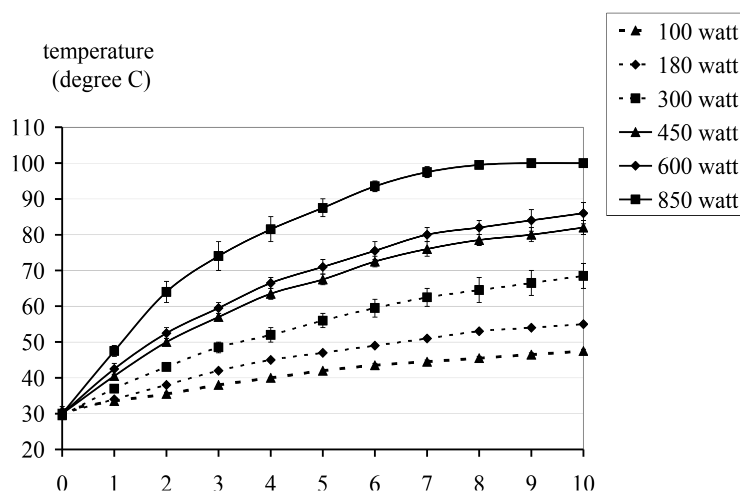


Figure 3.2 Temperature of water (50 mL) obtained from various microwave irradiation power. The data is representative of 3 independent repeats.

3.2.1 Effect of hydrolysis time

At chitin/conc. HCl ratio of 1:2 (w/w) under microwave irradiation 850 watts for 4-16 minutes, the effect of hydrolysis time was studied. Conc. HCl (50 mL) was pre-warmed by microwave oven for 30 seconds. Shrimp chitin (30 g) was added and microwave irradiation was continued for the designated period. GlcNHCl was isolated by precipitation, activated charcoal decolorization and ethanol washing. Overall, the yields of GlcNHCl are dependent on the hydrolysis time. In general, short reaction time leads to incomplete hydrolysis while prolong reaction time results in depolymerization. The isolated yield of GlcNHCl increases along with the hydrolysis time and reach the maximum of ~45% at 12 minutes. When the irradiation time was extended to more than 12 minutes, the yield of GlcNHCl gradually decreased probably due to the decomposition of the GlcNHCl (Figure 3.3).

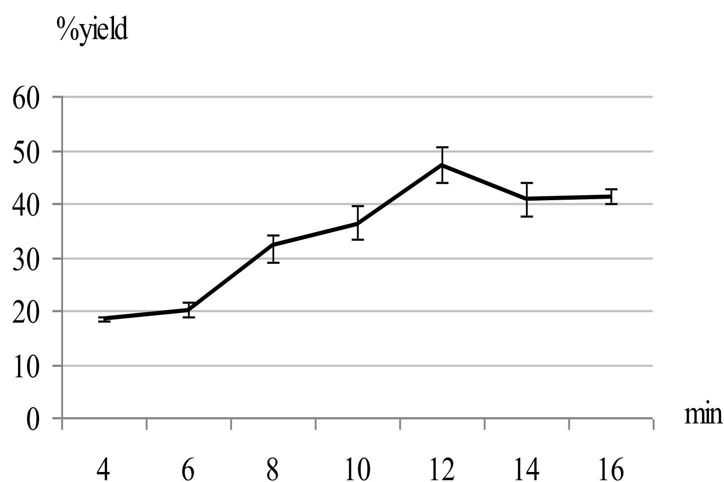


Figure 3.3 Percent yield of GlcNHCl obtained from acid (conc. HCl) hydrolysis under microwave irradiation (850 watts) for various periods using chitin/conc. HCl ratio of 1:2 (w/w)**.** The data is representative of 3 independent repeats.

3.2.2 The effect of chitin/conc. HCl weight ratio

To improve the yield of GlcNHCl, the effects of chitin/conc. HCl ratio was investigated. The percent isolated yields of GlcNHCl obtained from the hydrolysis at various weight ratios of chitin/conc. HCl were compared. The optimum chitin/conc. HCl ratio is 1:3 where GlcNHCl can be obtained more than 50% yield. At lower amount of conc. HCl, significant amount of chitin remained insoluble after the hydrolysis. The lost of HCl during the microwave heating may lead to inadequate acidity to dissolve chitin in the initial state of the hydrolysis that results in lower GlcNHCl yield. When higher amount of conc. HCl (at 1:4 ratio) was used, less GlcNHCl precipitated after cooling the reaction mixture down to room temperature as more water present in the mixture (Figure 3.4).

