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I am submitting herewith a dissertation written by Tao Wu entitled “Sonochemical and Hydrophobic Modification of Chitin and Chitosan.” I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Food Science and Technology.

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SONOCHEMICAL AND
HYDROPHOBICAL
MODIFICATION OF CHITIN
AND CHITOSAN

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Tao Wu
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Abstract

Chitin and chitosan are linear polysaccharides consisting of acetyl-glucosamine and glucosamine units with many potential applications in biomedicine, agriculture and food industry. In the food industry, chitosan can be used as antimicrobial agent and to create active packaging to improve food quality and extend shelf life. Commercial applications of chitosan are closely associated with its functional properties and biological activity, which are primarily governed by two structural properties: degree of acetylation (DA) and molecular weight (MW). The overall goal of this study was to develop methods for tailored modification of DA, MW and functionalities of chitosan.

The research was conducted in four phases. In the first phase, an accurate and rapid method to determine DA for both chitin and chitosan was developed. By employing concentrated phosphoric acid as a solvent for highly acetylated samples and by optimizing the analytical parameters through investigation of underlying chemical reactions, it was the first time that the DA of both chitin and chitosan could be analyzed by a single method in an accurate, rapid and economical way.

In the second phase, high intensity ultrasound (HIU) was investigated as a pretreatment method for the deacetylation process of chitin, and data indicated that HIU pretreatment of chitin flakes lasting up to 30 minutes was insufficient to change the DA of the resulting chitosan.

Since the HIU treatment has been considered as an efficient way to modify the MW of polysaccharides in general, the third phase of this study focused on tailored modification of chitosan MW with HIU process. The factors that influence the ultrasonic degradation process of chitosan were investigated and the results showed that the MW,

radius of gyration and polydispersity of chitosan were efficiently reduced, whereas the chain conformation and DA were unchanged after sonication. The degradation of chitosan by ultrasound was primarily driven by mechanical forces and degradation mechanism could be described by a random scission model. The degradation process was affected by ultrasound intensity, solution temperature, polymer concentration and ionic strength, while acid concentration had little effect. Additionally, the data indicated that the degradation rate coefficient was affected by the degree of deacetylation of chitosan and independent of the initial molecular weight.

In the fourth phase, hydrophobic modification of chitosan (HM-chitosan) was performed to probe new way to improve the functionality of chitosan. The physicochemical properties and antimicrobial activity of HM-chitosan and HM-chitosan based films were investigated, and the data indicated that hydrophobic modification introduced thermal responsive and self assembly properties, while maintained the antimicrobial activity of chitosan. Furthermore, the water vapor permeability and mechanical properties of HM-chitosan based films decreased.

Contributions of this research include developing a practical method for the DA analysis, elucidating the effects of HIU pretreatment on the deacetylation process and factors that affect the ultrasonic degradation process of chitosan, along with the effects of hydrophobic modification on the functionalities of chitosan and chitosan films.

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1 Literature Review

1-1 Overall introduction

As wellness and health become the top concerns of consumers today, the demand for food products free of synthetic preservatives has considerably increased. Application of natural compounds and biopolymers as food additives and packaging materials has been an important approach investigated by researchers in both academia and the food industry to address these concerns. Chitin, a natural polysaccharide consisting of acetylglucosamine, and chitosan, the deacetylated product of chitin, are such biopolymers that have received intensive interest for their potential applications as antimicrobial agents and antimicrobial packaging in the food industry (Ravi Kumar, 2000; Koide, 1998; Sashiwa and Aiba, 2004; Synowiecki and Al-Khateeb, 2003; Kurita, 2001; No and others 2007; Shahidi and others 1999).

The majority of commercial products of chitin origin are produced from shellfish waste and converted to chitosan by an alkali deacetylation process. The functional properties and biological activities of chitin and chitosan are tightly associated with their molecular parameters, the degree of acetylation (DA) and molecular weight (MW) (Berth and Dautzenberg, 2002; Chatelet and others 2001; Sorlier and others 2001). However, commercially available chitosan products generally do not have well controlled DA and MW due to the variation in sources and production methods. Therefore, many researches have been devoted to produce chitosan with controlled DA and MW to obtain desired functionalities.

In order to produce chitosan with controlled DA, most of studies have been focused on optimizing the deacetylation process to obtain highly deacetylated chitosan (Chang and others 1997; Tsaih and Chen, 2003; Weska and others 2007; Galed and

others 2005). However, since the crystalline structure of chitin is resistant to the concentrated alkaline deacetylation, highly deacetylated chitosan is only obtainable through long time deacetylation reaction or repeated deacetylation (Tolaimate and others 2003). High intensity ultrasound (HIU) has been investigated as a pretreatment method for the deacetylation process aiming to reduce the crystallinity of chitin and to improve the deacetylation efficiency (Serglo and others 2002). Additionally, HIU has been evaluated as a method to modify chitosan MW, along with many other methods including acid and enzymatic hydrolysis, microwave, and UV and gamma irradiation (Hasegawa and others 1993; Ilyina and others 2000; Wasikiewicz and others 2005; Chen and others 1997). Among them, HIU has been considered as advantageous due to its high efficiency and by-product free procedure (Kardos and Luche, 2001; Baxter and others 2005; Trzcinski and Staszewska, 2004).

Although the functionality of native chitosan depends on the DA and MW, it can be greatly improved by chemical modifications of chitosan (Kurita, 2001; Jayakumar and others 2005; Ravi Kumar, 2000; Sashiwa and Aiba, 2004). Hydrophobic modification is of special interest to the food industry due to the enhanced rheological properties and self-assembling tendency of chitosan obtained after modification (Rinaudo and others 2005; Desbrieres and others 1996; Chen and others 2003).

The main objective of this review is to provide readers with a brief introduction of chitosan research focusing on the application, physicochemical properties, and analytical production and processing method. Special attention will be paid to the application of high intensity ultrasound and hydrophobic modification for tailored modification of chitosan DA, MW and functionality.

1-2 Application of chitin and chitosan in the food industry

Although chitosan has not been approved as a generally recognized as safe (GRAS) material in the United States, it has been approved as a food additive in China, Japan and Korea (No et al., 2007). Similar to other polysaccharides, chitosan can simply act as a thickening and water binding ingredient in foods. However, it is only the unique polycationic characteristic of chitosan that distinguishes it from other polysaccharides in applications that aim to extend the shelf life and improve the quality of food products.

These applications have been investigated in numerous food products, including bread, egg, fruits and vegetables, juice, Kimchi (a traditional Korean fermented vegetable food), mayonnaise, meat, milk, noodle, rice cake, sausage, seafoods and seafood products, soybean curd and sprouts, starch jelly and vinegar (No et al., 2007). In general, when chitosan is applied in form of coatings or films, the improvement of quality is achieved by inhibiting the growth of microorganisms, retarding the oxidation, and by acting as a selective barrier to reduce respiration and transpiration rates (No et al., 2007).

1-2.1 Antimicrobial activity and applications in food preservation

1-2.1.1 Antimicrobial mechanism

Many studies have shown that chitosan and chitosan derivatives have strong antimicrobial activity against both gram-positive and gram-negative bacteria, including a number of foodborne pathogens, spoilage bacteria, and fungi. With the addition of 3% chitosan to ground beef and turkey, the potential risk of *Clostridium perfringens* has been effectively reduced (Juneja and others 2006). Chitosan films reduces the *Listeria monocytogenes* in bologna samples by 2 logs (Zivanovic and others 2005). Combined

with its biodegradable, biocompatible and non-toxic properties, chitosan is very promising to be used as a food antimicrobial agent. However, the practical application of chitosan as antimicrobial agent is rather limited at this time due to the insufficient understanding of underlying mechanism for antimicrobial activity.

One of the hypothesis have suggested that chitosan, similarly to other biologically active polycations, interacts with negatively charged components of cell envelope which results in cell death (Helander and others 2001; Rabea and others 2003). However, the exact interaction site(s), mechanism, and “killing mode” remain unclear. Several studies have demonstrated that the interaction site in gram-negative bacteria is at the outer membrane and the cytoplasmic membrane appears to be unaffected (Helander et al., 2001; Liu and others 2004b). Recent research has also showed that the permeability of both outer membrane and cytoplasmic membrane is increased after chitosan treatment, which is evidenced by the release of cytoplasmic material or intake of hydrophobic probe 1-N-phenylnaphthylamine (NPN) of cell (Je and Kim, 2006; Helander et al., 2001; Liu et al., 2004b). However, no release of lipopolysaccharide or other membrane lipids has been found, indicating that the cell membrane structure has not been affected(Helander et al., 2001). Therefore, it remains unclear whether chitosan creates pores in the cell membrane, resembling to the action mode of certain antimicrobial peptides(Shai, 1999), or simply neutralizes the membrane, subsequently distorting it, resembling to the action mode of other antimicrobial peptides (Hancock, 2001).

Beside the aforementioned hypothesis, other hypotheses have been proposed to explain the antimicrobial activity of chitosan. For example, chitosan has been found to “kill” the bacteria by flocculating the cells and disrupting cellular metabolism(Guo and

others 2006), or by coating cell surface and blocking nutrients mass transfer(Qin and others 2006). Two recent studies have suggested that chitosan actually can pass the cytoplasmic membrane and “kill” bacteria by formation of insoluble precipitate at the physiological pH(Zheng and Zhu, 2003; Qin et al., 2006). It is also hypothesized that chitosan heptamers can penetrate into the cell and interfere with the transcription of RNA and the synthesis of proteins in fungi thus resulting in antifungal activity(Hadwiger and others 1986).

1-2.1.2 Factors that affect the antimicrobial activity

The antimicrobial activity of chitosan differs with the type of tested microorganisms. In general, gram-positive bacteria are more sensitive than gram-negative bacteria, which is probably due to the presence of extra outer membrane in the latter(No and others 2002). The highly cationic mutants of *Salmonella typhimurium* are more resistant to chitosan treatment than the parent strain, which supports the hypothesis described before that chitosan “kills” the bacteria through the electrostatic interaction(Helander et al., 2001). Furthermore, the bacteria in different growth stages have different sensitivity to chitosan - the earlier growth stage chitosan is added the greater effect it has(Guo et al., 2006).

Many studies have shown that molecular weight of chitosan has profound effects on its antimicrobial activity (Zheng and Zhu, 2003; No et al., 2002; Jeon and others 2001). However, a number of these reports contradict each other, as summarized in Table 1. For example, No et al. has found that the antimicrobial activity is increased with the decrease of MW in the range of 1 k to 22 kDa (No et al., 2002), whereas Jeon et al. has found that the antimicrobial activity of chitosan is increased with the increase of MW in the range of

Table 1-1: Antibacterial activity of chitosan as affected by degree of acetylation (DA), molecular weight (MW) and concentration.

Microorganisms	DA (%)	Concentration (ppm)	Antibacterial activity and MW dependence
<i>E.coli</i> (Liu and others 2006)	20%	<20 ppm	No antibacterial activity
		50-100 ppm	55 k>.....>155 k
		>200 ppm	No MW dependence
<i>L. monocytogenes</i> <i>S. typhimurium</i> (Zivanovic and others 2004)	150 k (15-25%)	1000 ppm	5 k < 150 k
	5 k (10%)		
^A G-(No et al., 2002)	No-specified	1000 ppm	746 k> all others in the range of 28 k-1671 k
		10000 ppm	1 k>.....>22 k
		1000 & 10000 ppm	No MW dependence
<i>E. coli</i> (Zheng and Zhu, 2003)	11%	2500 - 10000 ppm	5 k>.....>300 k
		2500 - 10000 ppm	5 k<.....<300 k
<i>E.coli</i> (Jeon and Kim, 2000)	11%	1000 ppm	Chitosan > oligomers (DP 3-6)
^C G- & ^D G+ (Jeon et al., 2001)	11%	1000 ppm	1 k< 5 k < 10 k
<i>E. coli</i> & <i>S. aureus</i> (Qin et al., 2006)	12-15%	300 ppm	48 k and 78 k > all others In the range of 14 k -400 k

A: *Escherichia coli*, *Pseudomonas fluorescens*, *Salmonella typhimurium* and *Vibrio parahaemolyticus*

B: *Listeria monocytogenes*, *Bacillus megaterium*, *Bacillus cereus*, *Staphylococcus aureus*, *Lactobacillus plantarum*, *L. brevis* and *L. bulgaricus*

C: *Escherichia coli*, *Escherichia coli O-157*, *Salmonella typhimurium* and *Pseudomonas aeruginosa*

D: *Streptococcus mutans*, *Micrococcus luteus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*

1 kDa to 10 kDa (Jeon et al., 2001). These discrepancies are probably due to the difference of the experimental material and analytical methods. In general, for gram-negative bacteria, chitosan is more effective than chitosan oligomers, and low MW chitosan is more effective than high MW chitosan (Zheng and Zhu, 2003; No et al., 2002). For gram-positive bacteria, the trend is somewhat reversed – chitosan is more effective than chitosan oligomers but high MW chitosan is more efficient than low MW chitosan (Zheng and Zhu, 2003; No et al., 2002). These experimental observations has suggested that multiple antimicrobial mechanisms may exist(Zheng and Zhu, 2003).

The effect of DA on the antimicrobial activity of chitosan can not be ignored because DA is associated with the chitosan chain conformation in solution(Anthonsen and others 1993). Study of Ikeda et al. has pointed out that the chain conformation of biological active polycations affects their biological activity(Ikeda and others 1990). Park et al. has found out that 25% DA chitosan processes highest antimicrobial activity as compared to 10% and 50% DA chitosan(Park and others 2004).

Chitosan exhibits antimicrobial activity only in the acidic environments, which has been attributed to the low solubility of chitosan at higher pH and to the less protonation extent above pH 6.5, the pKa of amino group within chitosan(Chung and others 2003). However, 50% acetylated water-soluble chitosan does not process antimicrobial activity at all even in the acidic media(Qin et al., 2006). Thus, solubility and protonation may be a requirement to initiate the interaction between chitosan and cell, but not a cause of cell death.

The effect of ionic strength of solution on the antimicrobial activity of chitosan has been investigated by several groups (Chung et al., 2003; Devlieghere and others

2004). However, the results are contradictory. Chung et al. has suggested that with the antimicrobial activity of chitosan is enhanced with the increase of ionic strength from 0 to 0.6 M (Chung et al., 2003), while Devlieghere et al. suggest that addition of 0.5% and 2% sodium chloride have negative effect on the antimicrobial activity of chitosan (Devlieghere et al., 2004).

The effects of other environmental factors and food components on the antimicrobial activity have also been investigated (Chung et al., 2003; Devlieghere et al., 2004). The antimicrobial activity of chitosan is reduced with addition of metal ions, but promoted with the addition of EDTA (Chung et al., 2003). Starch and protein have negative effect on the antimicrobial activity of chitosan, while oil has no influence (Devlieghere et al., 2004).

1-2.1.3 Methods to improve the antimicrobial activity of chitosan

Several approaches have been investigated to improve the antimicrobial activity of chitosan. Among them, chemical modification of chitosan has been the most intensively studied. The antimicrobial activity of chitosan derivatives, such as quaternary ammonium chitosan (Jia and others 2001; Kim and others 1997), hydroxypropyl chitosan (Xie and others 2002), carboxymethylated chitosan (Liu and others 2001), sulfated chitosan (Huang and others 2004), and a series of aminofunctionalized chitosan (Je and Kim, 2006) have been investigated. Most of these modified chitosans have an increase of antimicrobial activity compared to the parent chitosan (Je and Kim, 2006; Jia et al., 2001; Kim et al., 1997).

Since chitosan alters the cell membrane permeability, which sensitizes bacteria to many external agents, combination of chitosan with external agents to obtain a

synergistic effect has been another way to promote the antimicrobial activity(Helander et al., 2001). A recent study has shown that chitosan sensitizes some gram-negative bacteria to lysis by SDS(Helander et al., 2001). The complex of chitosan and zinc exhibits wide spectrum of antimicrobial activity, which are 2-8 times and 4-16 times higher than those of chitosan and zinc sulfate alone, respectively(Liu and others 2004a). Another recent article has indicated that combination of nonionic surfactant alkyl β -glucopyranoside (AG) and chitosan has much higher antimicrobial activity than chitosan and AG alone(Liu et al., 2004a). It is worthy to note that the last two cited articles consider the formation of complex between chitosan and zinc or AG as the main reason for the synergistic effect.

1-2.2 Metal chelating ability and use as antioxidant

Recently, there has been an increased interest in the antioxidant ability of chitosan and chitosan derivatives(Kim and Thomas, 2007; Guo et al., 2006; Guo and others 2005; Xie and others 2001), but it may be arguable whether the antioxidant capability of chitosan is due to its metal chelating ability or due to formation of macromolecular radicals(Xie et al., 2001). Regardless of the mechanism of its antioxidant potential, chitosan has been applied in meat and meat products to retard lipid oxidation during storage. Lipid oxidation is effectively delayed in salmon fillets with coating of 1% and 2% chitosan(Sathivel, 2005). Chitosan coating substantially retards lipid oxidation in shelf stable intermediate moisture meat product after irradiation treatment(Rao and others 2005).

1-2.3 Application as edible films and coatings

As a polysaccharide, chitosan forms films with good gas barrier ability, but poor moisture ability. Chitosan films are basically hydrophilic with water vapor permeability

of chitosan films ranged from 0.26 to 0.69 ng·m/m²·s·Pa(Park and others 2002). The oxygen permeability of chitosan films is comparable with commercial polyvinylidene chloride (PVDC) or ethylene vinyl alcohol copolymer films(Butler and others 1996). Study of Bai et al. indicates that chitosan films are more selectively permeable to oxygen than to carbon dioxide, thus chitosan coating is likely to modify the internal atmosphere without causing anaerobic respiration(Bai and others 1988). The mechanical of chitosan film are varied with composition, chitosan nature and storage conditions (Butler et al., 1996; Park et al., 2002). Although certain chitosan films exhibits extremely high physical strength, the tensile strength of most chitosan films ranges from 6.7 to 150.2 MPa and is too brittle to be used as ordinary self-standing packaging films(Park et al., 2002). The addition of plasticizers results in decrease of tensile strength and increased film flexibility(Park and others 1999). The elongation at break values of chitosan films ranges from 4.1 to 117.8%, and is much lower than those of commercial high density polyethylene or low density polyethylene films(Park et al., 2002). Overall, the mechanical properties of chitosan films are comparable to medium strength commercial polymer films. Therefore, future research should be directed toward the improving of water vapor barrier ability and mechanical properties, whereas maintaining the oxygen barrier ability and antimicrobial activity.

1-2.4 Nutrition benefits and risks

Glucosamine, the monomer of chitosan, has been marketed as dietary supplement that prevents osteoarthritis, while chitosan has been marketed as dietary supplement for weight control. An additional beneficial effect of chitin and chitosan as dietary supplements is the reduction of cholesterol due to its ability to bind dietary lipids(Koide,

1998), although new research indicates that small amount intake of chitosan fails to meet the fat reduction claim in clinical tests(Gades and Stern, 2005).

Earlier experimental evidence has shown that the consumption of chitosan is safe at the regular exposure level(Knorr, 1984). More evidence from strict designed long term experimental data must be accumulated to fully evaluate the safety of chitin and chitosan. As indicated by nutrition studies, the potential risks of dietary chitosan are associated with the calcium metabolism, minerals, and fat soluble vitamins(Koide, 1998).

1-3 Chemical structures and sources of chitin and chitosan

Chitin is a homopolymer of N-acetyl-D-glucosamine monomer units covalently attached by β (1 \rightarrow 4) linkage and chitosan is a homopolymer of glucosamine (Figure 1-1). Compared to cellulose, the C2 hydroxyl group is replaced by an acetamide and amino group in chitin and chitosan, respectively. Commonly available chitin and chitosan are actually not pure homopolymers, but exist as co-polymers of acetyl-glucosamine and glucosamine. This results in occasional interchangeable usage of chitin and chitosan definitions in the literature. In general, chitin has degree of acetylation (DA, the mole fraction of N-acetyl-D-glucosamine in the polymer chain expressed as a percentage) ranged from 85 – 95% depending on the source and extraction method(Kurita, 2001). Chitosan usually has degree of acetylation less than 30%(Kurita, 2001). However, the more practical way to distinguish chitin and chitosan is by their solubility in dilute acids in which chitin is insoluble and chitosan is soluble. Thus the homogeneous deacetylated chitin with DA of 50% should also be considered as chitosan.

Chitin is a structural polysaccharide found in certain species of fungi, insects and crustacean animals. Similar as cellulose in plant cell wall, chitin is the major component

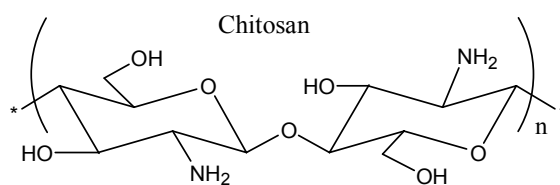
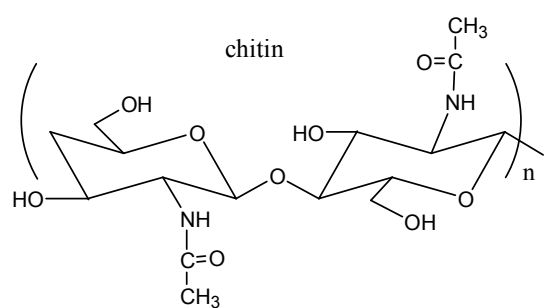
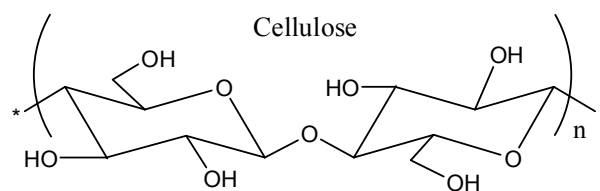


Figure 1-1: Chemical structures of cellulose, chitin, and completely deacetylated chitosan.

providing rigidity and strength for fungal cell walls(Ruiz-Herraera, 1992), which are primarily composed of chitin, glucans, mannans and glycoproteins. There is strong evidence showing that chitin, glucans and glycoproteins are covalently cross-linked together and that the cross-linking is a dynamic process that occurs extracellularly(Shaun M. Bowman, 2006). In animals, chitin mainly exists in the shells of crustaceans and mollusks, in the backbone of squids and cuticle of insects(Tharanathan and Kittur, 2003). Long chitin molecules are associated with proteins by covalent bonds and together they form a complex structural network(Tharanathan and Kittur, 2003). On crustacean shells, calcium carbonate deposits into the network contributing to strength of the shells and protection of the organisms(Tharanathan and Kittur, 2003).

Chitosan is not native to animal sources, but only presents in a small number of fungi, such as *Mucor*, *Absidia* and *Rhizopus* species(Ruiz-Herraera, 1992). The presence and amount of chitin in animals and fungi is specific to species, age and environmental conditions where the organism exists. Chitin content in the dry shells of crabs, lobsters and shrimps ranges from 14 to 27 %(Ashford and others 1977), while in the fungal cell wall it varies from 2 to 42 %, the lowest value corresponding to yeasts, and the highest values to Euscomycetes(Ruiz-Herraera, 1992).

Chitin is the second most abundant biopolymer on earth next to cellulose. It is estimated that its total annual biosynthesis reaches 100 billion tons(Tharanathan and Kittur, 2003). Total chitin produced by arthropods has been estimated to be $1,362 \times 10^6$ tons in freshwater, athalassohaline and marine ecosystem(Cauchie, 2002). The best available source of chitin is the seafood waste, primarily the crabs and shrimps shells.

The annual worldwide production of crustacean shells has been estimated as 1.2×10^6 tons (Synowiecki and Al-Khateeb, 2003).

1-3.1 Physico-chemical properties and analysis

Properly processed, highly purified chitin and chitosan are white and odorless solids. Their chemical structures are similar to those of cellulose (Fig1-1). The only difference is that the 2-hydroxy group of the cellulose has been replaced with an acetamide or amino group in chitin or chitosan, respectively. Therefore, the physico-chemical properties and research methodologies for all three biopolymers are similar. For example, chitin is insoluble in the common organic and inorganic solvents, but soluble in salt organic mixtures of N, N-dimethylacetamide (DMAc) with 5% LiCl or N-Methyl-2-pyrrolidone (NMP)-5% LiCl, which is a common solvent for cellulose (Rutherford III and Austin, 1977).

1-3.1.1 Degree of acetylation (DA) and analysis methods

Degree of acetylation (DA) is defined as the fraction (expressed in %) of acetylated glucosamine units in the polymer chain. The physicochemical behavior and biological activity of chitin and chitosan have been greatly affected by the DA value (Minagawa and others 2007; Gupta and Jabrail, 2006) apparently because the DA relates to the number of hydrophobic groups in chitosan chain.