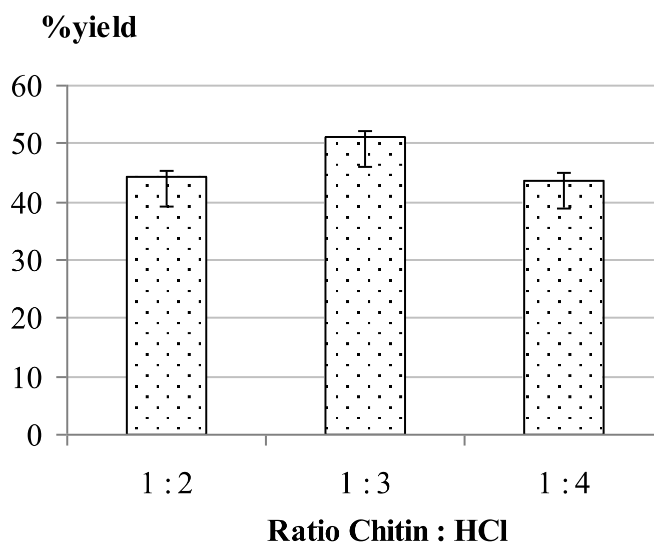


Figure 3.4 Percent yield of GlcNHCl obtained from acid hydrolysis under microwave irradiation (850 watts) for 12 minutes using different chitin/conc. HCl ratio. The data is representative of 3 independent repeats.

3.2.3 The effect of irradiation power and chitin/conc. HCl weight ratio

The chitin hydrolysis using microwave irradiation power varied from 180 to 850 watts. The increase of irradiation power from 180 to 450 watts resulted in the increase of GlcNHCl yield whilst the increase from 450 to 850 watts did not show significant effect to the yield (Figure 3.5) because at the high irradiation power, the path of reaction may be overheated and GlcNHCl product was decomposed. The results indicate that the hydrolysis of chitin may be performed at 450 watts instead of 850 watts to reduce the energy consumption of the process.

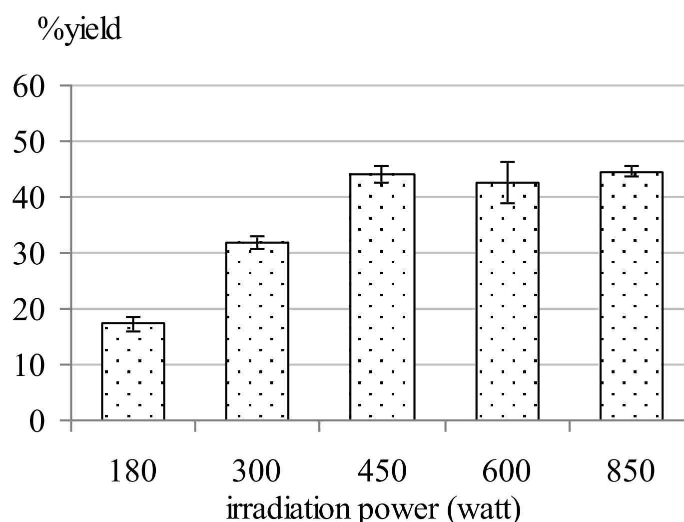


Figure 3.5 Percent yield of GlcNHCl obtained from acid hydrolysis at various microwave irradiation power; chitin/conc. HCl ratio of 1:2(w/w); hydrolysis time 12 minutes. The data is representative of 3 independent repeats.

When the chitin/conc. HCl to 1:3(w/w) increased, the percent yield of GlcNHCl at various time increased. The results at 450 to 850 watts did not show significant effect to the yield as same as the chitin/conc. HCl at 1:2(w/w) (Figure 3.6).

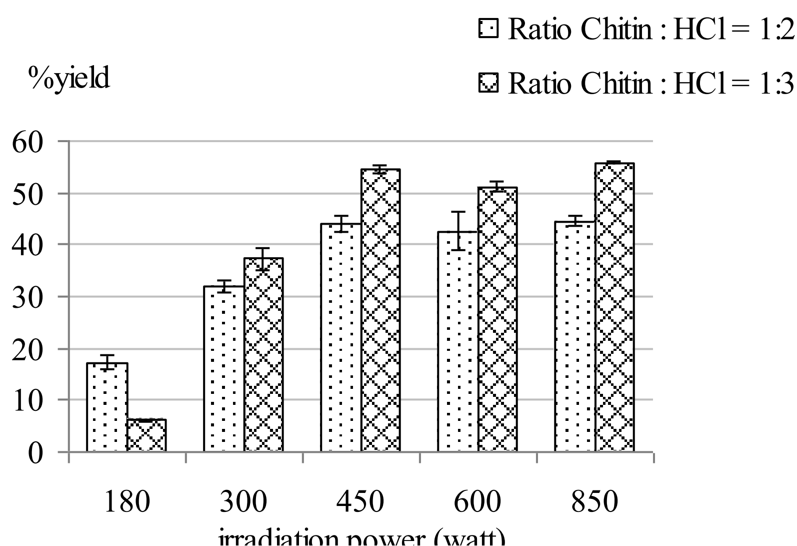


Figure 3.6 Percent yield of GlcNHCl obtained from acid hydrolysis at various microwave irradiation power; chitin/conc. HCl ratio of 1:2 and 1:3 (w/w); hydrolysis time 12 minutes. The data is representative of 3 independent repeats.

As described in the previous paragraph, the hydrolysis of chitin at microwave irradiation power 450-600 watts gives more important effect GlcNHCl yields than 850 watts. It is possible that at lower microwave irradiation power, the reaction mixer had lower temperature and the amount of decomposition GlcNHCl product is reduced. In this case, if reaction time is increased to 15 minutes, GlcNHCl yield may also be increased. In figure 3.7, GlcNHCl yield at 450 and 600 watts was slightly increased and there was no significant difference between 12 minutes.