Various techniques have been proposed to determine the degree of acetylation for chitin and chitosan. The methods include infrared spectroscopy (IR) (Duarte and others 2002; Brugnerotto and others 2001), ^{13}C solid-state NMR (Duarte and others 2001), first derivative ultraviolet spectrometry (Muzzarelli and Rocchetti, 1985), potentiometric titration (Jiang and others 2003), and high performance liquid chromatography

(HPLC)(Niola and others 1993). Among these techniques, IR is the most common because of its convenience in minimal sample preparation, but IR requires the precise calibration using a wide spectrum of chitin and chitosan standards with known DA. The ^{13}C solid-state NMR appears to be the most reliable technique and is often used as the reference method, but it is not available in many laboratories due to the high cost of the instrument. Ultraviolet spectrometry and potentiometric titration are techniques that require dissolved samples and, thus, are not applicable for chitins and chitosans with DA > 50 %. Although widely used, none of the above methods can achieve a timely and accurate analysis of DA for both chitin and chitosan.

1-3.1.2 Molecular weight (MW) and analysis methods

The most common ways to obtain the molecular weight of chitosan are viscometry and gel permeation chromatography. Both methods are not time consuming and easy to perform, compared to other commonly used methods, such as analytical ultracentrifugation(Terbojevich and Cosani, 1997). However, both methods are not absolute and rely either on determination of the Mark-Houwink equation constants or on the construction of calibration curve with chitosans of known MW, which are usually not available.

The methods based on viscosity determination measure the molecular weight using Mark-Houwink equation: $[\eta] = kM^a$, where η represents the intrinsic viscosity and M - the viscosity average molecular weight. The constants a and k are usually determined by static light scattering method in 0.1 M acetic acid and 0.2 M sodium chloride buffer and commonly reported with values of 1.81×10^{-3} and 0.93, respectively(Roberts and

Domszy, 1982). However, change of degree of acetylation may affect the values of the constants(Rinaudo, 2006).

The tandem of light scattering detection and gel permeation chromatographic (GPC) technique has been shown as a powerful tool in the analysis of polymer molecular weight, radius of gyration and molecular conformation. The theory of light scattering for macromolecule analysis can be found elsewhere(Wyatt, 1993). Two basic principles are utilized to calculate the molecular weight and radius of gyration.

- 1) The intensity of light scattered ($I_{\text{scattered}}$) is directly proportional to the product of the molar mass (M), concentration (c) and square of specific refractive index increment $\left(\frac{dn}{dc}\right)^2$ of the polymer.

$$I_{\text{scattered}} \propto Mc \left(\frac{dn}{dc}\right)^2 \quad (1)$$

- 2) The angular variation of the scattered light is directly related to the size of the molecule, where $P(\theta)$ is the particle scattering function describing the angular variation of the scattered intensity as a function of particle size.

$$I(\theta)_{\text{scattered}} \propto Mc \left(\frac{dn}{dc}\right)^2 P(\theta) \quad (2)$$

Although there are some limitations for this technique, for example the dn/dc for heterogeneous copolymers must be measured at each elution and is often overlooked by researchers, GPC with light scattering detector represents the most accurate and powerful technique for the macromolecular molecular weight analysis(Wyatt, 1993). It has been successfully applied in the analysis of chitosan molecular weight(Beri and others 1993;

Ottoy and others 1996). The optimized separation conditions, injection concentration and measurement uncertainty have all been discussed(Beri et al., 1993; Ottoy et al., 1996). It is found that ammonium acetate buffer is the best mobile phase to reduce the reversible interaction between chitosan and the chromatographic column(Ottoy et al., 1996). The technique is capable of providing reliable results for chitosan with molecular weight in the range of 10,000 – 50,000(Beri et al., 1993).

1-4 Chitin, chitosan and chitosan derivatives production methods

Crustacean chitin is naturally closely associated with proteins, minerals, lipids, and pigments. The process of industrial extraction of chitin consists of three basic steps (Figure 1-2): 1) demineralization - to remove calcium carbonate; 2) deproteinization - to remove proteins; and 3) decoloration - to remove pigments(No and Mayers, 1997). Demineralization is generally performed with HCl at a concentration of 0.275 – 2 M at a temperature of 0 - 100 °C for 1 - 48 h(No and Mayers, 1997). Deproteinization is typically performed with 1 M NaOH at 65 - 100 °C for 1 - 72 h(Roberts, 1992) and decoloration is carried out by ethanol, acetone, or hydrogen peroxide(No and Mayers, 1997).

Chitosan is converted from chitin by treatment with concentrated sodium or potassium hydroxide solution (40-50 %) at 80 - 150 °C(No and Mayers, 1997). Depending on the end application, chitosan can be converted to oligomers and monomers by acid catalyzed hydrolysis or to various derivatives by appropriate chemical modifications. The whole process of production of chitin, chitosan and chitosan derivatives from crustacean shells is shown in Figure 1-2 (No and Mayers, 1997).

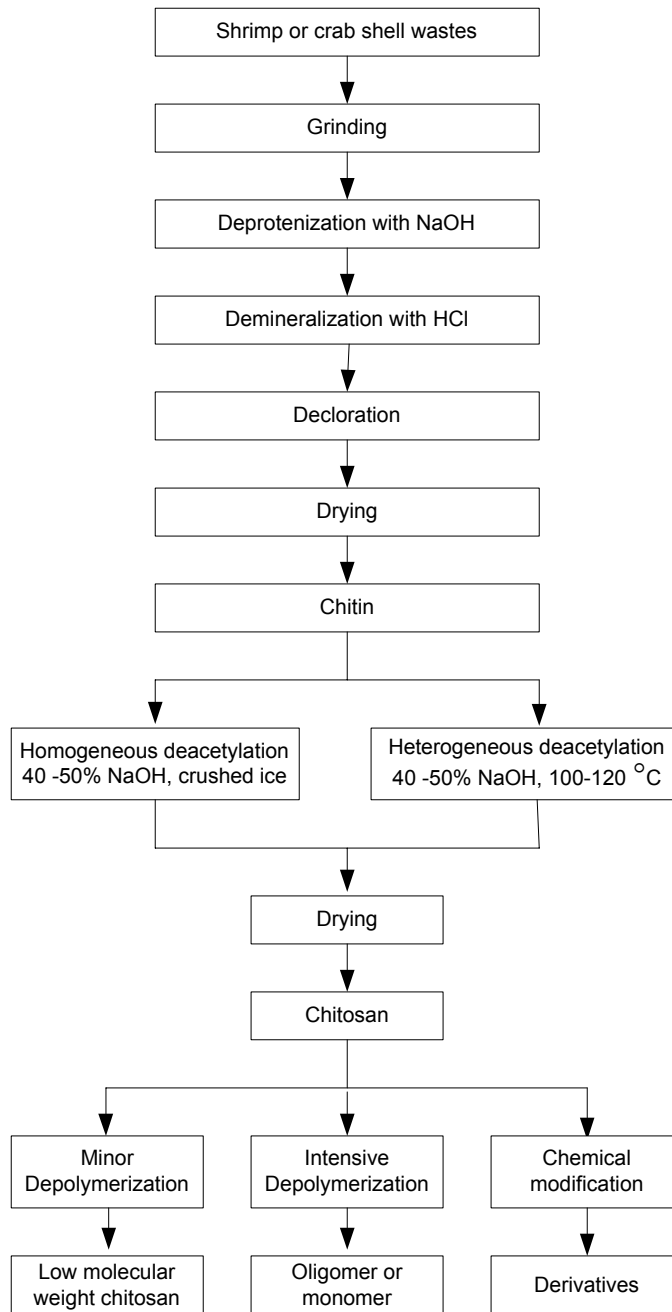


Figure 1-2: Manufacture process of chitin, chitosan and chitosan derivatives.

Many protocols have been studied to optimize the deacetylation process of chitin, including heterogeneous(Tolaimate et al., 2003) and homogeneous deacetylation(Sannan and others 1975; Kurita and others 1977). It is surprising that homogeneous deacetylation produced water soluble chitosan, which is considered to be a random-type copolymer of N-acetyl-D-glucosamine and D-glucosamine units(Kurita et al., 1977), whereas the heterogeneous process produces water insoluble chitosan which is believed to be a block-type copolymer of the N-acetyl-D-glucosamine and D-glucosamine units(Kurita et al., 1977).

Generally, the heterogeneous deacetylation can be carried out in an anhydrous or aqueous medium. Thus, Tolaimate et al. compared the anhydrous medium consisted of 50% (w/w) solid potassium hydroxide, 25% ethanol and 25% monoethylene glycol and aqueous medium with 40% sodium hydroxide(Tolaimate et al., 2003). Their results indicate that deacetylation of chitin from crustacean shell in anhydrous medium produces chitosan with better quality, represented by high MW and less DA in a shorter time(Tolaimate et al., 2003). However, another earlier study show that heterogeneous deacetylation in aqueous medium results in less degradation of chitosan and thus is better than the deacetylation in the anhydrous medium(Domard and Rinaudo, 1983). The addition of reducing agents, such as sodium borohydride and thiophenol reduces the extent of chitosan degradation in aqueous medium, but not in anhydrous medium(Domard and Rinaudo, 1983; Tolaimate et al., 2003). The influence of reaction temperature, reaction time, alkaline concentration and solution to chitin ratio on the deacetylation process of chitin has been the topic of several studies(Chang et al., 1997; Tsaih and Chen, 2003). However, no matter which method is utilized, it seems that a

complete deacetylated product cannot be obtained through a single heterogeneous deacetylation process.

The homogenous deacetylation is usually completed by treating alkaline suspension of chitin with crushed ice to dissolve chitin first and consequently carried out the deacetylation in a homogeneous condition(Sannan et al., 1975). Currently, the heterogeneous deacetylation in aqueous medium is widely used in industry.

Depending on the end applications, chitosan can be processed into high, medium, low molecular weight product, oligomers and monomers by chemical, enzymatic and irradiation methods. Chemical hydrolysis is generally performed at high temperature by concentrated acids, such as hydrochloric acid(Varum and others 2001) or nitrous acid(Allan and Peyron, 1995). It can be also carried out by other chemicals, such as hydrogen peroxide(Chang and others 2001) and redox radical initiator(Fedoseeva and others 2006). However, a disadvantage of chemical hydrolysis is the use of toxic chemicals, which is not desired in food industry. Additionally, hydrolysis by concentrated acid produces a large amount of monomer because the end residues are hydrolyzed 2.5 and 2.0 times faster as compared to the other residues(Einbu et al., 2007).

A number of enzymes including chitosanase, certain types of carbohydrases and proteases and several reactors, including batch reactor, tubular reactor containing immobilized with enzyme, ultrafiltration membrane reactors can be utilized to hydrolyze chitosan into oligosaccharides(Kim and Rajapakse, 2005). To reduce the cost, it appears that utilization of a dual reactor consisting of a tubular reactor and an ultrafiltration membrane reactor for a continuous operation is needed(Jeon and Kim, 2000). However, the high viscosity of concentrated chitosan solutions would limit the operability of

reactor for continuous production(Ming and others 2006). Therefore the reduction of viscosity before enzymatic hydrolysis seems necessary.

Recently, several studies have reported use of various irradiation technologies for chitosan degradation, including microwave(Guo et al., 2005), gamma and ultraviolet radiation(Wasikiewicz et al., 2005). Based on studies of Huang et al, it is clear that the irradiation technologies causes change of chitosan chemical structure due to deamination and formation of carbonyl/carboxyl groups(Huang and others 2007). On the other hand, a physical technique - the high intensity ultrasound, is considered a by-product-free method and promising, environment-friendly technique for the degradation of chitosan (Baxter et al., 2005; Trzcinski and Staszewska, 2004; Chen et al., 1997; Einbu et al., 2007; Guo et al., 2006; Tsaih and Chen, 2003).

1-5 Theory and application of ultrasound in the food industry

Ultrasound is defined in terms of human hearing and it presents sound waves with frequency higher than that to which the human ear can respond (i.e. > 20 kHz)(Mason and Lorimer, 2002). The use of ultrasound in industry can be divided into two categories. The first category involves low amplitude (higher frequency) sound, which is used for analytical purposes, in medical imaging and chemical analysis. The second category, known as power ultrasound or high intensity ultrasound, is commonly referred to the sound waves with intensities higher than 1 W/cm^2 and frequencies between 20 and 100 kHz(McClements, 1995), although frequencies above 100 kHz are used occasionally. It has been in applied in various field for thermoplastic component welding, surface cleaning and decontamination, biological cell disruption and activation of various of chemical reactions(Mason and Lorimer, 2002). It has also been used in the food industry

for homogenization, emulsification, tenderization of meat, filtration, drying, and as aid in extraction (McClements, 1995; Gallego-Juarez and others 2003; Roberts, 1993).

As an ultrasound wave travels through a liquid medium, it causes the molecules to oscillate about their mean position. During the compression and rarefaction cycle of ultrasound propagation, the average distance between the molecules decreases and increases alternatively. If such distance exceeds the critical molecular distance necessary to hold the liquid intact, the liquid will break down and generate cavitation bubbles (Mason and Lorimer, 2002). The growth and collapse of these bubbles produces shock waves and generates highly turbulent flow conditions, extremely local high pressures and temperatures, which induce the so called sonochemical effects including mechanical and chemical effects (Fig1-3) (Mason and Lorimer, 2002). The mechanical effect is responsible for the solid fragmentation, erosion and increased mass transfer in a heterogeneous reaction system. The chemical effect produced by the thermolysis of water results in the formation of free radical and initiation of free radical reaction in the system (Kardos and Luche, 2001).

Ultrasonic degradation of a polymer in solution is a special case of these sonochemical reactions. It is generally accepted that the degradation is primarily of mechanical nature at low frequencies (Basedow and Ebert, 1977). The stresses that stretch and eventually break the polymer are caused by the friction forces generated by the relative movement of the solvent and polymer molecules as a result of the cavitation bubble collapse (Basedow and Ebert, 1977). Based on this mechanism, earlier studies indicate that the polymer degradation by ultrasound has two important characteristics (Basedow and Ebert, 1977): (1) the degradation is a non-random process

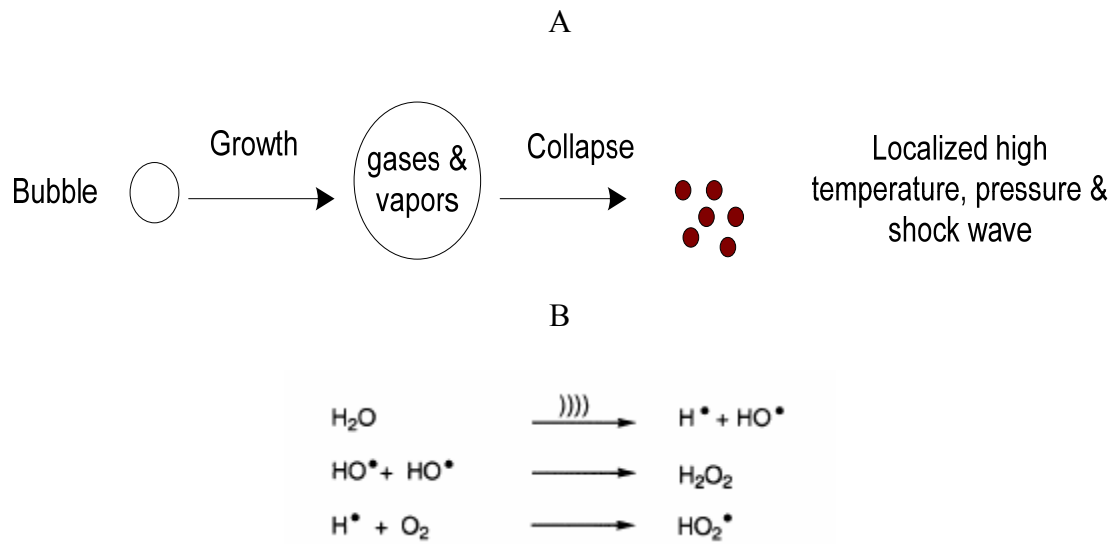


Figure 1-3: Mechanical effect (A) and chemical effect (B) of high intensity ultrasound.

Adjusted from (Kardos and Luche, 2001)

and the chain break occurred at the weakest points, (2) a final limiting molecular weight exists below which further degradation by ultrasound does not occur.

Many factors have profound effects on the polymer ultrasonic degradation process, including the acoustic parameters (frequency and intensity), the solvent properties and the nature of polymer. With the increase of frequency, the mechanical effects of ultrasound are decreased. This is because of the fact that high frequency waves provide insufficient time for cavitation bubble to grow and collapse (Mason and Lorimer, 2002). However, at frequencies higher than 100 kHz, the formation of free radical cannot be ignored and may play a role in the polymer degradation (Mark and others 1998; Kardos and Luche, 2001).

Many studies showed that the degradation is faster at higher intensities (Mason and Lorimer, 2002). It is suggested that the degradation occurs only at intensities above a threshold, at which ultrasonic wave energy is large enough to overcome the cohesive forces holding liquid together (Mason and Lorimer, 2002). It is also suggested that the degradation rate constant is a linear function of intensity (Baxter et al., 2005).

Most of the experimental data indicates that the polymer degradation rate decreases with increasing solvent vapor pressure (Mason and Lorimer, 2002; Madras and Chattopadhyay, 2001). The degradation is, however, less affected by the solvent density, viscosity and surface tension (Basedow and Ebert, 1977). With the increase of solvent vapor pressure, more solvent vapor will enter the bubble and cushion the collapse of the bubble resulting in a decrease of degradation rates (Kardos and Luche, 2001). Another way to increase the vapor pressure is to increase the solution temperature. Therefore, the increase of solution temperature leads to a decrease of degradation rate based on the “cushioning effect” (Kardos and Luche, 2001).

Most studies agree that the degradation rate is highest for sample with the highest initial molecular weight, lowest concentration, whereas there are contradictory evidences of the effect of polymer nature (Mason and Lorimer, 2002; Trzcinski and Staszewska, 2004; Chen et al., 1997).

1-6 Chemical modification of chitosan and potential application in the food industry

The commercial use of chitosan has been limited by its poor solubility at neutral and alkaline conditions. The chemical modification to improve solubility or to introduce a variety of functional groups is considered as a breakthrough in the utilization of chitosan(Sashiwa and Aiba, 2004). Most of studies have been subjected to recent reviews(Sashiwa and Aiba, 2004; Rinaudo, 2006; Jayakumar et al., 2005; Ravi Kumar, 2000; Kurita, 2001), which includes sugar modified chitosan, chitosan-dendrimer hybrid, cyclodextrin-linked chitosan, crown ether bound chitosan, acrylation, acetylation, N-phthaloylation, tosylation, alkylation, Schiff base formation, reductive alkylation, O-carboxymethylation and N-carboxyalkylation, silylation and graft copolymerization. Among these various modifications, the introduction of hydrophobic group on chitosan is of special interests to food industry due to their self aggregate behaviors which has potential application in food encapsulation. In general, the introduction of hydrophobic side group can be approached by three methods: (1) direct reaction with carboxylic acid anhydride(Jiang and others 2006), cyclic acid anhydride(Sashiwa and Shigemasa, 1999), or carboxylic acid chloride(Sashiwa and others 2002; Le Tien and others 2003), (2) amide formation between carboxylic acids and amine group on chitosan chain catalyzed

by water soluble carbodiimide (EDC)(Nakajima and Ikada, 1995; Guo et al., 2005), (3) reduction amination with aldehydes by reducing agents (Figure 1-4).

Water-soluble chitosan derivatives can be prepared by simple acetylation with acetic acid chloride or acetic acid anhydride(Sashiwa et al., 2002). Both moderate substitution of N, O-acetyl groups and moderate molecular weight is required for solubility in water(Sashiwa et al., 2002). Chitosan-based polymer micelles can be prepared by reacting swollen chitosan with palmitic anhydride in dimethyl sulfoxide (DMSO)(Jiang et al., 2006). The loading capability for the hydrophobic model drug, ibuprofen, is approximately 10% and the drug release is strongly depended on pH and temperature: low pH and high temperature considerably accelerated drug release (Jiang et al., 2006). Various N-acylated chitosan derivatives can be prepared via ring-opening reactions with various acid anhydrides in aqueous methanol system and a few of them have solubility over a wide range of pH(Sashiwa and Shigemasa, 1999). N-acylation of chitosan with various fatty acid chlorides prompts its hydrophobic character and the best mechanical characteristics and drug release properties is found for palmitoyl chitosan (substitution degree of 40-50%)(Le Tien et al., 2003).

Linolenic acid can be grafted on chitosan through a 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-mediated reaction(Guo et al., 2005). The bovine serum albumin (BSA) loading ability of this hydrophobically modified chitosan self aggregate is increased from 19.85 ± 0.04 to $37.57 \pm 0.25\%$ (Guo et al., 2005). Linoleic acid modified chitosan prepared by this method formed nanoparticles with size ranging from 200 to 600 nm(Chen et al., 2003). These nanoparticles encapsulates the lipid soluble model compound, retinal acetate, with 50% efficiency(Chen et al., 2003). Chitosan

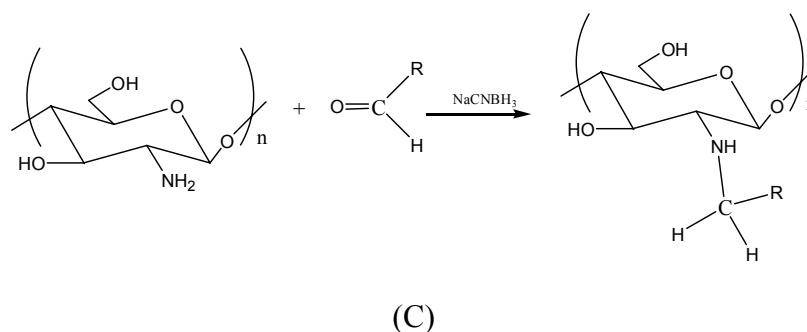
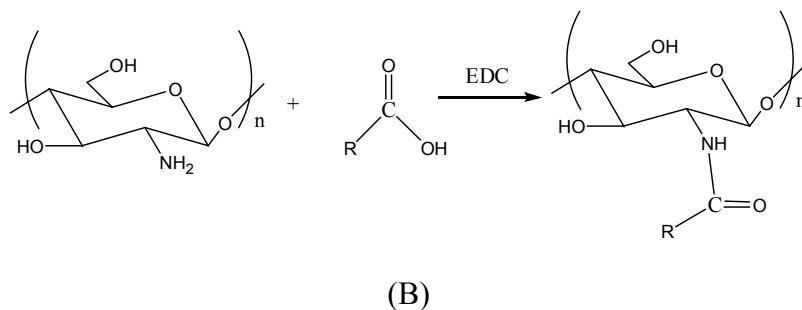
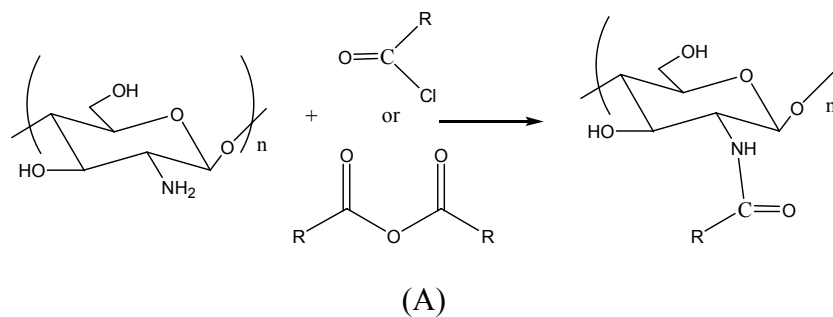


Figure 1-4: Hydrophobic modification of chitosan by (A) acid chloride and acid anhydride, (B) 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) mediated coupling, and (C) reduction amination.

derivatives, having 2.8-5.1% deoxycholic acid groups can form colloiddally stable self-aggregates in aqueous media with mean diameters of less than 200 nm and unimodal size distribution(Lee and others 1998). These colloidal self-aggregates has potential to be used as delivery tool for bioactive compounds.

Reductive alkylation of chitosan with various aldehydes can be easily catalyzed by reducing agents, such as sodium cyanoborohydride (NaCNBH_3) and sodium borohydride (NaBH_4)(Desbrieres et al., 1996). This is a simple way to prepare N-alkylated chitosan. Chitosan samples can be dissolved in acetic acid and react with an aldehyde at ambient temperature. The formed unstable Schiff bonds between aldehyde and amino group of chitosan can be reduced by titration with an excess aqueous reducing agents(Uragami and others 1997). Chitosan bearing alkyl groups at C2 to C6 with degree of substitution up to 0.08 produces membranes with altered water-permselectivity(Uragami et al., 1997). The characteristics of the membrane is corresponding to the change of physical and chemical properties(Uragami et al., 1997).

A series of alkylated chitosan having different alkyl chain lengths and different degree of grafting have been successfully prepared and process interesting thickening properties(Desbrieres et al., 1996; Rinaudo et al., 2005). The alkylated chitosan requires a minimum of six carbon atoms to demonstrate hydrophobic interactions in solution(Desbrieres et al., 1996), while the physical gelation requires alkyl chain length of C12 and the degree of grafting of 5%(Rinaudo et al., 2005). The hydrophobic interaction is increased with the degree of grafting, but the solubility of chitosan is limited by high degree of grafting eventually(Desbrieres et al., 1996).