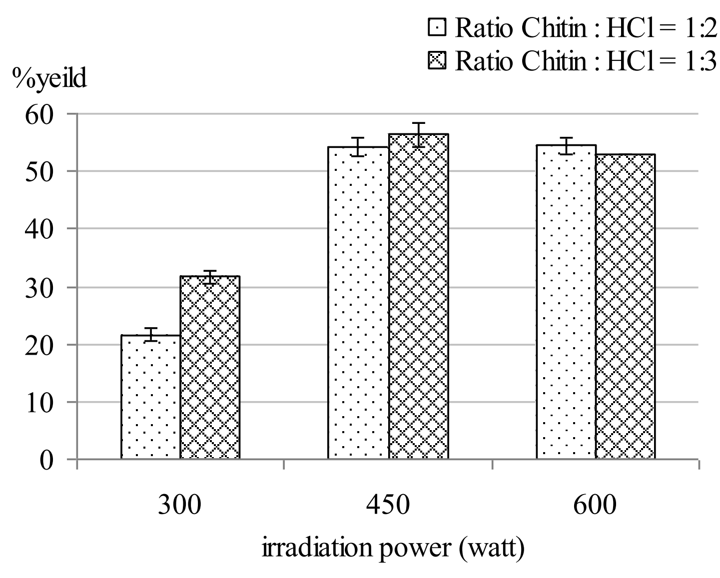


Figure 3.7 Percent yield of GlcNHCl obtained from acid hydrolysis at 300-600 watts microwave irradiation power; chitin/conc. HCl ratio of 1:2 and 1:3(w/w); hydrolysis time 15 minutes. The data is representative of 3 independent repeats.

Comparison figure 3.6 versus figure 3.7 at irradiation power 300 watt, GlcNHcl yield for 12 min hydrolysis time was more than 15 min hydrolysis time. It's possible that at 12 min hydrolysis time not only GlcNHCl monomer but also GlcNHCl dimer and GlcNHCl oligomer was found in the dry product.

3.2.4 The effect of mechanical stirrer

To evaluate the effect of heat transfer on the microwave assisted hydrolysis, the reaction was conducted with and without mechanical stirring. The stirring did have some small effects on the yield of GlcNHCl in comparison with that of the reaction without stirring (Figure 3.8). However, the GlcNHCl yield with mechanical stirrer can be obtained more than the GlcNHCl yield without mechanical stirrer and

the GlcNHCl yield gave a minimum error. Although the stirring slightly increased the yield of GlcNHCl, it was likely that the microwave heating already provided efficient heat transfer in the reaction.

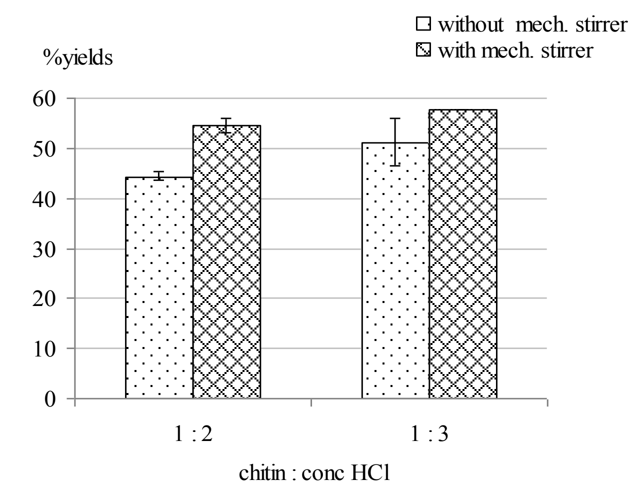


Figure 3.8 Percent yield of GlcNHCl obtained from acid hydrolysis of chitin using chitin/conc. HCl ratio of 1:2 and 1:3 (w/w) under microwave irradiation (850 watts) for 12 minutes with and without mechanical stirrer. The data is representative of 3 independent repeats.

Though, the microwave irradiations were varied to 450-600 watts, the percent yields of GlcNHCl was not increased more than previous experiment (figure 3.9).