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2 Determination of the Degree of Acetylation (DA) of Chitin and Chitosan by an Improved First Derivative UV Method

2-1 Abstract

An economical and accurate determination of degree of acetylation (DA) for highly acetylated chitin has always been a challenge for the researchers dealing with chitin and chitosan. A new protocol of the first derivative UV method employing concentrated phosphoric acid as a solvent for highly acetylated chitin was developed in this study. The solvent was proposed based on thorough investigation of the effects of associated reactions including chain degradation, monomer dehydration, and oxazolinium ion formation. The reproducibility and performance of the new protocol was evaluated using commercial samples and the results showed the DA values of both chitin and chitosan could be determined accurately and timely by a single analytical technique.

2-2 Introduction

Interest in the application of chitin and its derivative chitosan in the food industry and biomedicine is constantly increasing (Kumar and others 2004). The degree of acetylation (DA) is a key parameter that influences the physico-chemical properties of chitin and chitosan, such as solubility, chain conformation (Berth and Dautzenberg, 2002), electrostatic properties (Sorlier and others 2001) and biological properties of chitosan films (Chatelet and others 2001). Numerous analytical methods, including infrared red (IR) spectroscopy (Van de Velde and Kiekens, 2004; Duarte and others 2002), high pressure liquid chromatography (HPLC) (Frederic and others 1993), nuclear magnetic resonance (NMR) (Lavertu and others 2003; Duarte and others 2001), titration (Raymond and others 1993; Jiang and others 2003) and ultraviolet-visible (UV) adsorption spectroscopy (Muzzarelli and Rocchetti, 1985) have been proposed to precisely determine its true value. However, all the methods have some limitations. For example, expensive instruments are needed in case of IR, NMR and HPLC methods, extensive sample preparation (HPLC), or insufficient accuracy (titration, IR). The first derivative UV method developed in late 1980s, offered a simple and fast measurement of DA value with good accuracy and precision (Muzzarelli and Rocchetti, 1985). Although zero order of UV spectra can be used for DA measurement as well (Hsiao and others 2004), the first derivative of the spectra is less affected by the background noises of impurities and has been advocated by researchers as a standard method for routine determination of DA for chitosan (Tan and others 1998). Several modified first derivative UV methods have been proposed to improve the convenience and accuracy of the measurement (Liu and others 2006; Pedroni and others 2003). However, those methods use diluted acetic or

hydrochloric acid to dissolve chitosan prior to analysis. This unavoidably limits the determination scope to highly deacetylated chitosan samples only, which are soluble in diluted acids.

For highly acetylated chitin, concentrated phosphoric acid has been proposed as a good solvent and the DA determination is valid in the whole range of DA (Hsiao et al., 2004). Additionally, the results obtained by this method are well correlated with a solid state ^{13}C NMR method, so far the best but the most expensive method for the DA analysis(Hsiao et al., 2004). However, polysaccharides generally undergo complex reactions under the conditions of concentrated acids and heat. For example, cellulose can be hydrolyzed into glucose and consequently converted into 5-hydroxymethylfurfural (HMF) and levulinic acid by acid catalyzed dehydration(Girisuta and others 2007). Compared to these well investigated reactions of cellulose, little information is available regarding the effects of hot concentrated acid on chitin and chitosan. One study has reported the formation of HMF from fully deacetylated chitosan after nitrous acid depolymerization(Tommeraaas and others 2001). Apparently, the formation of HMF can introduce errors in the DA measurement because both acetyl-glucosamine and glucosamine can be converted to HMF. An intermediate glucofuranosyl oxazolinium ion of acetyl-glucosamine and similar products have been found in chitin solutions in concentrated phosphoric acid and anhydrous hydrogen fluoride (Vincendon, 1997; Bosso and others 1986). The intermediate ion is not stable and can be hydrolyzed into monosaccharide phosphate in diluted acids (Figure 2-1)(Vincendon, 1997; Bosso et al., 1986). Apparently, the formation and hydrolysis of such intermediate ions affect the DA measurements due to blocking or liberating acetyl group (Figure 2-1).

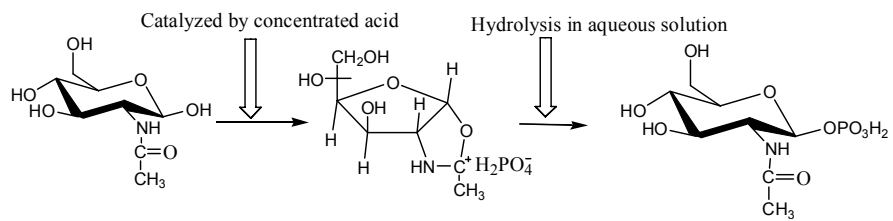


Figure 2-1: Formation and hydrolysis of glucofuranosyl oxazolinium ion from acetyl-glucosamine.

* adapted from (Vincendon, 1997)

Therefore, without thorough investigation of aforementioned reactions, DA determination method employing concentrated phosphoric acid as a solvent for chitin and chitosan should be used with caution. The objectives of this study were to evaluate the effects of chemical reactions associating with the utilization of phosphoric acid as a solvent on the DA determination by a first derivative UV method, and to improve the methodology to be used for determination of the whole DA range (0-100%) accurately and timely.

2-3 Experimental details

2-3.1 Materials and instruments

Acetyl-glucosamine (GlcNAc), D-glucosamine hydrochloride (Glc) and 85% phosphoric acid were purchased from Sigma (St. Louis, MO). Chitin and chitosan samples were provided by Primex (Primex, Iceland). A Shimadzu 2010 (Shimadzu, Columbia, MD) double beam UV-Vis spectrophotometer was used to collect the first derivative UV spectra of standards and samples under scan mode in the range of 400 to 190 nm. Sampling interval and slit width were both set at 1.0 nm. The pathway length was at 1cm.

2-3.2 Standard preparation and formation of standard curve

Standard solutions of GlcNAc and Glc were prepared in 0.85% phosphoric acid at concentrations of 0, 10, 20, 30, 40 and 50 $\mu\text{g/ml}$. The calibration curve was made by plotting the first derivative UV values at 203 nm (H_{203}) as a function of GlcNAc and Glc concentration.

2-3.3 Sample preparation and the DA determination

Chitin and chitosan samples were ground using Tomas Mill (sieve #40) and stored in desiccators at room temperature until analysis. A 3-step procedure was used to prepare a sample for the DA determination (Figure 2-2). Aliquots of 100 ± 10 mg chitin or chitosan were heated in 20 ml 85% phosphoric acid for 40 min at 60°C with constant stirring. After 40 min, when chitin/chitosan was completely dissolved, 1 ml clear solution was taken and diluted to 100 ml with deionized water. The dilution was necessary to get the chitin/chitosan concentration to the range detectable by a spectrophotometer. The diluted solutions were incubated at 60°C for 2 hrs prior the UV measurement. This was considered as a standard method. These parameters were applied in all experiments if not otherwise explained.

2-3.4 DA calculation method

The degree of acetylation of chitin and chitosan samples was calculated as

$$\text{DA (\%)} = \frac{\frac{m1}{203.21} \times 100}{\frac{m1}{203.21} + \frac{m2}{161.17}}$$

Where: $m1$ is the mass of acetyl-glucosamine in 1 ml chitin/chitosan solution (Step 2; Figure 2-2), calculated from the calibration curve by the corresponding H_{203} ; $m2$ is the mass of glucosamine in 1 ml chitin/chitosan solution (Step 2; Figure 2-2), calculated as $m2 = M - m1$. The mass of chitin/chitosan (M) in the 1 ml solution (Step 2; Figure 2-2) was calculated as: $M = (M_1 \times M_3)/(M_1 + M_2)$, where M_1 is mass of solid chitin/chitosan sample taken for analysis (100 ± 10 mg; Figure 2-2); M_2 is mass of 20 ml 85% phosphoric acid (Step 1; Figure 2-2); and M_3 is mass of 1 ml chitin/chitosan solution in concentrated phosphoric acid (Step 2; Figure 2-2).

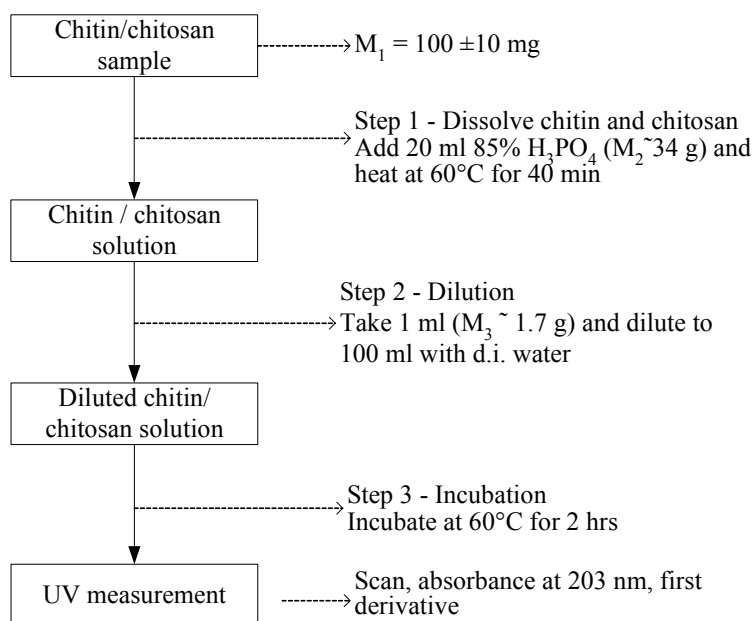


Figure 2-2: Diagram of the three-step procedure for determination the degree of acetylation (DA) values for chitin and chitosan.

2-3.5 Effects of chitin/chitosan solubilization on DA determination

Chitin and chitosan samples were dissolved in concentrated phosphoric acid by heating at 60°C for 10 to 40 min. After heating, the samples were diluted as explained earlier and incubated at room temperature for 0, 1, 2, 3 and 4 hrs before UV scans.

2-3.6 Effects of oxazolium ion formation on DA determination

Chitin and chitosan samples were dissolved in concentrated phosphoric acid by heating at 60°C for 40 to 180 min. After heating, the samples were diluted, incubated at room temperature for 0, 8 and 24 hrs before UV scan.

2-3.7 Effects of oxazolinium ion hydrolysis on DA determination

Chitin and chitosan samples were dissolved in concentrated phosphoric acid by heating at 60°C for 40 min. After heating, the samples were diluted with d.i. water (1 to 100 ml), and incubated at room temperature or 60°C for 0 to 24 and 0 to 5 hrs, respectively before UV measurements.

2-3.8 Effects of dehydration reactions on DA determination

Chitin and chitosan samples were dissolved in conc. phosphoric acid and heated at 60 or 80°C for 40, 60, and 100 min. After the heating, 5 ml sample solutions were diluted to 100 ml with d.i. water and the UV scans were immediately taken.

2-4 Results and Discussion

2-4.1 Standard spectra and calibration curve

The principle of the first UV derivative method for the DA determination is based on the absorbance intensity of acetyl group in chitin or chitosan (Muzzarelli and Rocchetti, 1985). Although acetyl-glucosamine absorbs in the range of 190 to 220 nm, the minimum interferences from glucosamine on the first derivative of acetyl-glucosamine was between

202 and 208 nm (Figure 2-3). By plotting the first derivative UV values at those wavelengths against the concentrations of GlcNAc to perform linear regression analysis, the best linear regression was specifically obtained at 203 nm. Thus, the first derivative value at 203 nm was chosen for DA measurements in this study and was represented by the symbol of H_{203} . A similar wavelength selection was made in a previous study where 202 nm was chosen when acetic acid was used as a solvent (Muzzarelli and Rocchetti, 1985). A plot of H_{203} values against the concentrations of N-acetyl-D-glucosamine in the range of 0 to 50 $\mu\text{g/ml}$ resulted in a good linear regression with the R^2 value of 0.996 (Figure 2-4). The H_{203} of glucosamine was also plotted against its concentration in the same graph clearly showing that the presence of glucosamine at the tested concentrations did not impose noticeable interference to the calibration curve of acetyl-glucosamine (Figure 2-4).

2-4.2 Effects of chitin/chitosan solubilization on DA determination

Although chitin/chitosan samples appeared visually to be dissolved in conc. phosphoric acid after a few minutes or so by heating at 60°C (Figure 2-2, Step 1), the determined DA values of the samples heated for 10 min were reasonable only if the UV scans were taken immediately after dilution (Figure 2-2, Step 3). If the diluted samples were incubated for an hour or longer, appearance of white haze was observed, and the resulting DA values were significantly lower (Figure 2-5). For samples dissolved by heating for 20 min, the DA values were correctly determined in samples incubated up to 1 hr after dilution. If the chitin samples were heated for 30 – 40 min in conc. phosphoric acid, the samples stayed clear under conditions examined in this study, and the DA values were the same regardless of how long the diluted samples were incubated (Figure. 2-5).

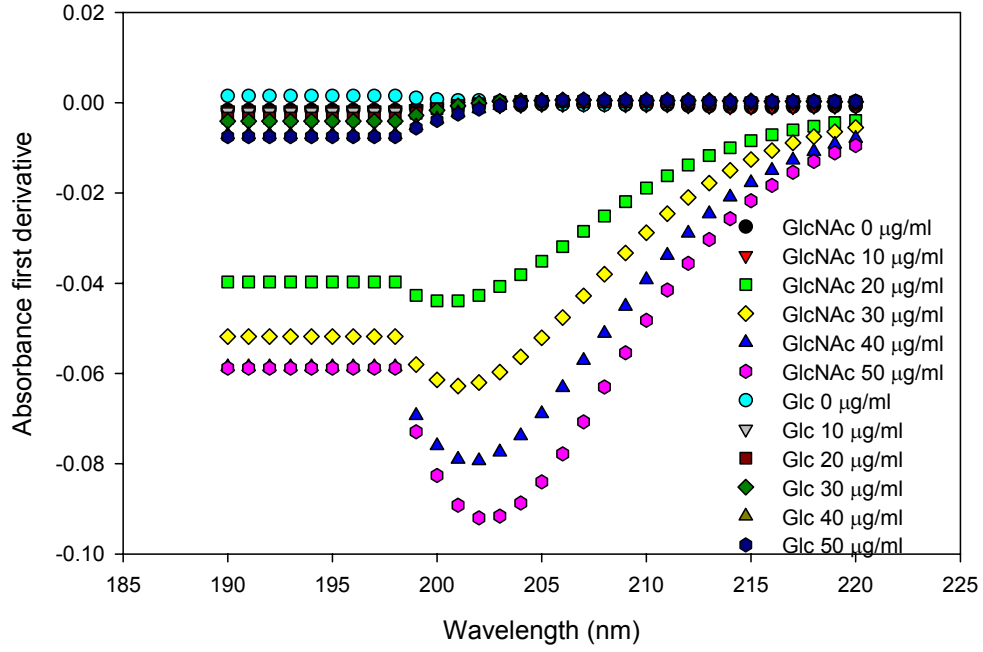


Figure 2-3: First derivative UV spectra of glucosamine (Glc) and acetyl-glucosamine (GlcNAc) standards at concentrations ranged from 0 to 50 µg/ml.

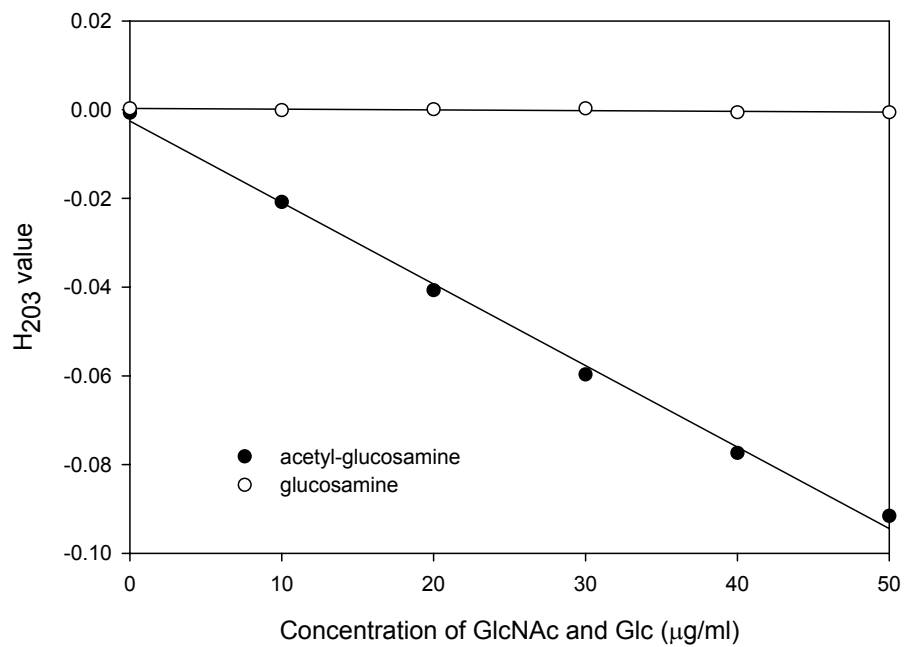


Figure 2-4: Plot of H_{203}^* values against concentrations of glucosamine (Glc) and acetyl-glucosamine (GlcNAc).

* H_{203} represents the first derivative value at 203 nm.

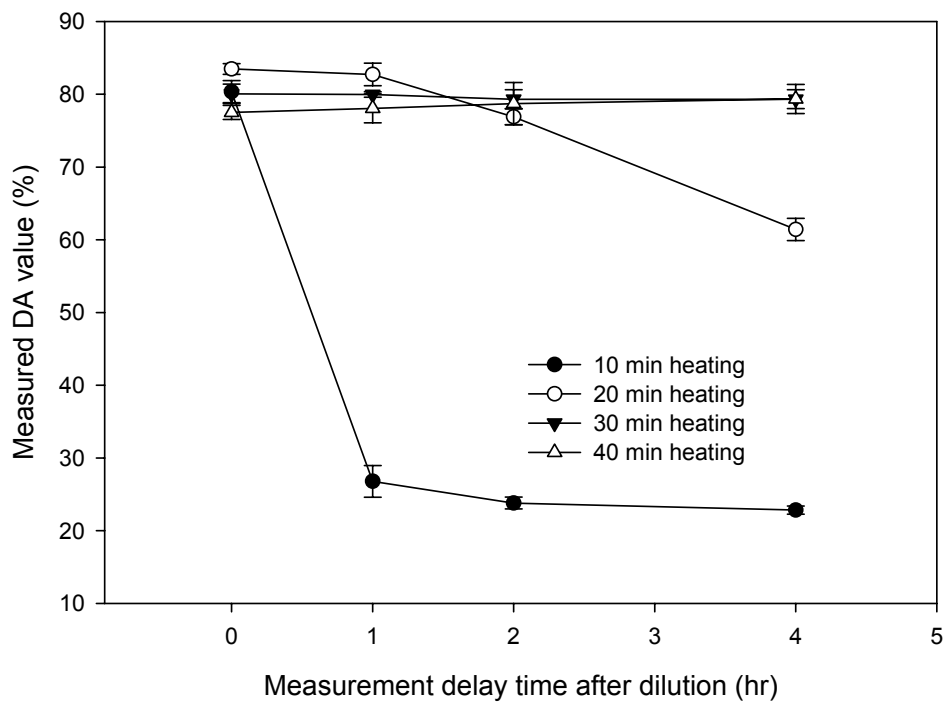


Figure 2-5: Variation of degree of acetylation (DA) values determined for the same chitin sample dissolved by heating at 60°C for 10 to 40 min and incubated for 0 to 4 hrs after dilution of the chitin solution.

* Values are represented as mean \pm standard deviation (n=3).

These results indicated that if the chitin was not completely dissolved in conc. phosphoric acid, it re-aggregated and precipitated out in diluted solutions causing underestimation of DA. Therefore, unless all samples can be analyzed in the exact time immediately after dilution (what is possible only if there is no more than one sample), minimum of 30 min of heating is necessary to achieve an accurate DA determination.

2-4.3 Effects of glucofuranosyl oxazolinium ion formation and hydrolysis on DA determination

The DA values determined after various length of heating at 60°C (to help dissolve chitin or chitosan) and different times of incubation after dilution are shown in Figure 2-6. The determined DA values for the samples which UV scans were taken immediately after dilution decreased as the time of heating increased over 40 min. This was probably caused by the formation of oxazolinium ions during prolonged heating of chitin/chitosan in conc. phosphoric acid. The oxazolinium ion blocked the acetyl-group (Figure 2-1) which resulted in significant reduction of absorbance at 203 nm and, consequently, underestimated DA value. On the other hand, if the measurements were taken after prolonged incubation of diluted samples (8 and 24 hrs), the determined DA values were higher than those obtained immediately after dilution. This was the result of hydrolysis of the oxazolinium ions in aqueous solutions over time and liberation of the acetyl-groups (Figure 2-1). Hsiao et al. made similar observations (Hsiao et al., 2004). However, they oversimplified the fact by saying that the ‘sample inhomogeneity’ was the reason for the inconsistent results without recognizing potential reactions during the analysis.

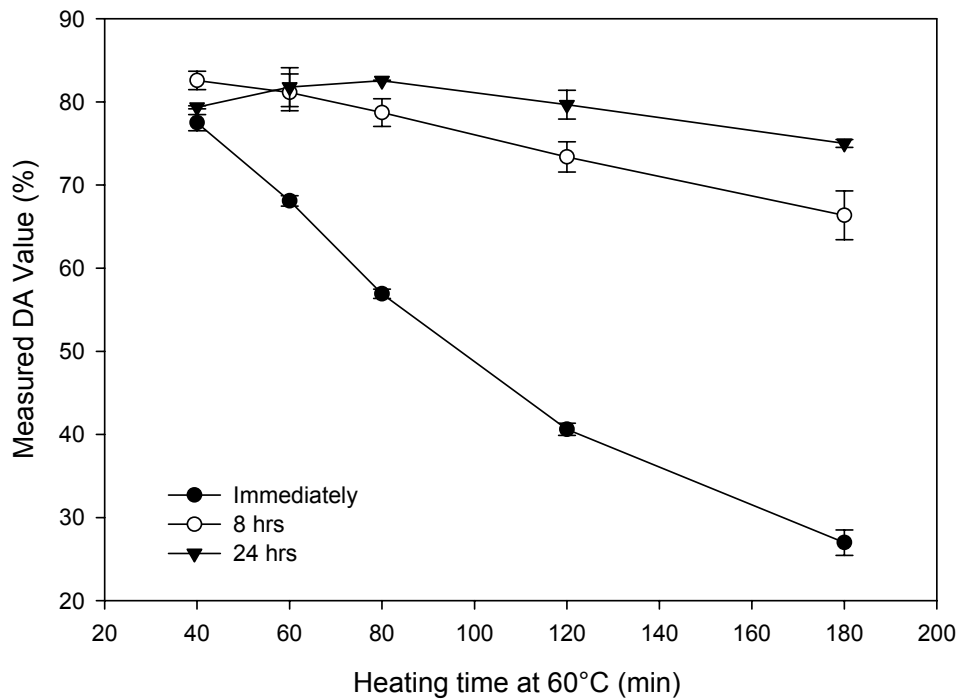


Figure 2-6: Degree of acetylation (DA) values of chitin measured immediately, 8 and 24 hrs after dilution.

* Values are represented as mean \pm standard deviation (n=3).

The hydrolysis of oxazolinium ions was accelerated by incubating the diluted chitin solution at elevated temperature, what significantly reduced analysis time. It can be seen from Figure 2-7 that if the diluted chitin solutions were kept at 60°C, the determined DA values achieved the highest value after 2 hrs (89.8%, for this sample) while the highest DA value of the samples incubated at room temperature was obtained after 5 hrs, and the value was still significantly lower (79.3%) compared to the values determined after 2 hr-incubation at 60°C.

2-4.4 Effects of possible dehydration reactions on DA determination

The UV spectra of chitin samples in diluted aq. phosphoric acid are shown in Figure 2-8. The samples prepared by dissolving chitin at 60°C for 40 and 60 min had the characteristic peak at 203 nm relevant to absorbance of the acetyl group. However, if the samples were heated for 100 min, a new peak appeared at 285 nm. This peak was also present in all the samples heated at 80°C. The area of the peak increased with the temperature and heating time. The maximum absorbance at 285 nm is characteristic for the 5-hydroxymethylfurfural (HMF), a dehydration product of hexoses, including glucosamine and acetyl-glucosamine (Jun and others 2003). As the formation of HMF can significantly reduce the number of acetyl-glucosamine units and/or altered the glucosamine/acetyl-glucosamine ratio in chitin or chitosan molecules, its formation during the assay (Step 1: dissolving of polymers, Figure 2-2) must be avoided. Therefore, solubilization of chitin and chitosan in conc. phosphoric acid must be achieved at temperatures not higher than 60°C for no longer than 60 min.

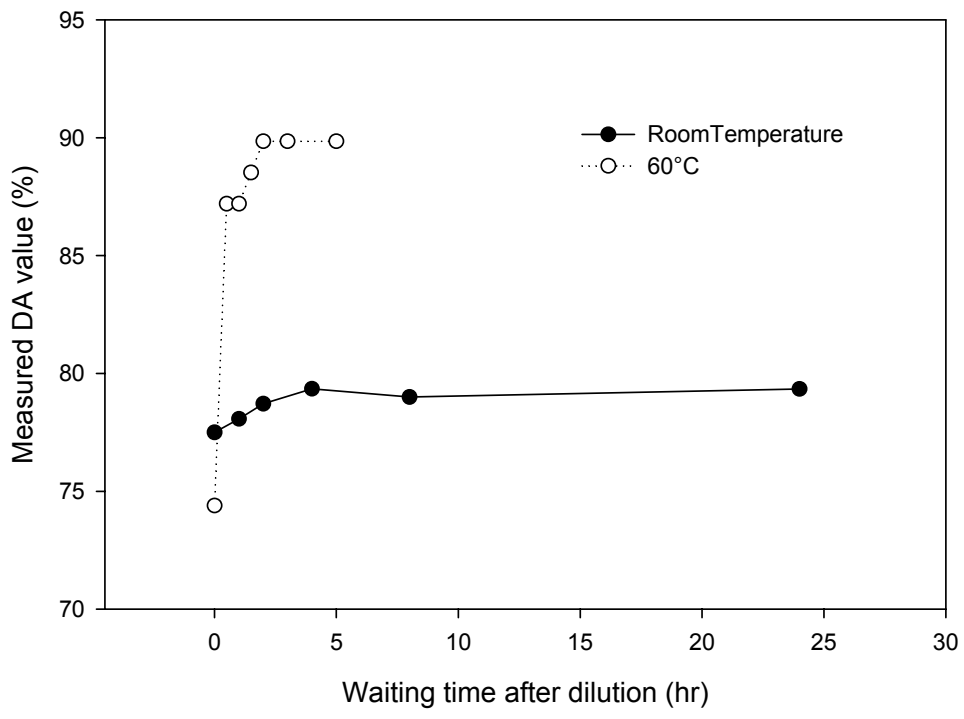


Figure 2-7: Effect of incubation temperature (room temperature and 60°C) on determined degree of acetylation (DA) value.

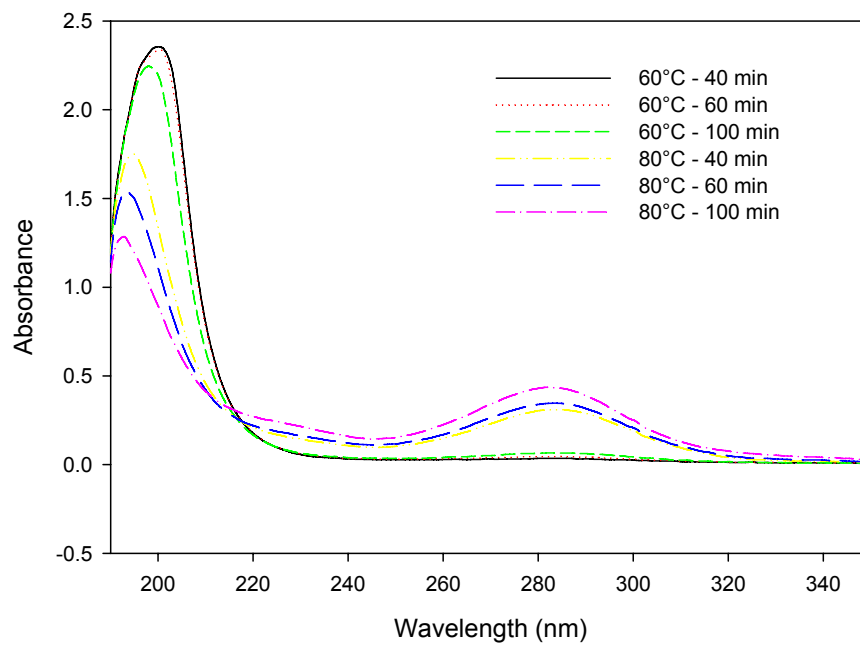


Figure 2-8: UV spectra of chitin in diluted phosphoric acid after heating.

2-4.5 Validity and reproducibility of the method

An accurate and rapid determination of DA values by the proposed first derivative UV method must satisfy two requirements: (1) a complete solubilization of chitin and chitosan in conc. phosphoric acid without significant loss of the monomers due to formation of HMF; (2) a full availability of acetyl groups by complete release of the oxazolinium ions in a short time. These two requirements can be met by dissolving chitin and chitosan samples in conc. phosphoric acid at 60°C for 40 min and incubating the diluted solutions at 60°C for 2 hrs. The reproducibility of new protocol was tested using commercial chitin and chitosan products with known DA values and the results are presented in Table 2-1. As it can be seen, error was generally less than 5% and the results correlated well with the values obtained from the manufacturer.

2-5 Conclusions

An improved first derivative UV method is proposed based on the evaluation of effects of chemical reactions possibly associated with the assay. Using the conc. (85%) phosphoric acid as a solvent for both chitin and chitosan, heat treatment at 60°C for 40 min to enhance solubilization, and incubation of diluted solutions at 60°C for 2 hrs, the method enables determination of DA in the whole range. The assay is simple and accurate, does not require expensive equipment, can be performed in less than 3 hrs, and what is most important, allows use of the same procedure for analysis of both chitin and chitosan.

Table 2-1: The degree of acetylation (DA) values of commercial samples.

Sample	Nominal DA*	Determined DA**
1 – chitosan	19	20.2 ± 0.1
2 – chitosan	29	30.3 ± 0.2
3 – chitosan	39	32.5 ± 0.8
4 – chitin	unknown	88.7 ± 2.6
5 - water soluble chitosan	unknown	9.1 ± 0.6

* Provided by manufacturer

** Values are represented as mean ± standard deviation (n=3).

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3 Effects of High Intensity Ultrasound Pretreatment and Chitin Particle Size on the Yield and Degree of Acetylation of Resulting Chitosan

3-1 Abstract

High intensity ultrasound (HIU) was investigated as a pretreatment method for the deacetylation process. The data indicated that sonication of chitin flakes dispersed in water up to 30 minutes did not change the DA values, but decreased the yields of resulting chitosan. Reduction of the chitin particle size, from flake to powder, promoted the efficiency of a deacetylation, but with the cost of lower yield.

3-2 Introduction

Chitin, a linear polysaccharide consisting of 2-acetamido-2deoxy- β -D-glucose, is the second most abundant polysaccharide on earth. Chitosan is the deacetylated product of chitin with many possible applications in various industries: as antimicrobial edible film in the food industry, smart drug delivery carrier in the pharmaceutical industry, and heavy metal binding agent in waste water treatments (Rinaudo, 2006; Shahidi and others 2005; Ravi-Kumar, 2000; Dodane and Vilivalam, 1998; Kurita, 1998).

Chitin can be deacetylated by homogeneous or heterogeneous reactions (Sannan and others 1975; Kurita and others 1977). The homogenous deacetylation is usually completed by treating alkaline suspension of chitin with crushed ice to hydrate chitin first and the deacetylation is consequently carried out in diluted alkali at low temperature (Sannan et al., 1975). The commercial manufacturing of chitosan is, however, usually done with heterogeneous reaction - by treating chitin with concentrated sodium- or potassium-hydroxide at elevated temperature (Methacanon and others 2003). Contrary to homogeneously deacetylated chitosan, where glucosamine and acetyl-glucosamine units are uniformly distributed along the chitosan molecule, heterogeneously deacetylated chitosan consists of blocks of various lengths composed of mainly glucosamine or mainly acetyl-glucosamine (Kurita and others 1977). Deacetylation reaction can be influenced by several factors, such as alkali concentration, solution to solid ratio, reaction time and temperature (Tsaih and Chen, 2003; Chang and others 1997). Recently, several new protocols have been proposed for the deacetylation process, for example, use of water-miscible organic solvents (Batista and Roberts, 1990), deacetylation under an anhydrous reaction medium (Tolaimate and others 2003), and alkaline deacetylation enhanced by

flash treatments (Focher and others 1990). Nevertheless, none of these methods can achieve a complete deacetylation by a single alkali treatment mainly due to the highly crystalline structure of chitin (Methacanon et al., 2003). As Kurita et al. has suggested, the heterogeneous deacetylation takes place preferentially in the amorphous region of chitin, and then proceeds from the edge to the inside of the crystalline region (Kurita et al., 1977). Use of repeated alkaline treatments to achieve complete deacetylation has been reported, but chitosan produced this way has reduced molecular weight (Tolaimate and others 2000).

Recently, Serglo et al. has suggested that the reactivity of chitin towards deacetylation can be improved by the high intensity ultrasound (HIU) pretreatment (Serglo and others 2002). Compared to 15% in control, the DA values of chitosan samples from sonicated chitin decreased to 7.3, 8.4 and 8% with 30, 60 and 90 min ultrasound pretreatment, respectively (Serglo et al., 2002). HIU has also been used in the extraction process of chitin from shrimps and the deprotenization efficiency was greatly enhanced (Kjartansson and others 2006a; Kjartansson and others 2006b). Additionally, although the degree of acetylation of chitin was unaffected by sonication (Dodane and Vilivalam, 1998), the DA values of chitosan produced from sonicated chitin decreased from 70.0 to 68.7 and 61.4% for 1 and 4 hrs sonicated samples, respectively (Kjartansson et al., 2006b). However, in all these studies sonication has been applied for extensive time during which ultrasound generator probe can be significantly eroded and enrich sample with metal pieces.

The objective of this study was to investigate the effects of short HIU pretreatment (<30 min) on the yield and deacetylation process of chitin. In addition, the

effect of chitin particle size on yield and degree of acetylation of resulting chitosan was examined.

3-3 Experimental details

3-3.1 Materials and instruments

Acetyl-glucosamine (GlcNAC), glucosamine hydrochloride (Glc) and 85% phosphoric acid were purchased from Sigma (St. Louis, MO). Flake-like chitin and chitosan samples were provided by Primex Company (Primex, Iceland). To prepare powder-like samples, the flakes were ground using Tomas Wiley Mill and sieved through sieve # 40 (opening 425 μ m). Dry samples were kept in desiccators at room temperature. A Shimadzu 2010 double beams UV-vis spectrophotometer was used to collect the 1st derivative UV spectra of standards and samples under scan mode.

3-3.2 DA measurement

The DA analysis was performed by a 1st derivative UV method after modification (Hsiao and others 2004). In short, 100 mg sample was dissolved with 20 ml 85% phosphoric acid at 60°C with stirring for 40 minutes. The solution was diluted with deionized water (1:100 W/V) and incubated at 60°C for 2 hours before UV scan. Standard solutions of GlcNAc and Glc were prepared in 0.85% phosphoric acid at concentrations of 0, 10, 20, 30, 40 and 50 μ g/ml. The calibration curve was made by plotting the 1st derivative UV values at 203 nm (H_{203}) as a function of the concentrations of GlcNAc and Glc.

3-3.3 Standard deacetylation process

Two gram aliquots of flake-like chitin, powder-like chitin, and powder-like chitosan were measured into 50 ml plastic centrifuge tubes. 40 ml 50% NaOH was added and samples were vortexed for 2 minutes. All samples were placed in a 95 °C water bath. Every 30 minutes, the samples were taken out, vortexed for 30 seconds, and placed back into the water bath. After designed heating time, the samples were chilled in an ice bath, filtered through Miracloth (Rayon-polyester; EMD bioscience, San Diego, CA), washed with D.I water to neutral pH, and lyophilized. The chitosan yields were calculated as the mass ratio of samples before and after the deacetylation process.

3-3.4 HIU pretreatment and deacetylation

Prior sonication, 2.00 g flake-like chitin was soaked in 80 ml water overnight. The hydrated samples were sonicated by a 20 kHz ultrasound generator (Sonics and Materials VC-750, Newton, CT) with a ½ inch titanium probe at power level of 70 W/cm² determined by calorimetric method(Bober, 1998) for 10, 20 and 30 minutes in 30 second on-30 second off pulse mode. Control was stirred at high speed for 30 minutes. The sonicated and control chitin samples were filtered through Miracloth and lyophilized. Aliquots of 1.00 g HIU pretreated chitin were transferred to 50 ml plastic centrifuge tubes, 30 ml 50% NaOH was added, and the deacetylation reaction was carried out at 95 °C for 30 minutes. After exactly 30 min, the samples were chilled in an ice bath, filtered through Miracloth, washed with D.I water to neutral pH, and lyophilized.

3-3.5 Statistics analysis

All experiments were repeated three times and significant difference between treatments was determined using Duncan's multiple range test by SAS program 9.13 (SAS institute Inc, 2003).

3-4 Results

3-4.1 The effect of HIU pre-treatment on chitin deacetylation

Chitin used in this experiment had original DA value of 84.7% and moisture content of 4.3% (Table 3-1). The control was only stirred in water for 30 minutes and the yield was recovered to the level of $94.4 \pm 0.1\%$. The lost mass of 5.6% was probably the combination of chitin mass loss during filtering and washing, and moisture loss during lyophilization. With HIU pre-treatment for 10, 20 and 30 minutes, the yields further decreased to 90.3 ± 1.0 , 90.9 ± 0.3 , and $91.1 \pm 1.1\%$, respectively, which could be attributed to the creation of ultra-fine chitin particles by HIU and loss of those particles during the filtering and washing. However, all DA values of chitin were in the range of 86.1 ± 1.2 to $86.6 \pm 0.9\%$ regardless of the pre-treatment. No significant difference was found compared to the original chitin. The result indicated that the ultrasound treatment itself did not cause any deacetylation reaction of chitin. With 10 to 30 minutes of HIU, all chitin samples demonstrated gray-dust discoloration, which suggested the erosion of ultrasound generator probe and deposition of metal pieces in the samples occurred.

All chitosan samples produced by the same deacetylation process (95°C , 50% NaOH, 30 minutes) with or without HIU pretreatments had recovery yields ranging from $72.9 \pm 1.0\%$ to $76.9 \pm 1.0\%$ (Table 3-1). With 30 minutes sonication pretreatment, the $72.9 \pm 1.0\%$ yield of resulting chitosan was lower than that with 10 and 20 minutes

Table 3-1: Effect of high intensity ultrasound pre-treatment on the deacetylation process of chitin.

Sample Name	HIU Pretreatment	Deacetylation Process	Recovery Yield (%)	DA (%)
1	—	—	95.7 ± 0.5 ^A	84.7 ± 1.8 ^A
2	Stirring for 30 min	—	94.4 ± 0.1 ^A	86.4 ± 0.3 ^A
3	80W/cm ² , 10 min	—	90.3 ± 1.0 ^B	86.2 ± 1.5 ^A
4	80W/cm ² , 20 min	—	90.9 ± 0.3 ^B	86.6 ± 0.9 ^A
5	80W/cm ² , 30 min	—	91.1 ± 1.1 ^B	86.1 ± 1.2 ^A
6	Stirring for 30 min	95°C, 30 min	74.3 ± 2.2 ^{xy}	25.3 ± 0.5 ^x
7	80W/cm ² , 10 min	95°C, 30 min	76.9 ± 1.0 ^x	25.1 ± 1.0 ^x
8	80W/cm ² , 20 min	95°C, 30 min	75.6 ± 1.0 ^x	25.9 ± 1.3 ^x
9	80W/cm ² , 30 min	95°C, 30 min	72.9 ± 1.0 ^y	25.2 ± 1.6 ^x

* Values are represented as mean ± standard deviation (n=3);

* Numbers with different letters are significantly different at $p \leq 0.05$;

sonication pretreatment, but was not significantly different from the one with 30 minutes of only stirring ($74.3 \pm 2.2\%$). Similar DA values, ranging from $25.1 \pm 1.0\%$ to $25.9 \pm 1.3\%$, were obtained for all chitosan samples (Table 3-1) regardless of the pre-treatment. The results showed that the HIU pretreatment caused slight decrease in yield of resulting chitosan, but had no effect on the degree of acetylation of the chitosan. This is in contradiction with a previous report where increased reactivity towards deacetylation has been found for chitin with ultrasound pretreatment with 30 minutes (Serglo et al., 2002).

3-4.2 The effect of chitin particle size on chitin deacetylation

The average yields of chitosan produced from flake-like chitin, powder-like chitin and powder-like chitosan during deacetylation of various times were shown in Figure 3-1. The highest chitosan yield was $82.1 \pm 1.3\%$ obtained from powder-like chitosan, followed by $78.0 \pm 1.7\%$ and $70.3 \pm 2.2\%$ from flake-like and powder-like chitin, respectively. The highest yield from powder-like chitosan was probably corresponded to a less remove of acetyl group from chitosan. However, higher yield from flake-like chitin compared to that from powder-like chitin seemed to be due to a lower mass loss during filtering and washing. Similar phenomena have been found in the extraction process of chitin where sonication renders lower yield due to losses of depolymerized materials in the wash-water (Kjartansson et al., 2006b). Although the significant difference in chitosan yield was observed from different forms of raw material, no effect of deacetylation time on the chitosan yield was found for the samples of the same form (Figure 3- 1).

As shown in Figure 3-2, the DA value of chitosan from flake-like chitin was reduced from $88.2 \pm 2.3\%$ to $28.8 \pm 0.6\%$ during the first 0.5 hour and from $28.8 \pm 0.6\%$ to $16.2 \pm 0.4\%$ during the next 4.5 hours. The DA values of chitosan samples from powder-like

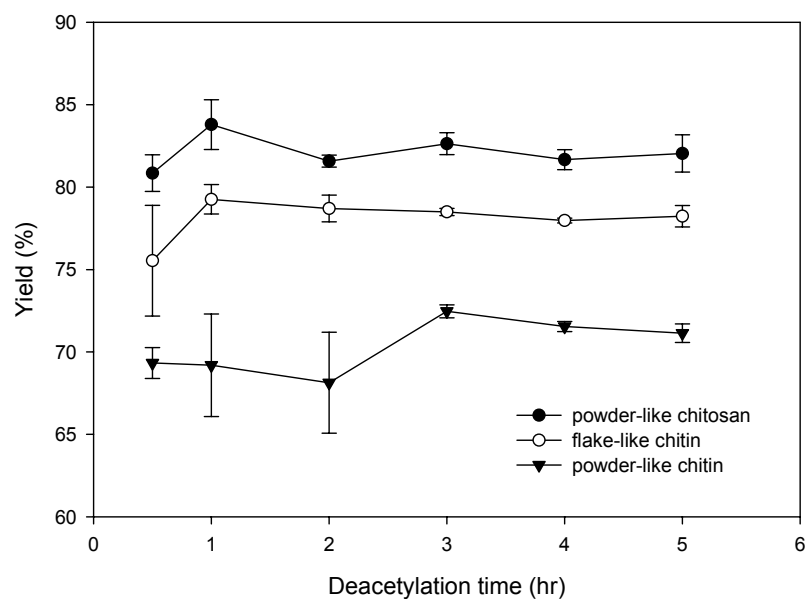


Figure 3-1: The average yields of chitosan produced from flake-like chitin, powder-like chitin and powder-like chitosan during various length of deacetylation (95°C, solid/solution ratio=1/20).

* Values are represented as mean \pm standard deviation (n=3).

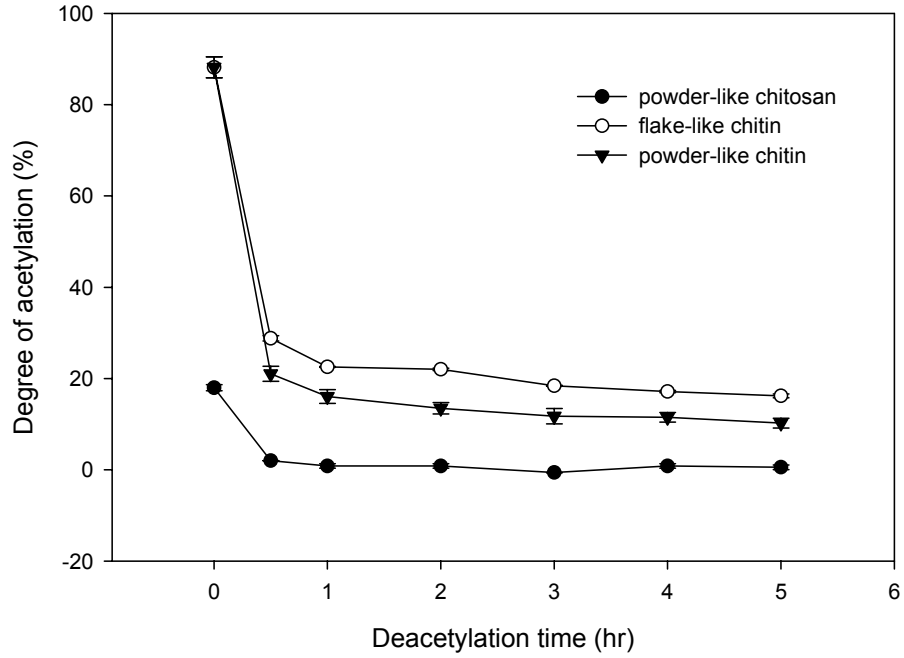


Figure 3-2: The degree of acetylation values of chitosan produced from flake-like chitin, powder-like chitin and powder-like chitosan as affected by the deacetylation time (95°C, solid/solution ratio=1/20).

* Values are represented as mean \pm standard deviation (n=3).

chitin decreased from $88.2 \pm 2.3\%$ to $21.0 \pm 1.6\%$ in the first 0.5 hour and from $21.0 \pm 1.6\%$ to $10.2 \pm 1.1\%$ during the remaining 4.5 hours. Thus, the deacetylation reaction of chitin occurred quickly at the initial stage and then leveled off. Similar facts have been observed by several previous studies (Kurita et al., 1977; Tsaih and Chen, 2003; Chang et al., 1997), although the exact time at which the deacetylation curve leveled off differed with deacetylation conditions. After reaching the stagnant stage of deacetylation, the resulting chitosan had lower DA value of $21.0 \pm 1.6\%$ from powder-like chitin compared to that of $28.8 \pm 0.6\%$ from flake-like chitin, which could be attributed to the increased surface area for deacetylation in powder-like chitin.

As it can be seen from Figure 3-2, the commercial chitosan was not completely deacetylated and had a DA value of $18.0 \pm 0.7\%$. The alkaline treatment resulted in a decrease to $2.0 \pm 0.0\%$ in the first 0.5 hour of deacetylation and to $0.6 \pm 0.5\%$ during next 4.5 hours. This result confirmed that a second alkaline treatment was required to achieve a complete deacetylation (Tolaimate et al., 2000).

3-5 Discussion and conclusions

This study showed that application of short high intensity ultrasound (HIU) pretreatment (<30 min) did not change the DA values of sonicated chitin and resulting chitosan. At the same time, reduction of chitin particle size from form of flakes to powder effectively increased the efficiency of deacetylation due to the increased surface area, but with decreased the yield.

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4 Manipulation of Chitosan Molecular Weight by High Intensity Ultrasound (HIU)

4-1 Abstract

The degradation of chitosan by high intensity ultrasound (HIU) as affected by ultrasound parameters and solution properties was investigated by gel permeation chromatography coupled with static light scattering. The molecular weight, radius of gyration and polydispersity of chitosan were efficiently reduced by ultrasound treatment, whereas chitosan remained in the same random coil conformation, and the degree of acetylation did not change after sonication. Our experiment showed that 1) the degradation of chitosan by ultrasound is primarily driven by mechanical forces and degradation mechanism can be described by a random scission model; 2) the degradation rate is proportional to M_w^3 ; and 3) the degradation rate coefficient is affected by ultrasound intensity, solution temperature, polymer concentration and ionic strength while acid concentration has little effect. Additionally, the data indicate that the degradation rate coefficient is affected by the degree of acetylation of chitosan and independent of the initial molecular weight.

4-2 Introduction

Commercial application of chitosan is closely associated with its functional properties and biological activities, which are primarily governed by two structural properties: the molecular weight (MW) and degree of acetylation (Trzcinski and Staszewska). However, the MW of commercially available chitosan is greatly affected by the sources, extraction, and production methods. It largely varies among manufacturers and even between the batches of the same manufacturer. Aiming to produce chitosan of desired MW, various methods, including acid and enzyme hydrolysis, microwave, UV and gamma irradiation, as well as high intensity ultrasound (HIU), have been investigated (Hasegawa and others 1993; Chen and others 1997; Ilyina and others 2000; Wasikiewicz and others 2005).

HIU has received much attention as a rapid, environmentally friendly, and by-product-free method. The mechanism, kinetics, and application of ultrasound in the degradation of various synthetic polymers have been widely investigated (Basedow and Ebert, 1977; Price, 1993; Suslick and Price, 1999; Mason and Lorimer, 2002). Cleavage of polymer chains by HIU with frequencies ranging from 20 to 100 kHz, has been attributed mainly to the action of shear forces formed due to the relative movement between solvent and polymer molecules during the collapse of cavitation bubbles (Basedow and Ebert, 1977). Thus, the degradation of a polymer by ultrasound is considered to be primarily of mechanical nature. However, at frequencies higher than 100 kHz, free OH radicals formed by ultrasound in an aqueous solution have significant role in the polymer degradation (Mark and others 1998). Czechowska et al. used 360 kHz ultrasound treatment to degrade chitosan and found that the chain scissions were induced

by both mechanical forces and free radicals(Czechowska-Biskup and others 2005). At the same time, side reactions with formation of carbonyl group were observed(Czechowska-Biskup et al., 2005).