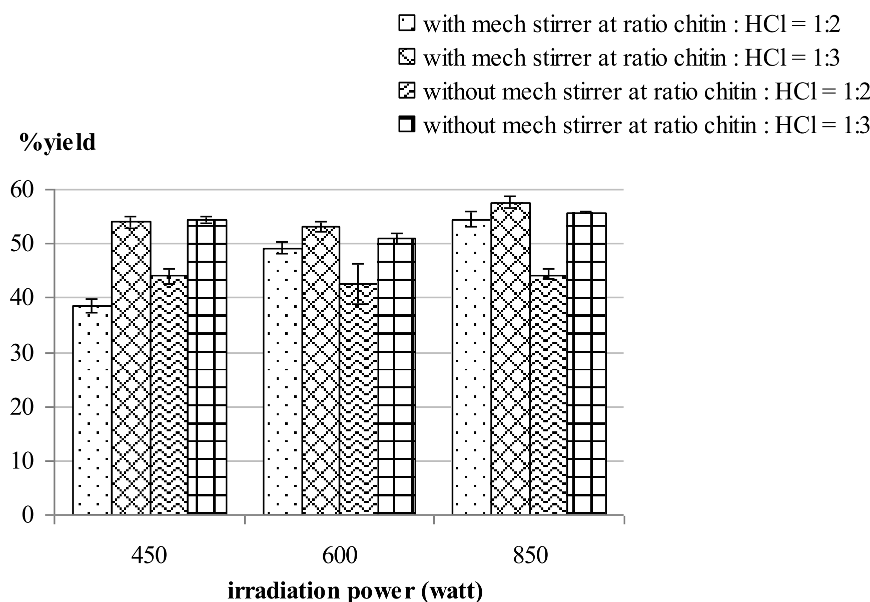


Figure 3.9 Percent yield of GlcNHCl obtained from acid hydrolysis of chitin using chitin/conc. HCl ratio of 1:2 and 1:3 (w/w) under various microwave irradiation for 12 minutes with and without mechanical stirrer. The data is representative of 3 independent repeats.

3.2.5 The effect of metal halide salts

As Kunlan *et. al.* have proposed that metal halide salts, such as NaCl, can effectively accelerate the acid hydrolysis to cause superheating of the solution [89]. In this experiment, NaCl solution containing 0.15 mol/L was added in the reaction mixer but NaCl solution happened to dilute conc. HCl and consequently chitin was not hydrolyzed. Then crystals NaCl were used instead of aqueous solution. At chitin/conc. HCl ratio of 1:2 and 1:3 (w/w) under microwave irradiation 850 watts, conc. HCl and crystal NaCl was pre-warmed by microwave oven for 30 seconds. Shrimp chitin was added and continued for the designated period. GlcNHCl was isolated by precipitation, activated charcoal decolorization and ethanol washing. Figure 3.8 demonstrated the NaCl effect on the percent yields of GlcNHCl based on weight of the isolated products. In order to measure the amount of NaCl, titration method with NaOH (0.1 M) was carried out (Figure 3.12). However, according to the titration results, even after withdrawing this 3% amount off the crude percent yield, the results were slightly better than the normal case without NaCl (Figure 3.10). In conclusion, the metal halide's ability to promote the acid hydrolysis was proved,

presumably due to the salt's ability to cause superheating of the solution and to increase dielectric interaction inside target molecules.

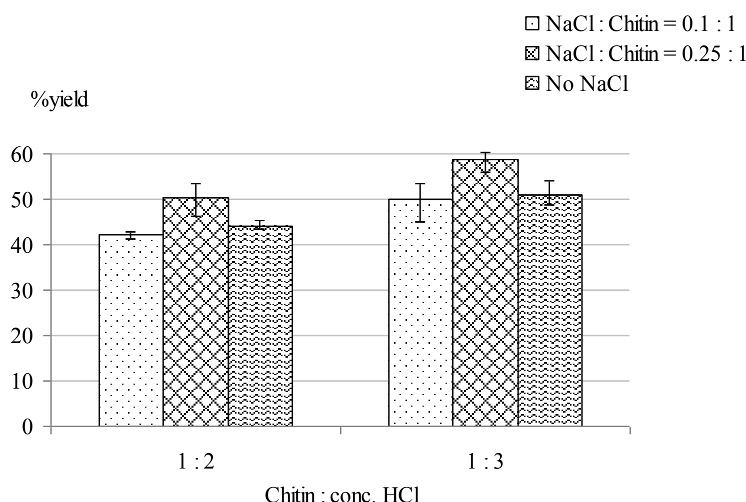


Figure 3.10 Percent yield of GlcNHCl obtained from acid hydrolysis at 850 watts microwave irradiation power; chitin/conc. HCl ratio of 1:2 and 1:3(w/w); NaCl/chitin ratio of 0.1 and 0.25 (w/w); hydrolysis time 12 minutes. The data is representative of 3 independent repeats.

3.3 Preparation of glucosamine sulfate

The reason for choosing preparation of glucosamine sulfate because in the previous experiment, metal halide salts affect, not only crystals NaCl put to the test but also the crystals Na_2SO_4. However the GlcNHCl yields are not better than the crystal NaCl case.

In order to synthesize it, glucosamine hydrochloride (30 g) and sodium sulfate (10 g), at glucosamine hydrochloride/sodium sulfate ratio of 2:1 (w/w), were taken in a flask and dissolved in water (50 mL). The flask was heated by microwave oven at 850 watts for 10 minutes. After stopping the irradiation, the mixer was stirred and added dropwise to vigorously stirred 95% ethanol (30 mL) at room temperature over a period of 3 hours. The precipitation occurs too quickly, with formation of crystalline agglomerates which may encapsulate some solvent and impurity, while temperature below 30 °C, the precipitation is completed. After overnight, stirred at 5 °C with the aid of an ice-water bath 1 hour and the crystalline mass obtained is filtered through a Buchner funnel. Glucosamine sulfate was obtained as creamy white crystal and was

further dried at 25 °C under vacuum. The result was shown in figure 3.9. Senin *et. al*. have reported that using an electrically heated bath 60 °C for 65 minutes provided 85.3% yield (Figure 3.11).

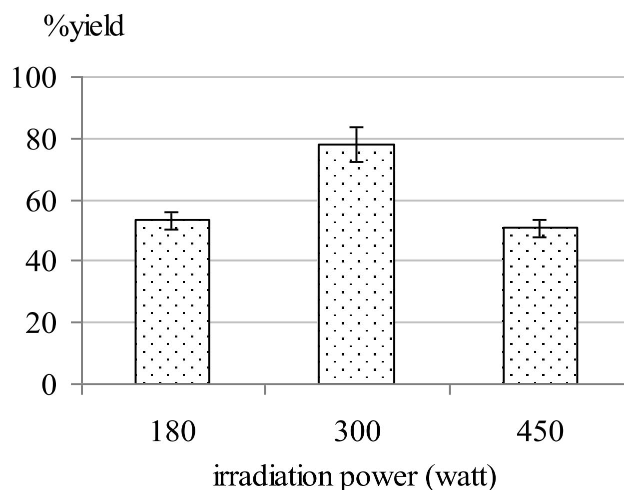


Figure 3.11 Percent yields of glucosamine sulfate obtained at 850 watts microwave irradiation power; GlcNHCl/Na_2SO_4 ratio of 2:1 (w/w). The data is representative of 3 independent repeats.

3.4 Product analysis

3.3.1 Purity analysis of GlcNHCl by acid-base titration

A white solid of GlcNHCl was dissolved in DI water for the NaOH titration. The NaOH solution was added into the GlcNHCl solution until the perpetual pink color of phenolphthalein will be observed. The titration was repeated two more times to obtain the average volume. From the titration curve (Figure 3.12), percent purity of GlcNHCl is 99-100% compared with GlcNHCl standard (Fluka Chemicals, Ltd., ≥ 99% HPLC).

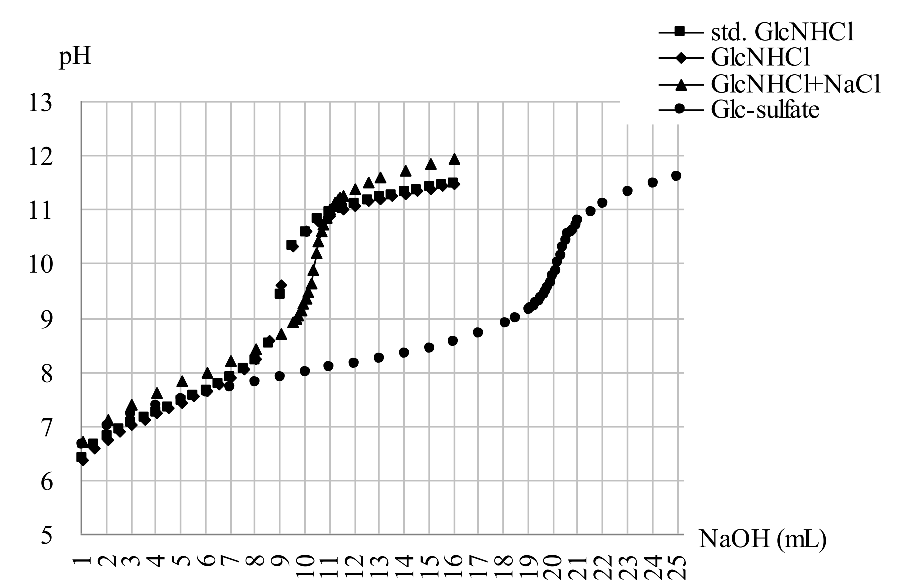


Figure 3.12 pH titration curves of Std. GlcNHCl, GlcNHCl, GlcNHCl+NaCl and Glc-sulfate by 0.1M NaOH standard solution.

3.4.2 Monitoring of GlcNHCl by ^{1}H-NMR and ESI-MS

The isolated GlcNHCl product obtained from the acid hydrolysis of chitin with microwave irradiation was evaluated by ^{1}H-NMR and ESI-MS. Using the optimum instrument conditions, the ^{1}H-NMR spectrum showed the same pattern (Figure 3.13) as the GlcNHCl standard (Fluka Chemicals, Ltd., ≥ 99% HPLC). MS scan mode was used to observe GlcNHCl as the signal of at $[GlcN\text{-}H_3O]^+$ m/z = 180 and $[GlcN+H]^+$ at m/z = 162 (Figure 3.14).