Two types of factors, ultrasound parameters (including frequency and intensity) and solution properties (solvent, temperature, nature of dissolved gas, nature of polymer, etc.) have been found to affect the degradation process of polymers (Basedow and Ebert, 1977; Mason and Lorimer, 2002). Due to the polydisperse nature in most polymers, an accurate analysis of the degradation kinetics is almost impossible without the information about the location of chain scission and the dependence of rate coefficients on the molecular weight of the polymer (Basedow and Ebert, 1977). Two simplified models, based on different assumptions of the location of chain scission, have been proposed to quantitatively describe the degradation process of polymers.

(I) Random Scission Polymer Degradation Model: One of the earliest models has been developed by Schmid in which the author assumes that the scission of polymer chains occurs at random and that the rate of degradation decreases with decreasing chain length (Schmid, 1940). By the same assumption, the rate of degradation reaches zero at M_e , the final limiting molecular weight, below which no further degradation can occur.

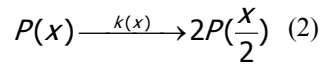
Thus:

$$\frac{M_e}{M_t} + \ln\left(1 - \frac{M_e}{M_t}\right) = -\frac{k_1}{c} \left(\frac{M_e}{M_t}\right)^2 \times t + \frac{M_e}{M_i} + \ln\left(1 - \frac{M_e}{M_i}\right) \quad (1)$$

where M_e , M_i , and M_t represent the final, initial and number average molecular weight after sonication time (t), respectively; m refers to the molecular weight of monomer, and

c to the initial molar concentration of the polymer and k_1 to the degradation rate coefficient.

(II) *Midpoint Chain Scission Polymer Degradation Model*: Assuming the degradation occurs at the midpoint of polymer chain, a continuous distribution model has recently been developed (Madras and others 2000). For a polymer $P(x)$ with chain length x and a molar concentration of $P(x, t)$, the overall degradation with a rate constant k can be described as:



The evolution of the number average molecular weight with sonication time is thus given by:

$$\ln \left[\frac{M_i - M_e}{M_t - M_e} \right] = k_2 M_e t \quad (3)$$

where k_2 refers to the degradation rate coefficient.

Baxter et al has indicated that the chain scission occurs randomly and follows the Schmid model (Baxter and others 2005), while Trzcinski and Staszewska has shown that a bimodal molecular distribution is obtained at early stages of degradation suggesting that the chain scission is not random, but occurs at the midpoint of the chain (Trzcinski and Staszewska, 2004). However, in both studies kinetics of ultrasonic degradation has been determined by using the viscosity average molecular weight although both models (Eq. 1 and 3) require that molecular weights are expressed as number average.

High intensity ultrasound has been widely investigated for degradation of chitosan. In general, it has been found that HIU reduces the molecular weight, radius of gyration and polydispersity of chitosan efficiently without affecting its degree of

acetylation (Chen et al., 1997; Baxter et al., 2005; Li and others 2007). Interestingly, it has been also reported that with intensive sonication, degree of acetylation (Trzcinski and Staszewska) of chitosan increases (chitosan is actually being acetylated) if the initial DA is $> 10\%$ and stays unchanged if it is $< 10\%$ (Liu and others 2006). Similarly to the degradation behavior of synthetic polymers, chitosan degrades more rapidly in dilute solutions and at low temperatures (Chen et al., 1997; Trzcinski and Staszewska, 2004; Li et al., 2007), whereas type of solvent has no significant influence on the degradation rate (Chen et al., 1997). However, Trzcinski et al. have found that the increase of acetic acid concentration from 0.1 M to 1 M results in a higher rate coefficient (Trzcinski and Staszewska, 2004), while Li et al. have found the optimal degradation conditions occurs at the lowest acetic acid concentration (Li et al., 2007). The initial molecular weight and degree of deacetylation have been found to affect the degradation process - chitosan samples with high molecular weight and low DA are easily degradable by HIU (Liu et al., 2006; Tsaih and Chen, 2003).

Despite that significant efforts have been focused in this area, contradictory results can be found in literature. In most studies, the actual ultrasound intensity has not been determined and, consequently, these results are not only hard to compare but are of little use for industry to scale up the process. Additionally, most of the published studies have monitored the degradation process by determination of viscosity average molecular weight, which lacks information of absolute molecular weight and can not be used to calculate the real kinetics. This is possibly the main reason for the conflicting results in literature. A comprehensive study was conducted here with objective to determine the effects of HIU parameters (intensity and treatment time) and solution properties

(temperature, chitosan concentration, acetic acid concentration, ionic strength, chitosan initial DA and molecular weight) on the ultrasound degradation of chitosan. Additionally, a simplified calculation to predict the change of molecular weight has been provided, which can be used as a guideline for the industrial application of HIU in the degradation of chitosan.

4-3 Experimental details

4-3.1 Materials

Chitosan samples with various degree of deacetylation (19%, 29% and 39% DA as labeled by manufacturer) were kindly donated by Primex (Primex Co., Iceland). Water soluble chitosan was purchased from EZ Life Science Co. Ltd. (Seoul, South Korea). Other chemicals were purchased from Fisher Scientific (Pittsburgh, PA). All chitosan samples were analyzed for weight average molecular weight (M_w) and DA by methods described latter in section 4-4.1.

4-3.2 Chitosan solution preparation

Chitosan solutions, 0.25%, 0.5%, 1% and 2% (w/v), were freshly prepared in 1% (v/v) aqueous acetic acid. Ionic strength of 1% chitosan solution was adjusted to 0.1M and 0.2M by adding suitable amounts of sodium chloride. All chitosan solutions were filtered through Miracloth[®] (Rayon-polyester; EMD bioscience, San Diego, CA) and kept in a refrigerator until sonication. Chitosan with 20.2% DA was used to investigate the effects of acoustic intensity and time, and 32.5% DA chitosan was used to investigate the effects of solution properties.

4-3.3 Ultrasound treatment procedure

100 ml of each chitosan solution was sonicated by a 20 kHz ultrasound generator (Sonics and Materials VC-750, Newton, CT) with a ½ inch titanium probe in pulse mode (30 second ON, 30 second OFF). For evaluation of effects of sonication time and amplitude, the temperature control at the generator was set at 30°C and the sample was kept in ice water bath during the experiment. For evaluation of effects of temperature, the temperature control was set at 30, 50, and 80°C and the beakers with samples were placed in an iced water bath, ambient temperature water bath, and no water bath, respectively. The sample temperature was monitored by a temperature probe during the entire ultrasound process. The temperature of solution increased when the sonication was ON and dropped a few degrees when the sonication was OFF, but the maximum temperature did not exceed 30, 50 and 80°C corresponding to the preset values at the generator. Sonication time was from 5 to 60 minutes. 1 ml sonicated sample solution was taken at specified time intervals, diluted with the solvent, and analyzed by gel permeation chromatography (GPC). All the presented data points were average of at least two independent sonication experiments.

The ultrasonic intensity can be measured calorimetrically by measuring the time-dependent increase in temperature of sample in the ultrasonic reactor (Kardos and Luche, 2001). However, the intensity of ultrasound can be simply controlled by setting the displacement (P_A) of ultrasound generator probe. As P_A increases, both the number and size of cavities increase, resulting in an increased overall chemical and mechanical activity. Based on the manufacturer's manual, for a 13 mm threaded probe with replaceable tip, the P_A set at 100% results in amplitude of 124 μm and the maximum

power output. Four ultrasound intensities, 47%, 57%, 67% and 87% were chosen for this study, which corresponded to P_A of 58, 70, 83 and 108 μm , respectively. The ultrasonic wave intensities at these four amplitudes were measured calorimetrically by determining the time-dependent change of sample temperature in the ultrasonic reactor as 31, 37, 48

and 62 W/cm^2 according to the next equation: $I = \frac{mC_p}{A} \left[\left(\frac{dT}{dt} \right)_a - \left(\frac{dT}{dt} \right)_b \right]$, where

$\left(\frac{dT}{dt} \right)_a$ is the slope of the initial temperature rise; and $\left(\frac{dT}{dt} \right)_b$ is the slope of heat loss

after the ultrasonic reactor was turned off; m is the sample mass, C_p is the heat capacity of the solvent, and A is the end surface area of sonicator probe. Unless specified, all experiments were carried out at intensity of 62 W/cm^2 and temperature of 30 $^\circ\text{C}$.

4-3.4 GPC coupled with multiangle laser light scattering detector (MALS)

GPC separations were performed by Waters 2596 module on three ultrahydrogel columns (Ultrahydrogel 500, 1000 and 2000, Waters, Milford, MA) with aqueous buffer (0.15M ammonium acetate/0.2M acetic acid with additional 0.02% sodium azide, pH 4.5) as mobile phase. The weight average molecular weight (M_w) was determined by a miniDAWN light scattering detector (Wyatt, Santa Barbara, CA) with a refractive index (RI) detector 410 (Waters, Milford, MA) to measure concentration and analyzed by ASTRA 4 software (Wyatt, Santa Barbara, CA). The cumulative and differential weight molecular distributions were obtained by ASTRA 5 software (Wyatt, Santa Barbara, CA). Results from the light scattering detector were analyzed by Zimm plots and known dn/dc and AUX calibration constant were used for the calculation of molecular weight and radius of gyration. The dn/dc values were adopted from literature as approximate 0.184,

0.184, 0.185 and 0.187 for chitosan samples of 32.5%, 30.3%, 20.2% DA and water soluble chitosan, respectively (Sorlier and others 2003). The GPC conditions were as follows: For chitosan samples with $M_w > 100$ kDa the concentration was 0.1% and for samples with $M_w < 100$ kDa the concentration was 0.2%, with an injection volume of 100 μ l and a flow rate of 0.8 ml/min. The column and RI detector temperature was 30°C, and the detector cells of MALS were kept at ambient temperature. Sample solution and mobile phase were filtered through a 0.45 μ m slightly hydrophobic polyvinylidene difluoride (PVDF) membrane (Whatman, Clifton, NJ) before use.

4-3.5 Overlap and entanglement concentrations

Overlap and entanglement concentrations of chitosan of 32.5% DA were estimated following the method of Cho et al. where the overlap concentration was at the concentration where $\eta = 2\eta_s$ (η_s is the viscosity of solvent) and entanglement concentration was at the concentration where $\eta = 50\eta_s$ (Cho and others 2006). The viscosity of chitosan solutions was determined by a Cannon-Fenske viscometer at 25°C with minimum of three replications.

4-3.6 Purification of sonicated chitosan for DA measurement

After 30 minutes sonication, the pH of chitosan solutions was adjusted to 10 using 1M NaOH. The precipitated chitosan was collected by centrifugation, dispersed in deionized water, and centrifuged again. The whole process was repeated three or four times until the pH of supernatant was 7. The pellets were freeze dried and stored in desiccator until further analysis.

4-3.7 DA measurement

The DA analysis was performed by the modified first derivative UV method (Hsiao and others 2004). In short, 100 mg sample was dissolved with 20 ml 85% phosphoric acid at 60°C with stirring for 40 minutes. The solution was diluted with deionized water (1:100 w/v) and incubated at 60°C for 2 hours before UV scan. Standard solutions of acetylglucosamine (GlcNAc) and glucosamine (GlcN) were prepared in 0.85% phosphoric acid at concentrations of 0, 10, 20, 30, 40 and 50 µg/ml. The calibration curve was made by plotting the 1st derivative UV values at 203 nm (H203) as a function of the concentrations of GlcNAc and GlcN.

4-3.8 UV spectra measurement

UV spectra of solutions of sonicated chitosan (sonication at 62 W/cm² for 30 minutes) were collected by a Shimadzu 2010 (Shimadzu, Columbia, MD) double beam UV-Vis spectrophotometer under scan mode in the range of 400 to 190 nm. Sampling interval and slit width were both set at 1.0 nm. Chitosan samples at concentration of 1% in 1% acetic acid were diluted with deionized water (1:25) before the UV measurement.

4-3.9 Statistics analysis

All experiments were repeated three times. ANOVA analysis and significant difference between treatments was determined using Duncan's multiple range test by SAS program 9.13 (SAS institute Inc, 2003).

4-4 Results and discussion

4-4.1 The effect of molecular weight on chitosan degradation by HIU

The weight average molecular weights (M_w) before sonication were determined approximately as 421, 307, 222 and 53 kDa for four chitosans, respectively (Table 4-1). The M_w decreased to 76, 72, 63 and 36 kDa after 30 minutes of sonication, respectively (Figure 4-1). The M_w decreased exponentially during sonication for samples with high initial M_w , while it decreased linearly for samples with low molecular weight. These results indicated that the ultrasound treatment was more efficient for the degradation of high molecular weight chitosans which was in agreement with previous reports (Liu et al., 2006; Chen et al., 1997). Earlier studies has developed the following equation to predict the change of polymer M_w during ultrasonic degradation (Tsaih and Chen, 2003; Portenlanger and Heusinger, 1997):

$$\frac{1}{(M_w)_t} = \frac{1}{(M_w)_i} + \frac{k'}{m}t = \frac{1}{(M_w)_i} + kt \quad (4)$$

where $(M_w)_t$ is M_w of the polymer after sonication time t , $(M_w)_i$ is the initial M_w of the polymer, m is the molecular weight of the monomer, k' and k are general rate coefficients. A plot of $\frac{1}{(M_w)_t}$ vs. the sonication time resulted in a non-linear relation

(data not shown). However, plots of $\frac{1}{(M_w)_t^2}$ vs. sonication time were all linear (Figure 4-

2), indicating that the change of molecular weight could be predicted by the following equation:

$$\frac{1}{(M_w)_t^2} - \frac{1}{(M_w)_i^2} = K't \quad (5)$$

Table 4-1: Degree of acetylation (DA) before and after sonication (30 minutes, 62 W/cm²) and initial weight average molecular weight (M_w) of chitosan samples used in this study.

Sample name	Nominal DA	Measured DA	Measured DA after sonication	Measured Mw
40% DA chitosan	39%	32.5 ± 0.8%	30.0 ± 0.1%	222 kDa
30% DA chitosan	29%	30.3 ± 0.2%	28.0 ± 0.7%	421 kDa
20% DA chitosan	19%	20.2 ± 0.1%	20.4 ± 0.5%	307 kDa
Water soluble chitosan	-	9.1 ± 0.6%	9.0 ± 0.1%	53 kDa

* Values are represented as mean ± standard deviation (n=3).

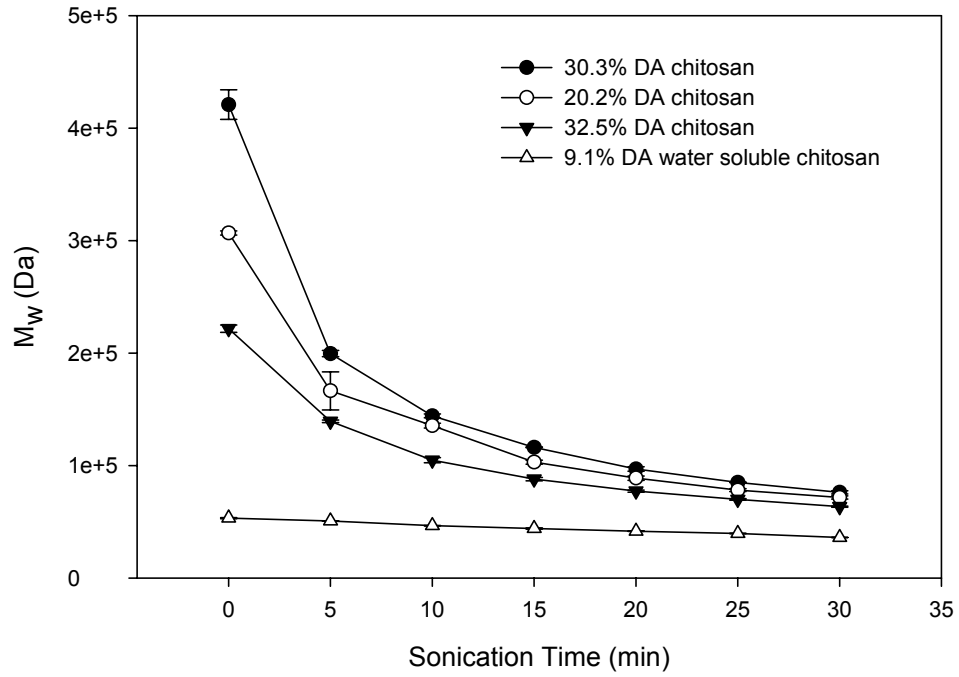


Figure 4-1: Variation of M_w with time of sonication for chitosan with different initial molecular weight and degree of acetylation.

* Values are represented as mean \pm standard deviation (n=3).

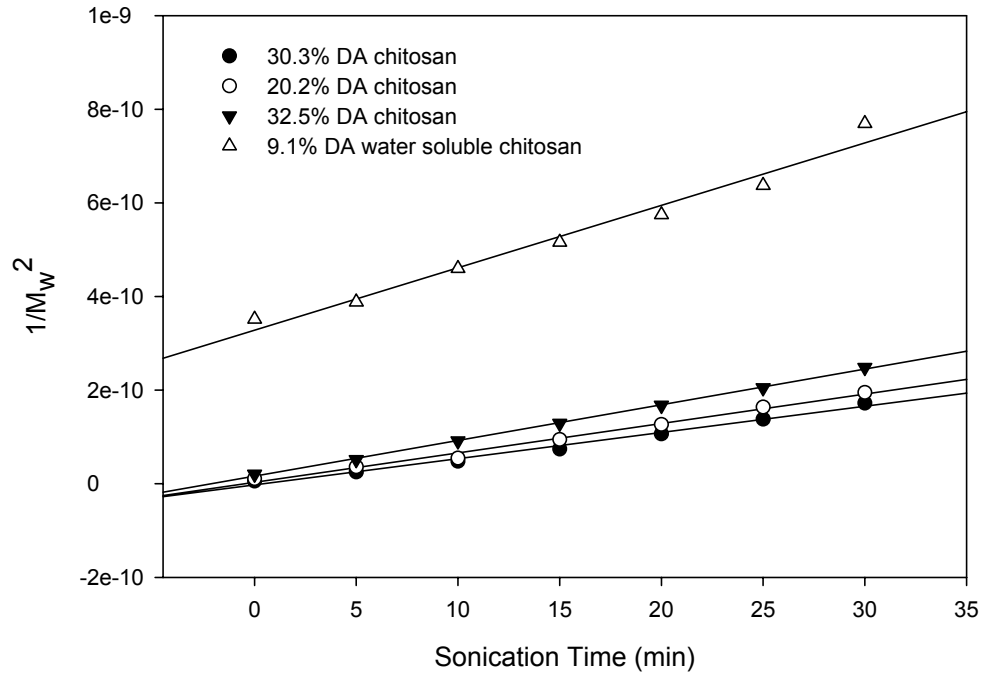


Figure 4-2: Variation of $\frac{1}{(M_w)^2}$ with sonication time for chitosan with different degree of acetylation and initial molecular weights.

Thus the rate of M_w reduction was actually proportional to the cube of initial molecular weight and could be described by:

$$\frac{dM_w}{dt} = -KM_w^3 \quad (6)$$

It is worth to note that the rate coefficients K' or K are not absolute rate constants to describe the rate of chitosan chain scission, but rather refer to processing parameters that are associated with the particular reactor, geometry and ultrasound frequency.

4-4.2 The effect of degree of acetylation on chitosan HIU degradation

The DA values before sonication were determined as 32.5, 30.3, 20.2, and 9.1% for 39%, 29%, 19% DA chitosan and water soluble chitosan, respectively (Table 4-1). The general rate coefficients for 32.5%, 30.3% and 20.2% DA chitosan were 7.62×10^{-12} , 5.60×10^{-12} and $6.28 \times 10^{-12} \text{ Da}^{-2} \cdot \text{s}^{-1}$, respectively and were relatively similar, whereas the rate coefficient of 9.1% DA water soluble chitosan was much higher, $1.33 \times 10^{-11} \text{ Da}^{-2} \cdot \text{s}^{-1}$. Contrary to this, the study of Trzcinski and Staszewska showed that the general rate coefficient decreased with the decrease of degree of acetylation (Trzcinski and Staszewska, 2004). However, a low intensity ultrasound generator was used in their study and results might not be directly comparable to our study using high intensity ultrasound generator. In another study the authors observed that the rate coefficients of ultrasound degradation increased with the decrease of chitosan DA and explained the findings with assumption that highly deacetylated chitosan molecules were more expanded and thus more vulnerable to the breakage by the shear force (Tsaih and Chen, 2003). The same study also suggested that the difference in bond energy of β -1, 4-glucosidic linkages among different monomer units may be responsible for the experimental

observation(Tsaih and Chen, 2003). A recent study in fact showed that the hydration energies in the 1, 4- β -glucosidic bonds were in the order of GlcNAc – GlcNAc > GlcN – GlcNAc \approx GlcNAc – GlcN > GlcN – GlcN and the authors proposed that the higher the hydration energy of the bond, the more energy would be needed to break the bond(Liu and others 2007). According to this study, chitosan with lower DA values was more vulnerable to degradation by ultrasound due to lower bond energy(Liu et al., 2007).

At this point we could not conclude that the difference of the rate coefficients for our chitosans (Figure 4-2) was caused purely by the difference in DA since our initial chitosans, besides variations in the DA, differed in initial M_w . However, the results showed that although the degradation rate was proportional to M_w^3 , the rate coefficient was independent of initial molecular weight. When a sonicated sample was collected, freeze-dried and sonicated for the second time, decrease of its molecular weight followed the trend as if the first sonication treatment was just prolonged. As shown in Figure 4-3, the degradation of sample with initial M_w of 72 kDa maintained the degradation trend of its ‘parent’ molecule with initial M_w of 307. The data were horizontally transpositioned to show the trend in M_w reduction (Figure 4-3) and $1/M_w^2$ vs. time plot (insert in Figure 4-3) shows how similar the rate coefficients were (6.28×10^{-12} and 5.93×10^{-12} Da⁻²·s⁻¹ for the original and re-sonicated sample, respectively). Thus, our results indicate that the degradation rate coefficient of chitosan sonication is affected only by the DA values. The results showed that only highly deacetylated chitosan (DA 9.1%) was easily degraded while chitosans with DA values in the range of 20.2 – 32.5% degraded at the slower rate. Similarly, Vijayalakshmi and Madras found that the degradation rate coefficient of

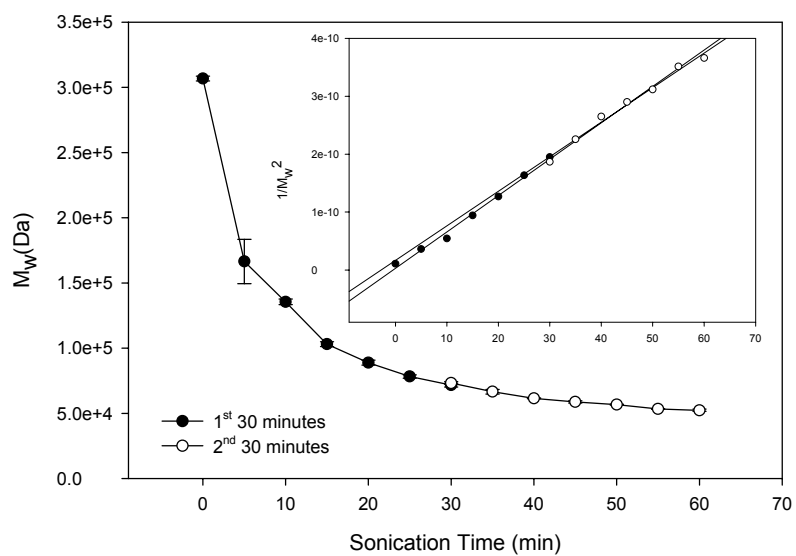


Figure 4-3: Variation of weight average molecular weight (M_w) and $\frac{1}{M_w^2}$ with sonication time for 20.2% degree of acetylation chitosan receiving 30 minutes sonication treatment twice.

* Values are represented as mean \pm standard deviation (n=3).

sonication degradation was nearly independent of the initial molecular weight of poly(ethylene oxide)(Vijayalakshmi and Madras, 2005).

4-4.3 Development of models that describes sonolysis of chitosan

As shown in Figure 4-4, the number average molecular weight, M_n decreased from 149, 129, 106 and 30 kDa to 50, 48, 41 and 23 kDa for 30.3%, 20.2%, 32.5% DA and 90.9% water soluble chitosan after 30 minutes of sonication, respectively. The determined values of M_n had a relatively large standard deviations compared to the results for M_w (Figure 4-1). This was commonly recognized as the inevitable interaction of a GPC column and a polymer (Barth and others 1996; Barth and others 1998).