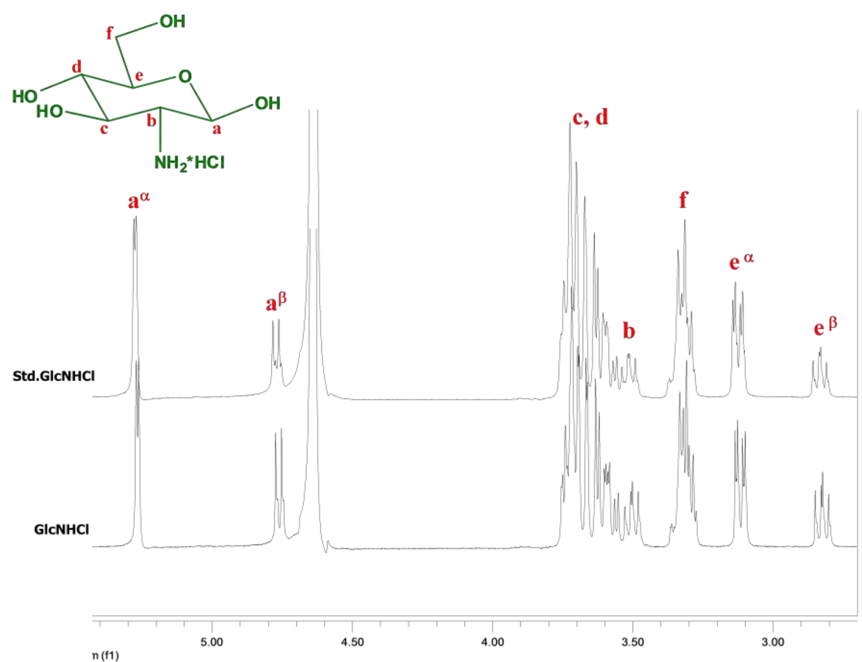


Figure 3.13 ^{1}H NMR spectra of standard GlcNHCl and GlcNHCl in the presence of an aliquot of D_2O

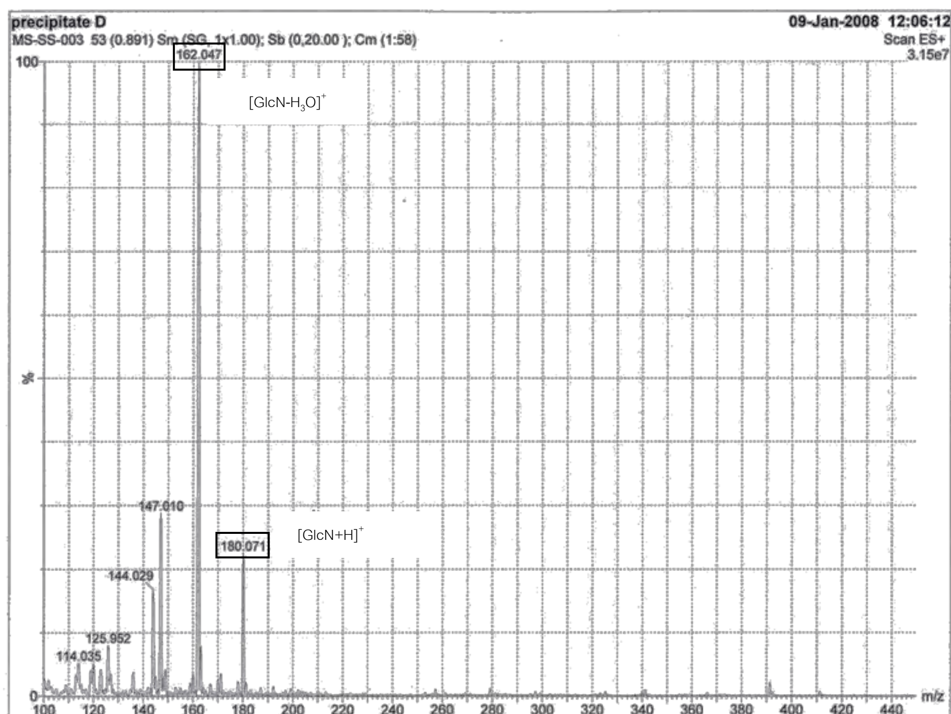


Figure 3.14 Mass spectra of GlcNHCl

CHAPTER IV

CONCLUSIONS

The acid hydrolysis with microwave irradiation of α-chitin from shrimp shell with conc. HCl seem to accelerate only the speed of heat transfer without any specific effect. The temperature was then much quickly reached and heat transfer in the medium was more efficient than the conventional heating process enabling shorter reaction times but there was no improvement in the product yield comparing to the conventional heating. In the case of chitin/conc. HCl ratio of 1:3 (w/w) conditions of 850 watts, 12 minutes, 55% isolated yield with 99-100% purity of GlcNHCl was obtained. When NaCl was added, the metal halide's ability to promote the acid hydrolysis was proved, presumably due to the salt's ability to cause superheating of the solution and to increase dielectric interaction inside target molecules. The stirring did not give significantly different yield of GlcNHCl in comparison with that of the reaction without stirring.

References

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APPENDICES

PREPARATION OF GLUCOSAMINE HYDROCHLORIDE FROM α-CHITIN BY MICROWAVE ASSISTED ACID HYDROLYSIS

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Abstract: Glucosamine hydrochloride (GlcNHCl) is a well known neutrapharmaceutical agent prescribed for osteoarthritis patients. Hydrolysis of shrimp shell α-chitin in concentrated hydrochloric acid under elevated temperature is a general method for production of GlcNHCl. To speed up the hydrolysis process, microwave assisted hydrolysis is studied in this work. With microwave irradiation, the hydrolysis is faster comparing to the conventional heating. Only 12 minutes of reaction time is required to complete the hydrolysis when the microwave is utilized while 90-120 minutes is generally required for conventional heating.