In order to determine which model describes sonolysis of chitosan the best, it is necessary to find the final limiting molecular weight M_e of chitosan. In our preliminary studies, 31 kDa chitosan was sonicated for 3 hours resulting in a final molecular weight of 17 kDa which was used as the M_e value to calculate the degradation kinetics.

Plots of $\ln\left(\frac{M_e - M_i}{M_t - M_i}\right)$ vs. sonication time for the Midpoint Scission Model and $-\frac{M_e}{M_t} - \ln\left(1 - \frac{M_e}{M_t}\right)$ vs. sonication time for Random Scission Model are shown in Figure 4-5-A and -B, respectively. All molecular weights (M_i , M_e , M_t) were determined as number average molecular weights. Plotting the values for Midpoint Scission Model, only the 9.1% DA chitosan gave a straight line (Figure 4-5-A), while all the analyzed chitosans gave straight line plots ($R^2 > 0.99$) based on the Random Scission Model (Figure 4-5-B). These results indicated that the ultrasonic degradation of chitosan was not midpoint scission based but rather happened randomly along the chitosan molecule. Similarly, Baxter et al found that chitosan was randomly degraded by sonolysis(Baxter et al., 2005)

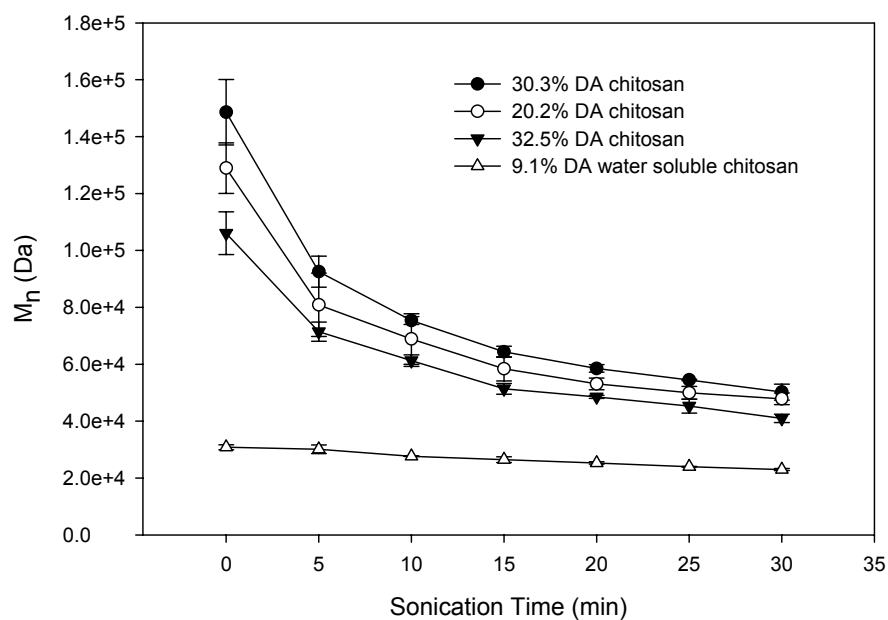


Figure 4-4: Variation of number average molecular weight (M_n) with time of sonication for chitosan with different degree of acetylation and initial molecular weights.

* Values are represented as mean \pm standard deviation (n=3).

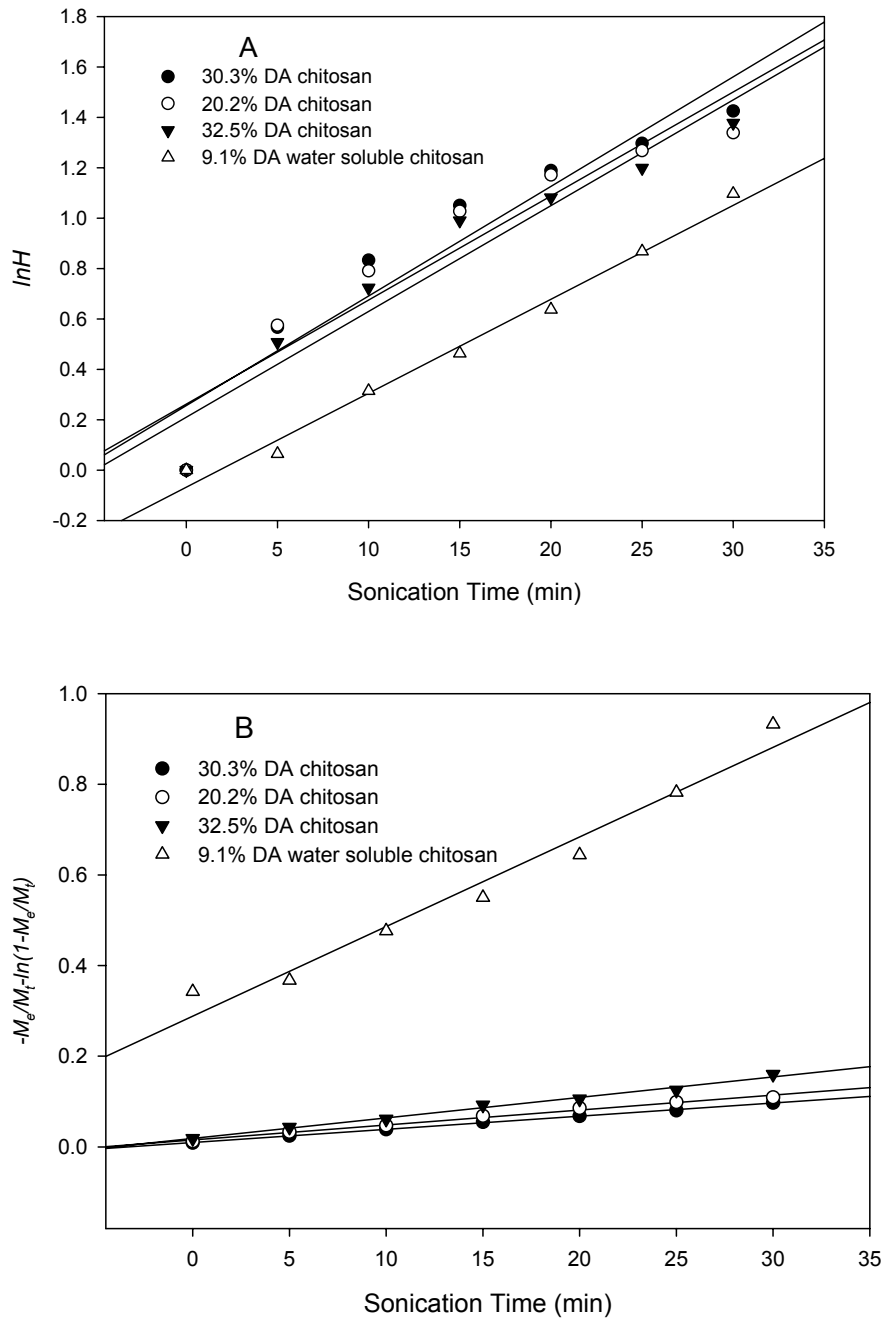


Figure 4-5: Evaluation of the mid-scission model (A) and random scission model (B) of chitosan degradation ($\ln(H) = \ln\left[\frac{M_i - M_e}{M_t - M_e}\right]$).

and Tayal and Khan found that ultrasonic degradation of a water-soluble guar galactomannan also followed the random scission model(Tayal and Khan, 2000).

The midpoint scission has been accepted as a general mechanism for ultrasonic degradation of synthetic polymers. However, most of these studies have been focused on homopolymers, whereas chitosan is considered to be a copolymer of N-acetylglucosamine and N-glucosamine. For a homopolymer, the solvodynamic forces are assumed to be the greatest near the midpoint of the chain and preferentially rupture the chain there. For a copolymer, especially block copolymer, such as chitosan which is not completely deacetylated(Kurita and others 1977), the cleavage may occur at the point other than midpoint of the chain. Thus, it was reported that ultrasound selectively induced site specific cleavage of azo-functionalized poly(ethylene glycol) at the weakest site of the macromolecule(Berkowski and others 2005). Another study suggested that the degradation of chitosan by ultrasound was not truly random but was related to the sequence of bond energies: $\text{GlcN-GlcN} > \text{GlcNAc-GlcN} \approx \text{GlcN-GlcNAc} > \text{GlcNAc-GlcNAc}$ (Liu et al., 2007). However, since the distribution of these bonds in a chitosan chain is still almost impossible to determine, we cannot propose the exact site of chain scission. Nevertheless, the Random Scission Model fitted our data better than Midpoint Scission Model. Following this reasoning, we can postulate that if being a copolymer is the main reason that leads to a non-midpoint chain scission of chitosan, data for 9.1% DA chitosan, which is closest to N-glucosamine homopolymer, should fit the midpoint chain scission model well. Examining the plots in Figure 4-5-A, it can be observed that this was really true – the midpoint scission model fitted well only the 9.1% DA chitosan, while did not fit data for more heterogeneous chitosans.

It should be also kept in mind that in addition to chitosan being a copolymer, the geometry of the ultrasonic reactor may contribute to the fact that scission occurs at random rather than at the midpoint. The multiple reflections of ultrasound by the rigid walls of the reactor can result in a heterogeneous stress distribution in the reaction chamber and further complicate the ultrasonic degradation(Cravotto and others 2005).

4-4.4 Mechanism of chitosan degradation by sonolysis

As shown in Figure 4-6, the degradation processes 9.1% DA and 20.2%DA chitosan were identical regardless on the presence of 0.005 mol/L *tert*-butanol in the solutions. *Tert*-butanol is an effective OH radical scavenger and addition of 0.005 mol/L of this compound into the chitosan solution would significantly eliminate the formation of OH radical without affecting the cavitation behavior of ultrasound(Czechowska-Biskup et al., 2005). Czechowska et al. found that the degradation of chitosan by a 360 kHz ultrasound was greatly inhibited with the addition of 0.005 ml/L *tert*-butanol, and suggested that ultrasonic degradation at this frequency was the result of both mechanical forces and free radical reactions(Czechowska-Biskup et al., 2005). However, as the addition of *tert*-butanol did not affect the degradation kinetics of chitosans during 20 kHz sonication in our experiments, we conclude that the ultrasonic degradation under these conditions was only mechanically induced.

4-4.5 Effect of ultrasound intensity on the degradation process

The influence of ultrasound intensity on the degradation of 20.2%DA chitosan is presented in Figure 4-7-A and the rate coefficients vs. ultrasonic intensities are shown in Figure 4-7-B. As expected, the results showed that the rates of ultrasonic degradation increased with the increase in ultrasonic intensity. Similar observation was found by

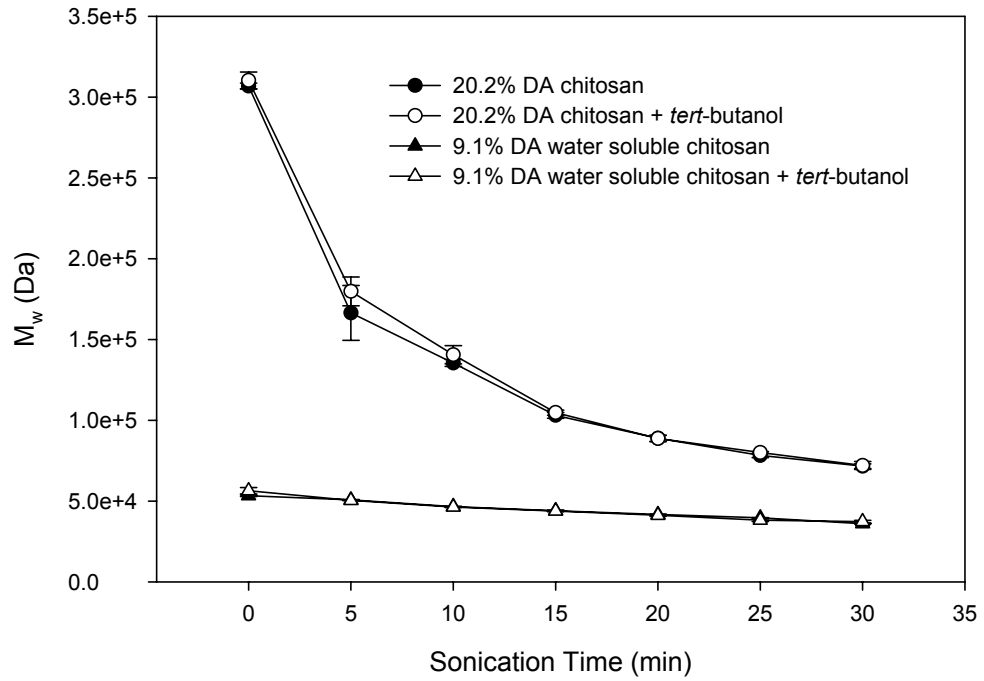


Figure 4-6: Degradation of 1% chitosan in 1% acetic acid in the presence and absence of 0.005 mol/L *tert*-butanol.

* Values are represented as mean \pm standard deviation (n=3).

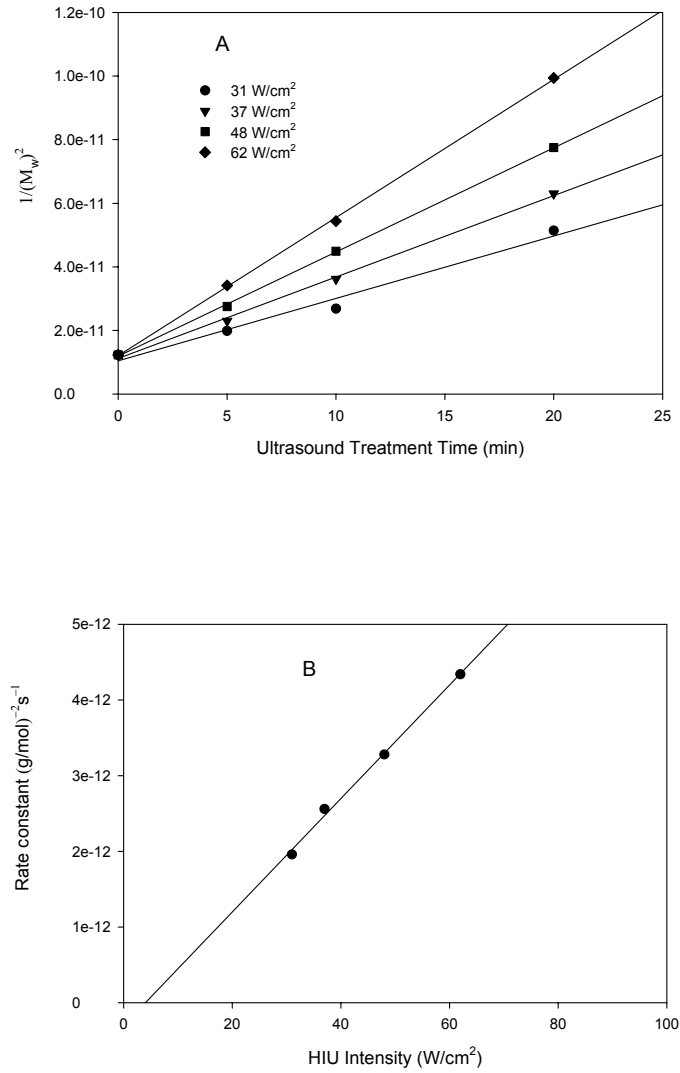


Figure 4-7: Effect of ultrasonic intensity on the ultrasonic degradation: (A)- variation of $\frac{1}{M_w^2}$ with sonication time at four intensities; (B) – plot of rate coefficients against the ultrasonic intensities.

Price and Smith for the degradation of polystyrene(Price and Smith, 1993). A linear relationship was found between the rate coefficient and the ultrasound intensity and a similar relationship was suggested for chitosan in an earlier study based on data of viscosity average molecular weight(Baxter et al., 2005). The non-zero intercept of the regression line is consistent with fundamental cavitation physics stating that cavitations are generated only above a certain intensity threshold, the so called cavitation threshold(Kardos and Luche, 2001).

4-4.6 Effect of temperature on the degradation process

The influence of solution temperature on the degradation rate of ultrasound was investigated at intensity of 48 W/cm^2 at 30, 50 and 80°C . Samples were either sonicated in ice water, room temperature water bath, or sonicated without any water bath. The effect of reaction temperature is shown in Figure 4-8-A. The degradation rate decreased with increasing temperature. These results are in agreement with published reports for synthetic polymers and chitosan(Price and Smith, 1993; Vijayalakshmi and Madras, 2004; Chen et al., 1997; Trzcinski and Staszewska, 2004). According to the cavitation physics, cavitations are more active in solvents with lower vapor pressure. Since the vapor pressure of solvents increases with increasing temperature, more solvent molecules may diffuse into the cavities at higher temperatures thereby dampening the collapse, an effect referred to as ‘cushioning’. The dependence of the degradation rate coefficients with temperature is shown in Figure 4-8-B. A similar dependence of degradation rate coefficients on temperature has been reported for the degradation of polyacrylamide and poly(ethylene oxide)(Vijayalakshmi and Madras, 2004).

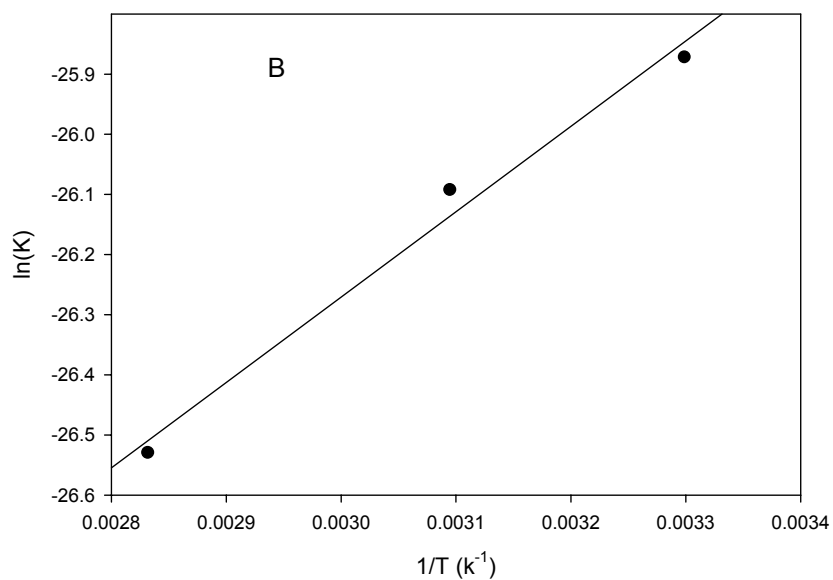
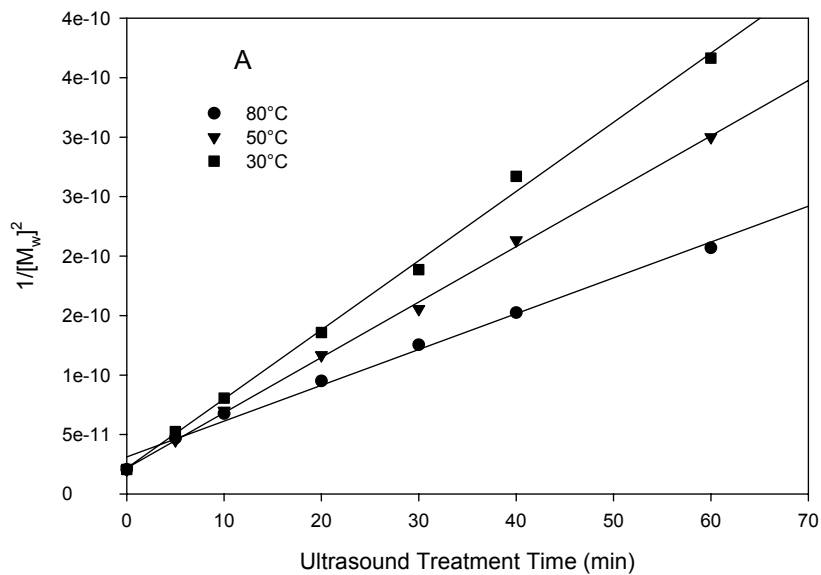


Figure 4-8: Effect of solution temperature on the ultrasonic degradation: (A)- alteration of $\frac{1}{M_w^2}$ with sonication time at three temperatures; (B)- plot of $\ln(K)$ against the $1/T$.

4-4.7 Effect of solution properties on the degradation process

The effects of solution properties were investigated by varying the polymer concentration (0.25%, 0.5%, 1% and 1%), ionic strength (1% chitosan prepared in 1% acetic acid with 0.1 and 0.2 M NaCl) and acetic acid concentration (1% chitosan in 1%, 2% and 4% acetic acid).

As presented in Figure 4-9-A, the calculated rate coefficients were 1.09×10^{-11} , 9.99×10^{-12} , 6.66×10^{-12} and $3.16 \times 10^{-12} \text{ Da}^{-2} \cdot \text{s}^{-1}$ for 0.25%, 0.5%, 1%, and 2% chitosan solutions, respectively. The rate coefficients decreased with increasing polymer concentration. This is consistent with published studies on synthetic polymers and chitosan (Chen et al., 1997; Basedow and Ebert, 1977; Trzcinski and Staszewska, 2004). With increasing polymer concentration, the viscosity of the solution increases thereby reducing the extent of the cavitation activity and thus reduces the polymer scission rate (Kuijpers and others 2005).

The rate coefficients for 0.25% and 0.5% chitosan were similar (1.09×10^{-11} and $9.99 \times 10^{-12} \text{ Da}^{-2} \cdot \text{s}^{-1}$) suggesting that the increase of the degradation rate due to the decrease of polymer concentration has a limit below which a further reduction in polymer concentration has no effect on the degradation rate. Is this limiting concentration the overlapping (C^*) or entanglement concentration (C_e)? The overlap concentrations of chitosan were reported to be 1.05 g/L (Desbrieres, 2002) and 2.8 g/L (Hwang and Shin, 2000) depending on the source of chitosan, whereas the entanglement concentration were reported as 5.0 and 7.4 g/L for chitosan with M_w 8.5×10^5 g/mol depending on the measurement methods (Cho et al., 2006). In this study, the overlap and entanglement concentrations of the investigated chitosan were determined as 0.27 and 8.87 g/L,

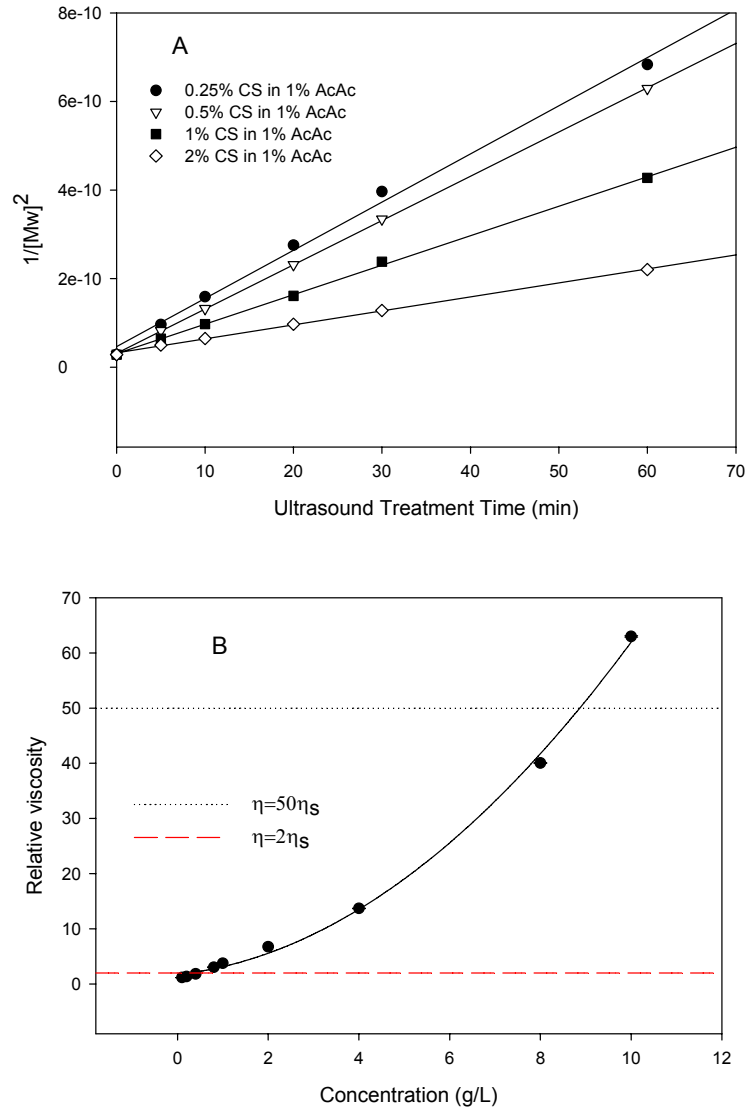


Figure 4-9: Effect of chitosan concentration on the ultrasonic degradation: (A) – alteration of $\frac{1}{M_w^2}$ with sonication time for chitosan with four concentrations; (B) - overlap concentration ($\eta=2\eta_s$) and entanglement concentration ($\eta=50\eta_s$) of chitosan.

respectively (Figure4-9-B). We therefore suggest that the limiting concentration of chitosan is between the overlap concentration and entanglement concentration, but closer to the latter value. It is likely that as soon as the polymers act as individual molecules that the effect of polymer concentration on ultrasonic degradation becomes insignificant.