Introduction

Chitin is the second most abundant polymer found in nature. It is a by-product or a marine biomass from crab, shrimp and squid processing industries. Furthermore, 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. It consists of homopolymer chains of β-1,4'-linked 2-acetamido-2-deoxy-D-glucose. It may be regarded as a derivative of cellulose, the most abundant natural polymer, in which the hydroxyl group (-OH) at the second carbon position of the pyranose ring is replaced in chitin by an acetamide group ($-NHCO-CH_3$)[1]. Chitin can be considered as an abundant and renewable source of nitrogen containing organic substances.

There are two hydrolytic processes, chemical hydrolysis and enzymatic hydrolysis, which may be used for chitin degradation to chitooligosaccharides and monomer. The acid hydrolysis of chitin in concentrated hydrochloric acid generally gives the monomeric salt, glucosamine hydrochloride (GlcNHCl).[2,3] GlcNHCl has wide range of applications and it most commonly use is to treat osteoarthritis patients. It is also used in cosmetic, antiviral, anti-cancer, anti-free radical, and substrate in synthesis of glycoprotein and glycolipid[4].

Microwave irradiation has been widely used in modern chemical reaction because of its high heating efficiency, enhance selectivity, improve reaction rates and give cleaner products with higher yields in shorter reaction times. The heating by microwaves is induced by the interaction of the radiation with the dielectric field associated with polar molecules and ions[5].

To our knowledge, there are no reports on GlcNHCl production using microwave irradiation. In this study, chitin was depolymerized to GlcNHCl using microwave assisted acid hydrolysis of shrimp chitin. Various hydrolysis parameter including irradiation power, irradiation time, and chitin/acid ratio, were optimized.

Materials and Methods

Staring material: Shrimp chitin flakes were purchased from Ta-ming Enterprises, Thailand and it is pulverized to fine particle by a 500 watts food blender (Philip, HR 1791/6). The moisture and ash content of powder chitin were measured at the Metallurgy and Materials Science Research Institute, Chulalongkorn University, Thailand.

Microwave heating procedure: Concentrated hydrochloric acid (50 mL) was pre-warmed by home microwave oven (Samsung, M183GN) for 30 second. Shrimp chitin (30g; chitin/acid ratio = 1:2 w/w) was added into the pre-warmed acid. The microwave irradiation was continued for the designated period. After stopping the irradiation, the resulting slurry was allowed to cool to room temperature and filtered. The precipitate containing glucosamine hydrochloride (GlcNHCl) was collected and then dissolved in distilled water (90 mL), stirred for 30 minutes with activated charcoal (60 mg) for decolorization. The solution was filtered to remove insoluble residue and activated charcoal. The clear filtrate was evaporated to recover crude GlcNHCl as light yellow solid. The solid obtained was then dispersed in absolute ethanol (30 mL), stirred for 30 minutes at room temperature and filtered to provide a white solid of GlcNHCl. The solid was dried under vacuum for 24 hour and weigh to determine the yield. The purity of the GlcNHCl was analyzed by acid-base titration and ^{1}H-NMR.

Purity analysis of GlcNHCl by titration: NaOH solution was standardized by potassium hydrogen phthalate (KHP) solution (~0.01 M, 0.5 g/250 mL MilliQ-water) in 50 mL flasks using a few drops of phenolphthalein. The NaOH solution (~0.01 M, 0.5 g/500 mL MilliQ-water) was filled into a burette and slowly added into KHP solution (10 mL) and the titration was repeated two more times. GlcNHCl solution was preparation by dissolving GlcNHCl salt (~0.01 M, 0.1 g/250 mL) in MilliQ-water into 50 mL

volumetric flasks and added a few dropped of phenolphthalein. The NaOH solution was slowly added into the GlcNHCl solution and the titration was repeated 2 more times.

^{1}H NMR of products: In standard NMR tube, a sample solid was added a drop of deuterium oxide (D_2O) prior to the spectrum acquisition. The spectra of standard GlcNHCl was acquired from the solutions prepared in a similar manner.

Results and Discussion

Figure 1 shows the hydrolysis of shrimp chitin with concentrated HCl at 1:2 (w/w) ratio under microwave irradiation at 850 watts for 4-16 minutes. The isolated yield of GlcNHCl increases with the hydrolysis time and reach the maximum of ~45% at 12 minutes. When the irradiation time was extended to more than 12 min, the yield of GlcNHCl gradually decreased probably due to the decomposition of the GlcNHCl.

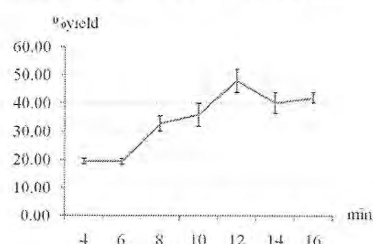

Figure 1. %Yield of GlcNHCl obtained from acid (concentrated HCl) hydrolysis under microwave irradiation (850 watts) for various periods using chitin: concentrated HCl ratio of 1:2 (w/w).