As presented in Figure 4-10, the rate coefficients decreased from 6.66×10^{-12} , to 3.64×10^{-12} and $3.37 \times 10^{-12} \text{ Da}^{-2} \cdot \text{s}^{-1}$ with the addition of 0.1M and 0.2M NaCl, respectively. Addition of more than 0.5M NaCl to the chitosan solution resulted in formation of precipitates, possibly due to electrostatic shielding. With chitosan being prepared in 1% acetic acid, the ionic strength for this solvent system was calculated as: $I = 10^{-\text{pH}} + I_{\text{NaCl}}$. Since the pH in all samples was 3.99 ± 0.01 , the influence of pH on ionic strength was negligible and thus ignored. With an increase in the ionic strength to 0.1M, the degradation rate decreased by approximately 50%. Further increases in ionic strength did not cause significant decreases in the rate coefficient.

It was recently reported that chitosan chains may assume a more compact structure with increase of ionic strength(Cho et al., 2006). The radius of gyration, the derived Kuhn length (b), and the persistence length (l_p) decreased with increasing ionic strength due to salt screening of the electrostatic repulsive interactions. Cho et al. reported that the radius of gyration decreased from 162 nm to 113 nm and 105 nm, and the persistence length decreased from 81 nm to 40 nm and 35 nm when the ionic strength increased from 0.009 to 0.12 and 0.24 M, respectively(Cho et al., 2006). Similarly to our observations, a white precipitate was found in chitosan solutions if the ionic strength was increased above 0.46M. Formation of precipitates was attributed to increased

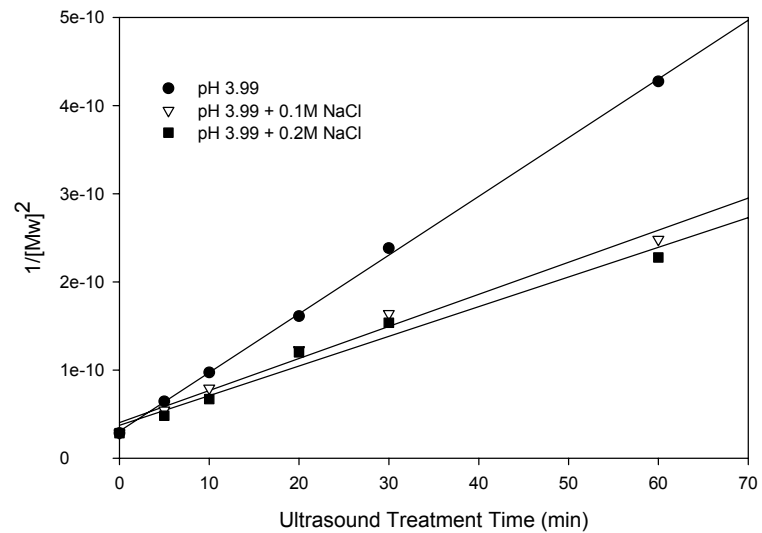


Figure 4-10: Effect of ionic strength on the ultrasonic degradation – alteration of $\frac{1}{M_w^2}$ with sonication time at three ionic strengths.

hydrophobic interactions, hydrogen bonding, and a decrease in electrostatic repulsion(Cho et al., 2006).

Although the effects of ionic strength on the radius of gyration could not be determined by GPC-MALS due to the strength of the mobile phase itself (0.15M ammonium acetate/0.2M acetic acid), the reduction of the rate coefficient with increasing ionic strength can be explained by the change in the molecular configuration. When the ionic strength increased from 0.0001 to 0.1 M, the radius of gyration of chitosan decreased from 162 nm to 113 nm, as reported by Cho et al(Cho et al., 2006), while we determined a decrease in rate coefficient for almost 50% (from 6.66×10^{-12} to 3.64×10^{-12} $(\text{Da})^{-2} \cdot \text{s}^{-1}$) under the same conditions. However, a further increase of the ionic strength from 0.1 to 0.2M caused only minor changes in the radius of gyration from 113 nm to 105 nm and consequently resulted in only a minor change of the rate coefficient (from 3.64×10^{-12} to 3.37×10^{-12} $\text{Da}^{-2} \cdot \text{s}^{-1}$)(Cho et al., 2006). An ionic strength of 0.05M is generally sufficient to screen electrostatic interactions in polyelectrolytes, and further increases do not have a significant effect on the radius of gyration of polymers(Cho et al., 2006). Similar results have been found for the degradation of dextran(Basedow and Ebert, 1977).

The rate coefficients of ultrasonic degradation were 6.66×10^{-12} , 6.19×10^{-12} and 6.41×10^{-12} $\text{Da}^{-2} \cdot \text{s}^{-1}$ for 1% chitosan in 1%, 2% and 4% acetic acid, respectively (Figure 4-11). The difference between these values is very small and the effect of acetic acid concentration in this range on the ultrasonic degradation of chitosan appears to be insignificant. As mentioned earlier, the rate of ultrasound degradation was found to be primarily affected by the vapor pressure of a solvent, while the effects of solvent

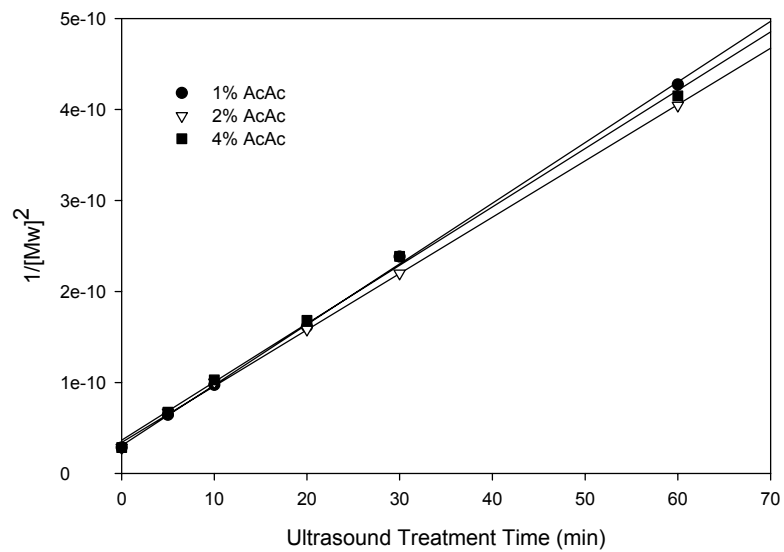


Figure 4-11: Effect of acid concentration on the ultrasonic degradation –alteration of $\frac{1}{M_w^2}$ with sonication time at three acid concentrations.

viscosity and surface tension are not as pronounced (Basedow and Ebert, 1977). Since the concentration of acetic acid in our study was never more than 4%, we concluded that the effect of acetic acid concentration on the solvent vapor pressure was probably too small to affect the rate of ultrasonic degradation. Furthermore, since the pKa of chitosan is around 6.3 and the pH for 1% chitosan in 1% acetic acid was 3.99 ± 0.01 , the majority of amino groups on chitosan were protonated and further increases in the acid concentration to 4% did not significantly affect chitosan conformation. Our results were similar to study of Chen et al (Chen et al., 1997) while Trzcinski et al reported that the increase of acetic acid concentration caused the increase of general rate parameters (Trzcinski and Staszewska, 2004). Contradictory results of Trzcinski et al (Trzcinski and Staszewska, 2004) may be caused by different behavior of the system with application of low power ultrasound emitter with frequency of 35 kHz and sonic intensity of $2\text{W}/\text{cm}^2$.

4-4.8 Effect of ultrasound on radius of gyration, polydispersity, conformation, molecular weight distribution and degree of acetylation of chitosan

As shown in Figure 4-12, the z average radius of gyration decreased from 117, 95, 67 and 27 nm to 29, 30, 25 and 21 nm (Figure 4-12-A) and the corresponding polydispersity decreased from 2.8, 2.4, 2.1 and 1.7 to 1.5, 1.5, 1.5, and 1.6, respectively (Figure 4-12-B). As a result of the molecular weight decrease, the decrease of radius of gyration was expected. The decrease of polydispersity with passage of sonication time has been reported and was attributed to the fact that large molecules are easily degraded (Chen et al., 1997).

The differential molecular weight and cumulative molecular weight distribution of 20.2% DA chitosan sonicated at $62\text{ W}/\text{cm}^2$ are shown in Figure 4-13-A and -B. The

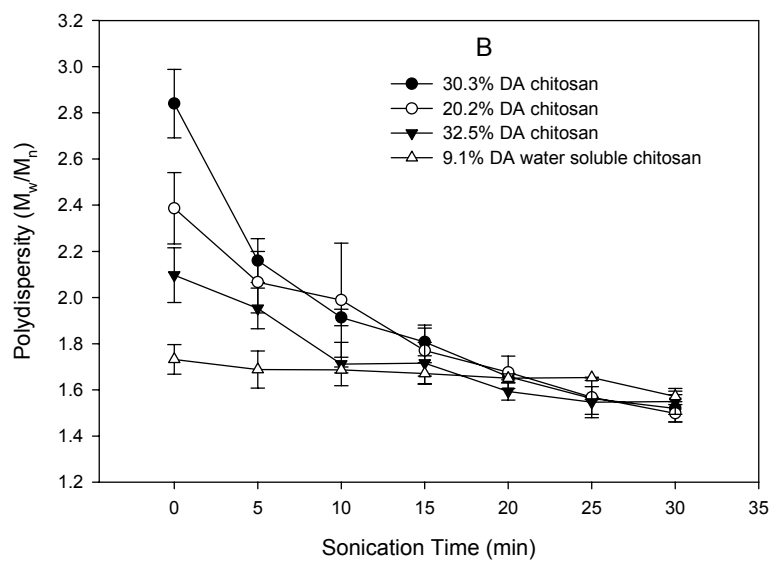
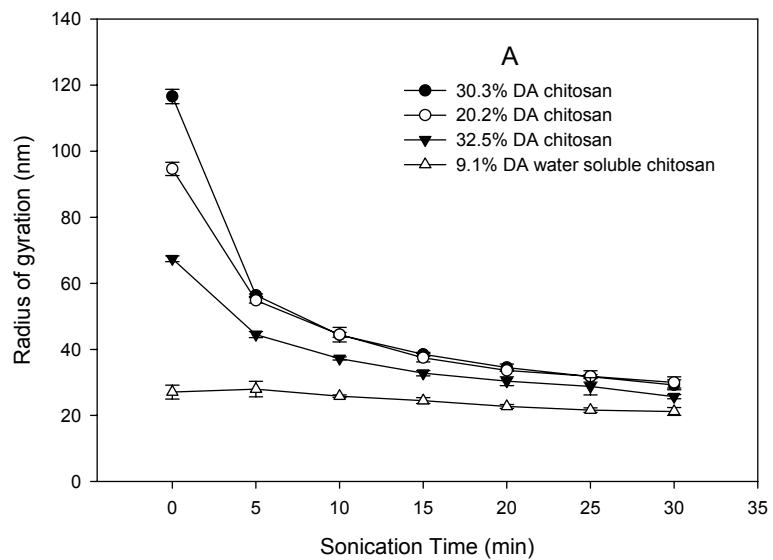


Figure 4-12: Alteration of radius of gyration and polydispersity of chitosan with the sonication time for chitosan with different molecular weight and degree of acetylation.

* Values are represented as mean \pm standard deviation (n=3).

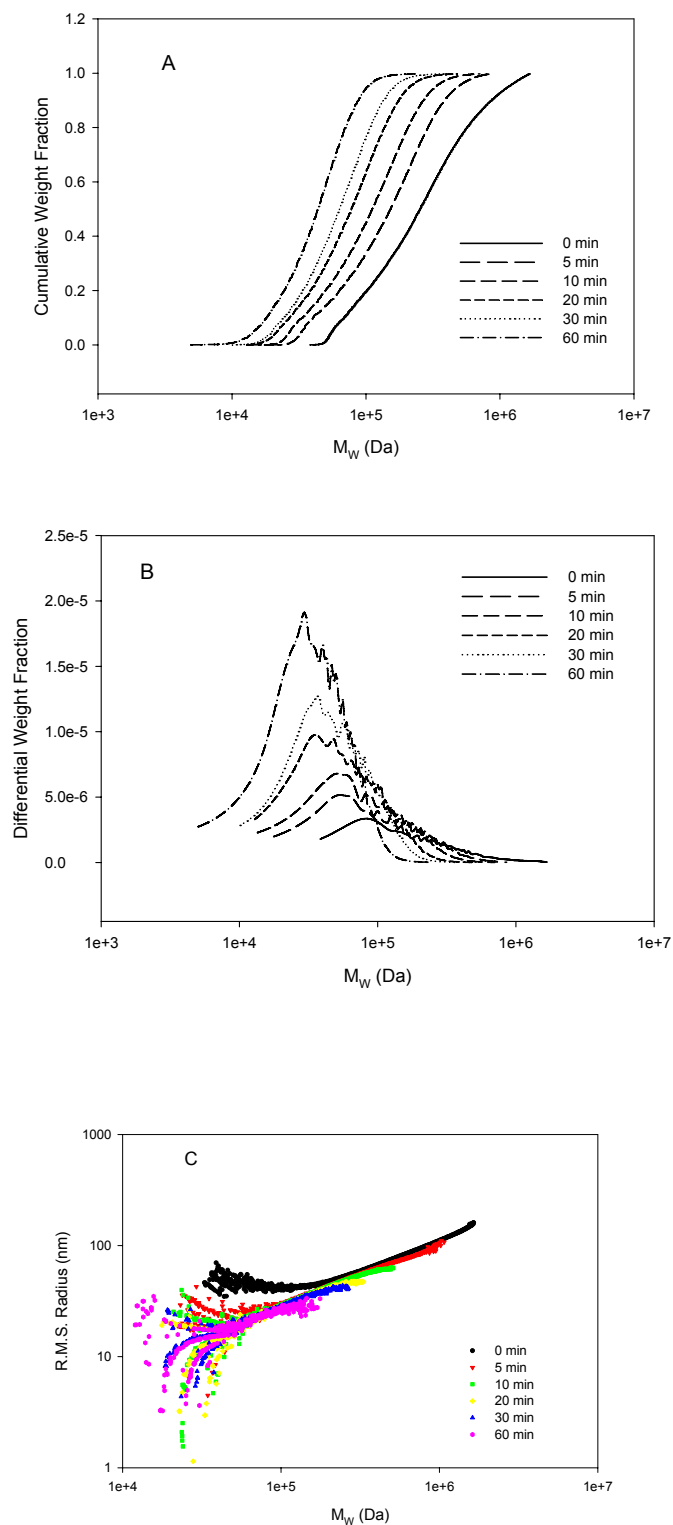


Figure 4-13: Alteration of cumulative (A) differential molecular weight distribution (B) and conformation of chitosan (C) with sonication time (30.3% DA chitosan).

cumulative distribution $W(M)$ is defined as the weight fraction of sample having molar mass less than M :

$$W(M) = \frac{\sum_{M' < M} C_{M'}}{\sum C_M} \quad (7)$$

where C_M is the mass concentration for the fraction having a molar mass of M' . The differential distribution is defined as:

$$X(M) = \frac{dW(M)}{d(\text{Log}M)} \quad (8)$$

As seen from both plots, the fractions of low molecular weight chitosan increased and chitosan with lower polydispersity was obtained with the passage of sonication time. The evolution of the mass molecular weight distribution using M_w in this study is consistent with the results using M_n (Madras et al., 2000).

Although the molecular weight distribution shifted toward lower molecular weights with increasing sonication time, conformation plots (Figure 4-13-C) showed that the majority of chitosan molecules remained in the same conformation after sonication. If a macromolecule of mass M is composed of i elements m , the mean square radius $\langle r_g^2 \rangle$ can be expressed as:

$$\langle r_g^2 \rangle = \frac{\sum_i r_i^2 m_i}{\sum_i m_i} = \frac{1}{M} \sum_i r_i^2 m_i \quad (9)$$

where r_i is the distance of element m_i to the mass center of the macromolecule with mass M . The radius can be related to the molar mass M_w by:

$$r_g = kM_w^\alpha \quad (10)$$

The plot of $\langle r_g^2 \rangle$ vs. the logarithm of the molar mass can be used to determine the slope α , which can provide valuable information about the polymer conformation. Theoretical slopes of 0.33, 0.50 and 1.0 have been described for spheres, random coils, and rigid rods, respectively. The slope α of this plot is related to the Mark Houwink parameter a by

$$\alpha = (a + 1)/3 \quad (11)$$

Most real random coils have an “a” value in the range of 0.55–0.60. The calculated slope of the regression lines for the plots in Figure 4-C were 0.50, 0.47, 0.52, 0.52, 0.49 and 0.52 for chitosan with 0, 5, 10, 20, 30 and 60 minutes sonication, respectively. The results showed that chitosan remained in a random coil conformation after the sonication regardless of sonication time. This result suggested that the degradation was not free radical induced because free radical degradation would result in the formation of macromolecule free radicals, and the recombination of the macromolecular free radicals would possibly lead to formation of side chain and the conformation change.

The UV spectra of chitosan before and after sonication also suggested that degradation was not free radical induced (Figure 4-14). Chitosan samples had almost same spectra after the ultrasound treatment. This was different from the degradation that carried out by a 360 kHz ultrasound, where by-products containing carbonyl group were formed and new absorbance peak at 265 nm occurred in UV spectra (Czechowska-Biskup et al., 2005). This further confirmed that at the low frequencies, the degradation was mainly mechanic force.

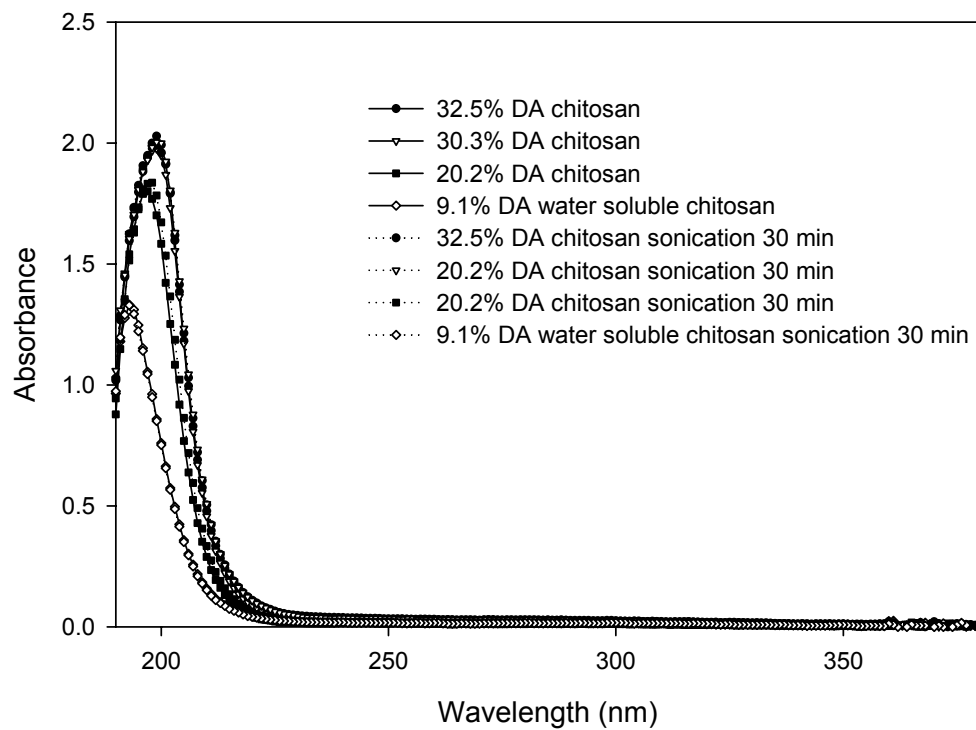


Figure 4-14: UV spectra of chitosan spectra before and after high intensity ultrasound treatment for 30 minutes at 62 W/cm².

The DA values of chitosan before and after sonication are shown in Table4-1. The DA values before sonication were determined as $20.2 \pm 0.1\%$, $30.3 \pm 0.2\%$, $32.5 \pm 0.8\%$, and $9.1 \pm 0.6\%$ for 19%, 29%, 39% DA and water soluble chitosan, respectively. It was noticed that the actual DA value was 32.5% for the chitosan with nominal DA of 39%. After sonication, the DA values were determined as $20.4 \pm 0.5\%$, $28.0 \pm 0.7\%$, $30.8 \pm 0.7\%$ and $9.0 \pm 0.1\%$. ANOVA analysis showed that high intensity ultrasound treatment had no significant effect on the DA values of chitosan. Our results were similar to the report of Baxter et al (Baxter et al., 2005), but differed to the report of Liu et al¹⁹. Lie et al used relative long sonication time compared to our study, which may be the reason for these discrepancies (Liu et al., 2006).

4-5 Conclusions

The molecular weight, radius of gyration and polydispersity of chitosan were efficiently reduced by ultrasound treatment while molecular conformation and degree of deacetylation did not change after sonication. Our experiment showed the degradation of chitosan by ultrasound is primarily driven by mechanical forces and degradation mechanism can be described by a random scission model. The degradation rate is proportional to M_w^3 and the degradation rate coefficients are significantly influenced by ultrasound intensity, solution temperature, polymer concentration and ionic strength while acid concentration has little effect. Additionally, the degradation rate coefficients are affected by the degree of acetylation of chitosan and independent of the initial molecular weight.

Results of this study confirmed that HIU offers a mean to tailor the molecular weight of chitosan without affecting molecular configuration. HIU degradation of

chitosan can be achieved at low solvent strengths (1% acetic acid) within relatively short time, which is a significant improvement over conventional timely acid hydrolysis procedures.

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5 Physicochemical Properties and Antimicrobial Activities of Hydrophobically Modified Chitosan (HM-chitosan) and Resulting Films

5-1 Abstract

Hydrophobically modified chitosan (HM-chitosan) was prepared by reacting chitosan with palmitoyl-chloride and blend films with different chitosan to HM-chitosan ratios were prepared by solution casting method. The physicochemical properties and antimicrobial activity of HM-chitosan and the blend films were investigated. The results showed that (1) hydrophobic modification of chitosan alters its solubility and thermal properties, introduces self assembled and thermal responsive properties, and has no effect on its antimicrobial activity against tested bacteria; (2) blend films have heterogeneous internal structure, rougher surface morphology, decreased water vapor permeability and mechanical properties compared to pure chitosan film, whereas the antimicrobial activity of blend films remains unchanged.

5-2 Introduction

Chitin, the second most abundant biomass on earth, is a linear polysaccharide consisting of 2-acetamido-2deoxy- β -D-glucose. Chitosan, the deacetylated product of chitin, is predicted to have many potential applications in various industries: as antimicrobial edible film in the food industry, smart drug delivery carrier in the pharmaceutical industry, and heavy metal binding agent in waste water treatments (Shahidi and others 2005; Rinaudo, 2006; Kumar and V., 2000; Dodane and Vilivalam, 1998; Kurita, 1998).

Possibility for chemical modifications of chitin and chitosan has recently drawn increased interests in order to provide advanced functionalities (Kumar and V., 2000; Kurita, 2001; Sashiwa and Aiba, 2004; Rinaudo and others 2005). Among these studies, the hydrophobic modification (HM) of chitosan was found to enhance its rheological performance (Rinaudo et al., 2005), and was evaluated for use as a self-assembled nanoparticle for controlled release of food additives and drugs (Liu and others 2005; Le Tien and others 2003). In general, the hydrophobic modification can be carried out on C2 amino group by reductive alkylation with aldehyde (Kurita, 2001), by reaction with fatty acid chloride or fatty acid anhydride (Hirano and others 2002), or by a water-soluble carbodiimide mediated conjugation with fatty acids (Liu et al., 2005). However, evaluation of effects of hydrophobic modification on antimicrobial activity of chitosan has not been a part of the published studies. Several studies on water-soluble chitosan derivatives observed decreased antimicrobial activities (Xie and others 2002; Avadi and others 2004), whereas another study showed an increased antimicrobial activity of water-soluble chitosan derivative prepared by grafting at the C6 position (Je and Kim, 2006). It

is hypothesized that chitosan, as a polycationic polysaccharide, binds to negatively charged phospholipids of the cell membrane changing the membrane permeability and ultimately leads to cell death(Helander and others 2001). The hydrophobic modification of chitosan may have a negative impact on its antimicrobial activity because the substitutions are usually made at the protonation site - C2 amino group. On the other hand, enhanced hydrophobic interactions between HM-chitosan and cell membrane lipids may promote its antimicrobial efficiency.