The effect of chitin/HCl ratio on the yield of GlcNHCl is illustrated in Figure 2. The optimum chitin: concentrated HCl ratio is 1:3 where GlcNHCl can be obtained at higher than 50% yield. At lower amount of HCl, significant amount of water insoluble chitin was remained after the hydrolysis. The lost of HCl during the microwave heating may lead to inadequate acidity to dissolve chitin in the initial state of the hydrolysis that results in lower GlcNHCl yield. When higher amount of concentrated HCl (at 1:4 ratio) was used, less GlcNHCl was precipitated after allowing the reaction mixture to cool to room temperature as more water present in the mixture.

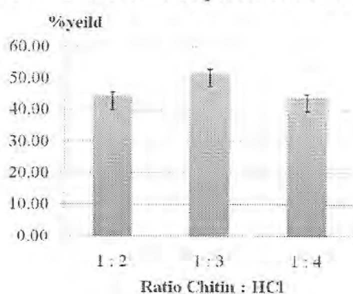

Figure 2. %Yield of GlcNHCl obtain from acid hydrolysis under microwave irradiation (850 watts) for 12 minutes using different a chitin: concentrated HCl ratio

The effect of irradiation power on the yield of GlcNHCl was studied by performing the chitin hydrolysis using microwave power varied from 180 to 850 watts. The increase of irradiation power from 180 to 450 watts resulted in the increase of GlcNHCl yield whilst the increase from 450 to 850 watts did not show significant effect to the yield (Figure 3). The results indicate that the hydrolysis of chitin maybe performed at 450 watts instead of 850 watts to reduce the energy consumption of the process.

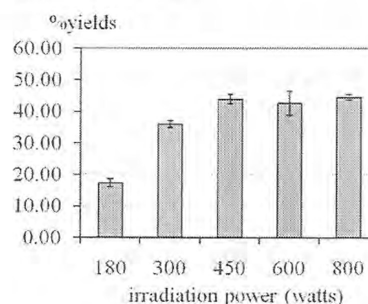

Figure 3. %Yield of GlcNHCl obtains from acid hydrolysis at various microwave irradiation power; chitin: concentrated HCl ratio of 1:2(w/w); hydrolysis time 12 minutes.

To evaluate the effect of heat transfer on the microwave assisted hydrolysis, the reaction was conducted with and without mechanical stirring. The stirring did not give significantly different yield of GlcNHCl in comparison with that of the reaction without stirring (Figure 4). Since the stirring did not increase the yield of GlcNHCl, it was likely that the microwave heating already provided efficient heat transfer in the reaction.

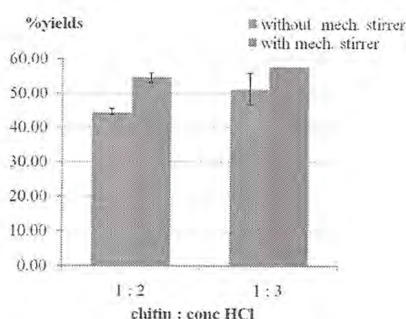

Figure 4. %Yield of GlcNHCl obtained from acid hydrolysis of chitin using chitin:concentrated HCl ratio of 1:2 (w/w). under microwave irradiation (850 watts) for 12 minutes with and without mechanical stirrer

After 12 minutes of irradiation, the resulting brown mixture was allowed to stand at room temperature that GlcNHCl precipitate from the mixture. The precipitate was redissoved in DI water, decolorized and reprecipitated in absolute ethanol from aqueous solution. GlcNHCl was obtain as a white crystalline powder. The isolated GlcNHCl product obtained from the hydrolysis show the same ^{1}H-NMR spectrum pattern (Figure 5.) as the GlcNHCl standard (Fluka Chemicals, Ltd., ≥ 99% HPLC). The %purity of GlcNHCl by acid-base titration is 94-95%. Compared with convention heating, oil bath, using 90 minutes for hydrolysed chitin to get 60-65 %yields.

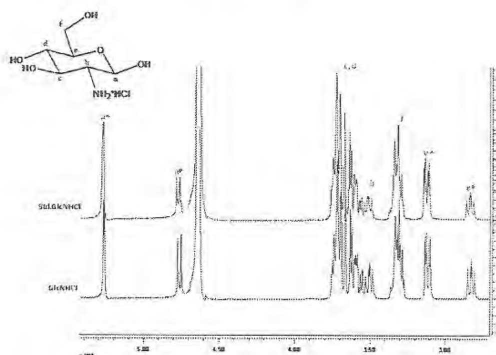

Figure 5. ^{1}H NMR spectra of standard GlcNHCl and GlcNHCl in the presence of an aliquot of D_2O

Conclusions

The results described here indicate that acid hydrolysis with microwave irradiation seems to act only on the speed of heat transfer without any specific effect on the chitin. The temperature is then faster reached and heat transfer in the medium is more efficient than the conventional heating process and yielding shorter reaction times but there is no improvement in the yield comparing to the conventional heating.

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VITAE

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