Since chitosan films have the potential to be used as antimicrobial packaging, their physicochemical properties have been well investigated regarding the effects of solvent type and pH of film forming solution, molecular weight and degree of acetylation of chitosan, age and thermal treatment of the films (Zivanovic and others 2005; Butler and others 1996; Morillon and others 2002). However, direct application of chitosan films in food industry is still limited due to the high moisture permeability and undesired mechanical properties(Butler et al., 1996). Blending of polysaccharides or proteins with lipids to prepare emulsion-type films has been shown as an effective way to reduce the water vapor permeability (WVP)(Wong and others 1992). The incorporation of lauric acid decreases WVP of chitosan film by 49.0%, while the incorporation of other fatty acids, such as palmitic acid, octanoic acid and butyric acid has no such effect(Wong et al., 1992). Another study has shown that the incorporation of palmitic or stearic acid actually increases the WVP by 26% (Srinivasa and others 2007). Therefore, blending with lipids does not always reduce WVP. The reason is that WVP can be affected by many factors, such as type, size and distribution of lipids, film orientation and film forming temperature (Perez-Gago and others 2005; Jesus-Alberto and others 2000).

The objectives of this study were to investigate the physicochemical properties and antimicrobial activity of HM-chitosan, along with the surface characteristic, mechanical properties and WVP of HM-chitosan based blend films.

5-3 Experimental details

5-3.1 Materials

Chitosan samples with degree of acetylation of 80% and molecular weight of 307 kDa (weight average) were kindly provided by Primex Company (Primex, Iceland) and palmitoyl chloride was purchased from Sigma Aldrich (St. Louis, MO). Other chemicals and materials were purchased from Fisher Scientific.

5-3.2 Chitosan purification

Chitosan solutions were prepared with 1% acetic acid at concentration of 1% and filtered through Miracloth® (Rayon-polyester; EMD bioscience, San Diego, CA) to remove suspended impurities. Filtered chitosan solutions were neutralized with 30% ammonia solutions overnight to precipitate all chitosan. Chitosan flocculates were collected, rinsed with D.I water until neutral, and lyophilized to obtain the purified chitosan.

5-3.3 Preparation of hydrophobically modified chitosan

Preparation of HM-chitosan followed the reference method with modification (Le Tien et al., 2003). Palmitoyl chloride 5 ml was dissolved in 100 ml D.I. water and emulsified with 0.1 ml Tween 20 to obtain an emulsion by homogenization. 5 g purified chitosan was dissolved in 500 ml 0.5% acetic acid, mixed with palmitoyl chloride emulsion and reacted at room temperature for 4 hours. The post-reaction mixture was

neutralized with 20 ml 10N NaOH overnight to precipitate HM-chitosan. HM-chitosan was collected by centrifugation, washed with D.I. water to a neutral pH, washed with acetone three times and lyophilized.

5-3.4 Thermal analysis

Thermogravimetry analysis (TGA) was performed by a Thermal Analysis TGA50 (TA instruments, New Castle, DE) at a heating rate of 10°C/min under air atmosphere. The gas flow was 100 ml/min. Differential scanning calorimetry (DSC) analysis was performed with a Thermal Analysis DSC Q1000 (TA instruments, New Castle, DE) at a heating and cooling rate of 10°C/min under nitrogen atmosphere. Heating was starting from -10 to 160°C for HM-chitosan and from -10 to 200°C for chitosan. The cooling process was the reverse process of heating. Two cycles were run for each sample in total.

5-3.5 Preparation of HM-chitosan based film

5-3.5.1 Preparation of chitosan and HM-chitosan solutions

5 g lyophilized HM-chitosan was ground using Tomas Mill (sieve 40), dispersed in 500 ml 1% acetic acid and submitted to ultrasound treatment (Sonics and Materials VC-750, Newton, CT) at 80% amplitude, pulse mode (30s on and 30s off) and temperature control of 30°C for 30 minutes. HM-chitosan solution was diluted to 0.1% with 1% acetic acid and dynamic light scattering (DLS) was performed using a ZetaPlus particle sizer (Brookhaven Instruments, New York, NY). 500 ml purified non-modified chitosan solution was prepared in 1% acetic acid at concentration of 1%. For the determination of antimicrobial activity, chitosan and HM-chitosan solutions were both prepared with D.I. water pH 5.0 adjusted by 1N HCl instead of 1% acetic acid.

5-3.5.2 Preparation of chitosan/HM-chitosan blend films

Varied volumes of chitosan and HM-chitosan solutions were mixed at ratios of 100/0, 75/25, 50/50, 25/75 and 0/100 (chitosan/HM chitosan) to obtain a total volume of 200 ml each. The mixed solution was purged with nitrogen for 10 minutes, degassed by vacuum for 15 minutes, and filtered through Miracloth® to remove foams. Approximate 14.5 g mixed solutions were cast into 60×15 mm polystyrene Petri-dish and dried at 35°C under vacuum for 72 hours to form uniform films. All films were stored in desiccators and carefully peeled from the Petri-dishes right before analysis.

The kinematic viscosity of all chitosan solutions were measured using a Cannon-Fenske viscometer (Cannon Instrument Company, State College, PA) at 25°C with three replications on each solution. The predicted viscosity was calculated by the following equation: $\eta = \phi A \times \eta A + \phi B \times \eta B$, where ϕA and ϕB , ηA and ηB are the volume fraction and viscosities of HM-chitosan and chitosan respectively.

5-3.6 Analysis of physicochemical properties of the films

5-3.6.1 Film thickness

Film thickness was measured with a microcaliper (Mitutoyo Corp, Japan) on five films per ratio treatment and ten random spots per film.

5-3.6.2 Swelling of the films and acetic acid content

Round disk shape samples were cut from the films using a single pore paper punch and immersed in 100 ml PBS buffer (10 mM, pH 7.4). The released acetic acid quantities were measured periodically using HPLC- (photodiode array) PDA detector (Dionex, Sunnyvale, CA) with a mobile phase of 0.05 M KH₂PO₄, pH 4.5 on a C18 column (Waters Nova-Pak, 4.6 × 250 mm) and calculated in a film weight basis. The

diameters of the disks were measured before and after immersion at 20 minutes. The swelling ratio (%) was calculated by the ratio of diameters before and after immersion.

5-3.6.3 Polarized microscopy

The morphological characteristic of blend films was examined with an Olympus BX51 polarized microscope (Olympus, Melville, NY) equipped with a DP 70 camera with normal and polarized light at 40X optical magnification.

5-3.6.4 Analysis of surface topography

The atomic force microscopy (AFM) images of blend films were analyzed by a DI Nanoscope IIIA Multimode Scanning Probe Microscope (Digital Instruments, Veeco Metrology Group, Santa Barbara, CA) with a standard 125 μm single-crystal silicon cantilevers (Model TESP; Digital Instruments) at ambient conditions. Sample films were attached onto iron AFM substrate disks using double sided tape. Three random points at both sides of film surface (polymer air surface and polymer-Petri dish surface) with area of 1 and 100 μm^2 were scanned. All images shown here were height images. The AFM images and roughness were analyzed through WSXM, free software downloadable at <http://www.nanotec.es>.

5-3.6.5 FTIR analysis

Fourier transform infrared (FTIR) measurements of HM-chitosan powder and blend films (two surfaces) were performed using FTIR with attenuated total reflection infrared (ATR) accessory (Nexus 680, Thermo Nicolet Corp, Madison, WI) in the wavenumber range of 700-4000 cm^{-1} with resolution 8. Every sample was analyzed six times to generate a statistic average spectrum.

5-3.6.6 Water vapor permeability (WVP) test

WVP was measured with the American Society for Testing and Materials (ASTM) E96-05 method. For each treatment, three films were mounted on Plexiglas test cups containing 5 ml of water with film top (chitosan-air surface) or film bottom (chitosan-Petridish surface) facing the high RH environment. The cups were placed in an environmental chamber (25°C and 50% RH) and weights were measured periodically after steady state was achieved. The weight losses were plotted against elapsed time and water vapor permeance were calculated after corrections with Schirmer's equation for resistance of still air layer in the cup. The thickness of still air layer under the film surface was measured by a ruler as 8 mm.

5-3.6.7 Mechanical properties

The puncture strength (PS), tensile strength (Hirano et al.) and percent elongation at break (%E) were measured with a TA XT plus texture analyzer (Texture Technologies, Surrey, U.K.). The PS was measured on three films per treatment using the TA-108S fixture and 2 mm diameter cylinder probe (TA-52) moving with a speed of 1mm/s. The PS was calculated by dividing the maximum force at break (N) with the thickness (mm) at the broken areas. TS and %E were measured based on ASTM D882 method. Sample films were cut by specimen cutting die (Qualitest USA LC, Plantation, FL) to the strips with uniform width of 6 mm and tested by a double clamp (TA-96) at a test speed of 1mm/s. Initial grip separation was set as 20 mm. The TS was expressed in MPa and was calculated by dividing the maximum load (N) with the cross-sectional area (m²). Percent elongation at break was calculated by dividing the extension at the moment of rupture with the initial gage length of the samples and multiplying by 100.

5-3.7 Antimicrobial activity of chitosan, HM-chitosan and blend films

5-3.7.1 Antimicrobial activities of chitosan and HM-chitosan

The *Listeria innocua* ATCC 51742 and *Escherichia coli* K12 were maintained on tryptic soy agar (TSA) (Becton Dickinson, Sparks, MD) slants at 4°C and activated at 35°C for 24 hr before experiments. Chitosan and HM-chitosan solutions were added into Tryptic Soy Broth with 0.6% yeast extract (TSB-YE; Becton Dickinson) to give a final chitosan concentration of 0.1%. The pH of broth was adjusted to 5.0 with 1N sterile HCl before autoclaving at 121°C for 15 minutes. Aliquots of 0.1 ml bacterial culture (10^6 CFU/ml) was added into 9.9 ml broth supplemented with chitosan solution or suspensions and incubated aerobically at 35°C for 3, 6, 12 and 24 hours. The enumeration was carried out by stepwise dilutions with 0.1% peptone water followed by plating on TSA-YE and incubated at 35°C for 48 hour.

5-3.7.2 Antimicrobial activities of chitosan-and HM-chitosan films

0.2 g films were peeled from Petri-dishes and added into 99 ml 0.1% peptone water blanks followed by adding aliquots of 1 ml bacterial culture (10^6 CFU/ml) and incubated aerobically at 35°C for 24 hours. The enumeration was carried out by stepwise dilutions with 0.1% peptone water followed by plating on TSA-YE agar and incubated at 35°C for 48 hours.

5-3.8 Statistical analysis

All experiments were repeated three times and significant difference between treatments was determined using Duncan's multiple range test by SAS program 9.13 (SAS institute Inc, 2003).

5-4 Results and discussion

5-4.1 Physicochemical properties and biological activity of HM-chitosan

5-4.1.1 Preparation and characterization of HM-chitosan by FTIR, TGA, DSC and DLS

The HM-chitosan became insoluble in diluted acids and thus the characterization by $^1\text{H-NMR}$ to determine the degree of substitution was impossible. However, successful grafting of the hydrophobic group was clearly observed from the FTIR spectra (Figure 5-1). The appearance of new peaks at 2914 and 2850 cm^{-1} were attributed to the palmitoyl alkyl chain. The new peaks at 1800, 1740, 1696 and 1464 cm^{-1} showed that both N-acylation and O-acylation occurred (Le Tien et al., 2003).

TGA analysis showed that prepared palmitoyl chitosan was probably a mixture consisted of chitosan, N-acylated and O-acylated chitosan. Compared to a single decomposition temperature of chitosan centered at 317.30 $^{\circ}\text{C}$, two other peaks centered at 225.94 and 392.03 $^{\circ}\text{C}$ were found for HM-modified chitosan (Figure 5-2). The N-acylation of chitosan has been reported to increase the decomposition temperature of chitosan (Choi and others 2007). Thus the decomposition temperature centered at 392.03 $^{\circ}\text{C}$ was assigned to the N-acylated chitosan. The decomposition temperature centered at 225.94 $^{\circ}\text{C}$ was tentatively assigned to the O-acylated chitosan. The corresponding weight losses for O-acylated chitosan, chitosan and N-acylated chitosan were 20.53%, 33.20% and 26.33%, respectively. At the end of decomposition process, the weight losses for chitosan and HM-chitosan were 73.11 and 85.24%, respectively. It showed the hydrophobic modification accelerated the thermal decomposition process of chitosan.

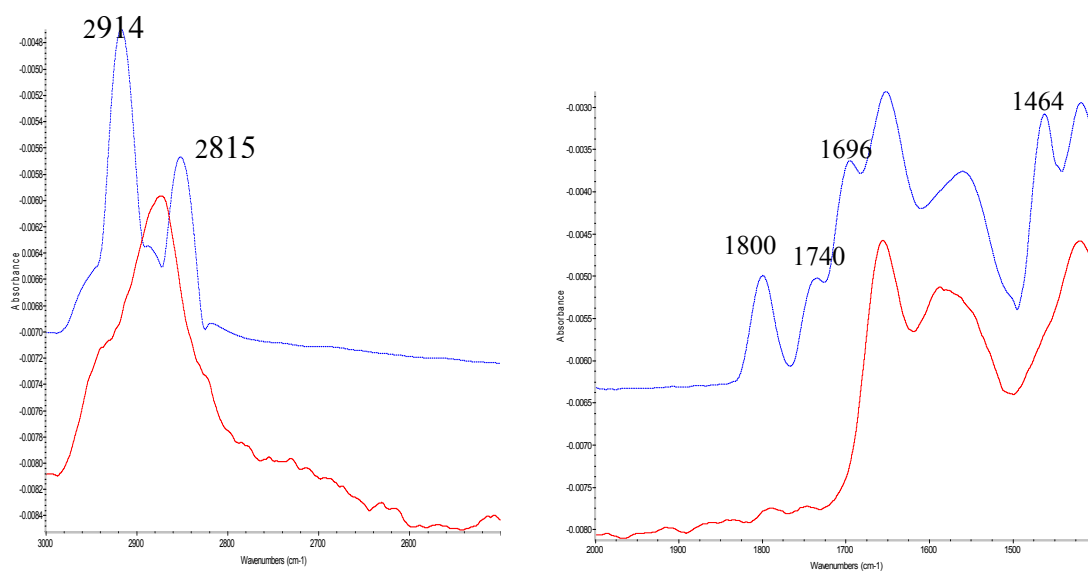


Figure 5-1: FTIR spectra of non-modified (bottom) and HM-chitosan (top) in the range of 3000 to 2500 cm^{-1} (left) and 2000-1400 cm^{-1} (right).

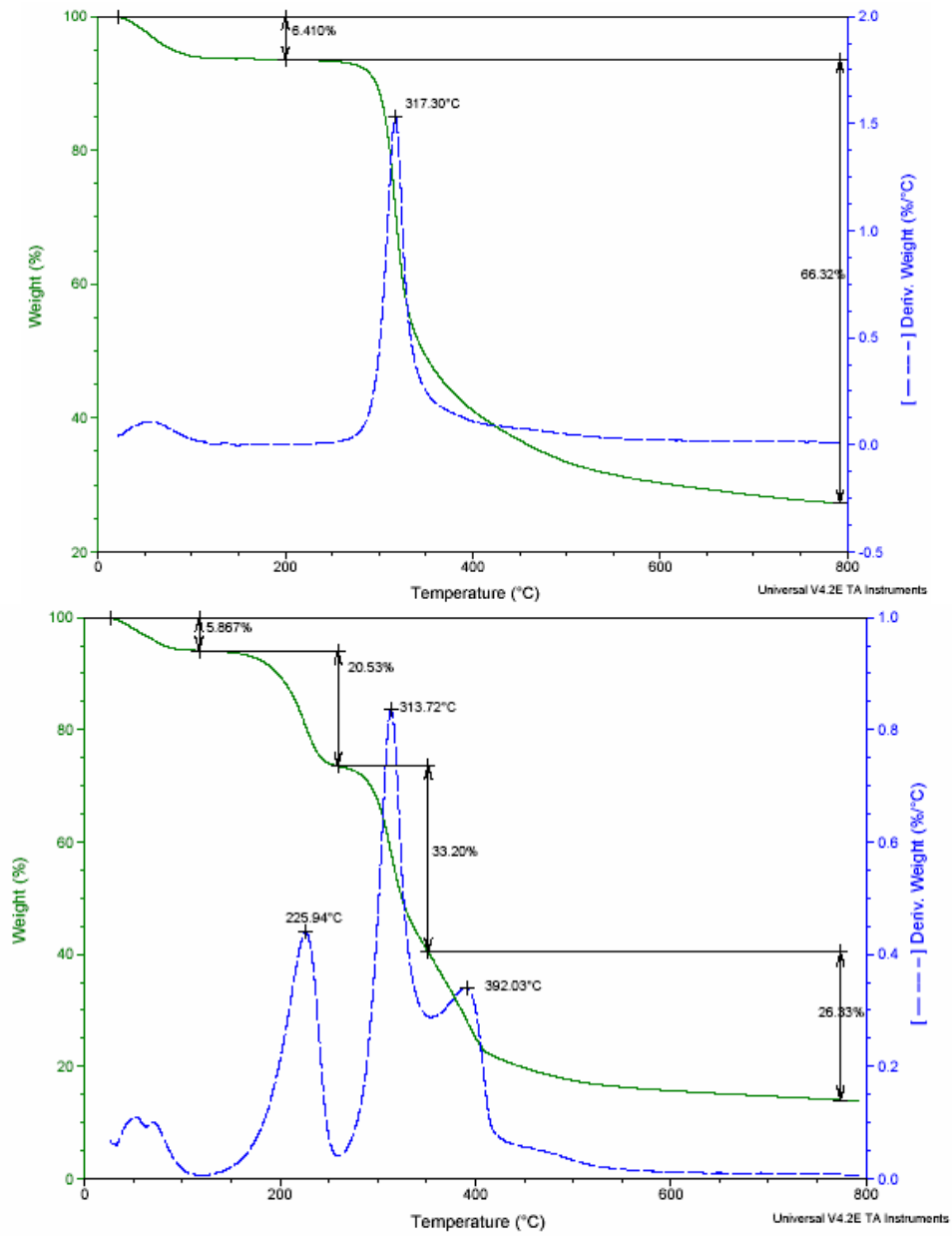


Figure 5-2: TGA thermograms of chitosan (top) and HM-chitosan (bottom).

In the first heating cycle of DSC, a broad negative peak from 60 to 200°C was found for chitosan (Figure 5-3). In the latter heating cycle, this peak disappeared and suggested it was related with the evaporation of water. For HM-chitosan, beside this very broad peak, another narrow endothermic peak was found in the first heating cycle and did not disappear, but become narrower in the latter run. This second peak, centered at 62°C, corresponded to the melting point of palmitic acid. Thus, it seemed that the grafting of palmitoyl group introduced a phase transition temperature for HM-chitosan. However, it was also possible that the reaction by-product palmitic acid was not completely removed from the HM-chitosan. Nevertheless, when dissolved in 1% acetic acid, HM-chitosan formed self-assembled particles. The particle size decreased with the increased temperature from 1200 nm at 30°C to 450 nm at 70°C (Figure 5-4).

5-4.1.2 Antimicrobial activity of chitosan and HM-chitosan

At concentration of 0.1% both chitosan and HM-chitosan effectively inhibited the growth of tested bacteria. After 24 hrs, the cell number of *L. innocua* was reduced from 4.25 to 1.19 and from 4.06 to 1.00 log CFU/ml for HM-chitosan and chitosan, respectively, whereas the cell number increased from 4.26 to 7.16 log CFU/ml in the control (Figure 5-5). The number of *E. coli* cells was reduced from 4.08 to 1.96 and from 3.87 to 2.14 log CFU/ml for HM-chitosan and chitosan, respectively, whereas the cell number increased from 4.15 to 8.49 log CFU/ml in the control (Figure 5-5). The initial plateau stage of both survival curves from 0 to 6 hrs also suggested that chitosan and HM-chitosan did not have immediate killing effects, but inhibited the growth of the test

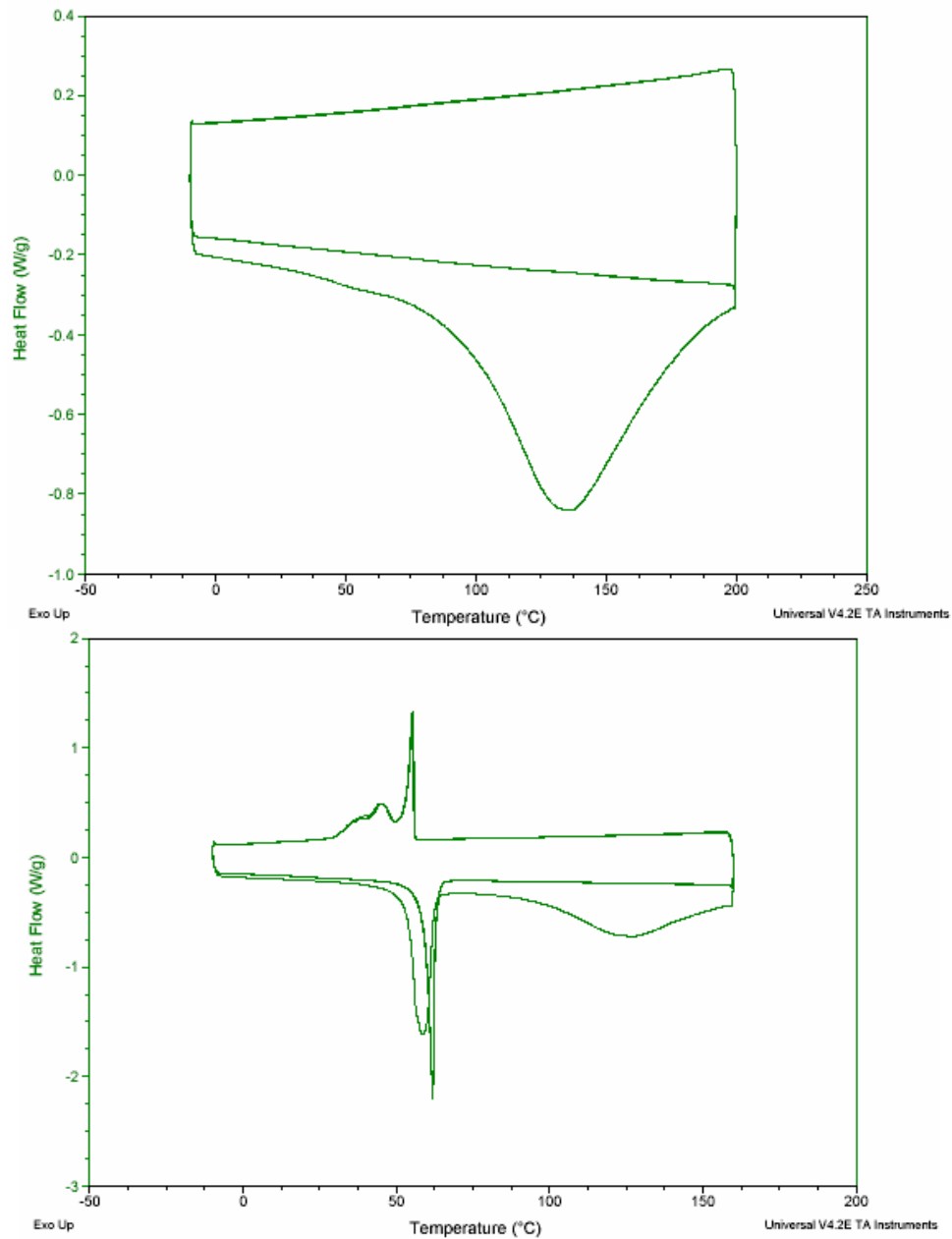


Figure 5-3: DSC thermograms of chitosan (top) and HM-chitosan (bottom).

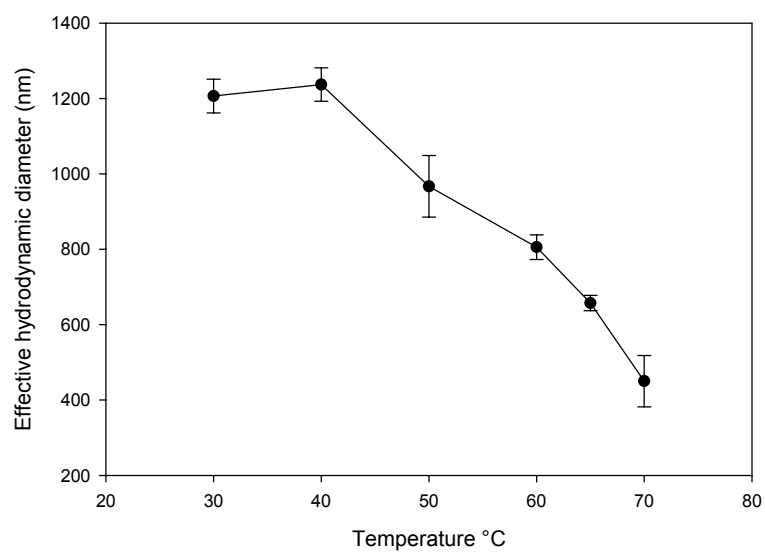


Figure 5-4: Variation of particle size of HM-chitosan (0.1%) in 1% acetic acid with temperature.

* Values are represented as mean \pm standard deviation (n=3)

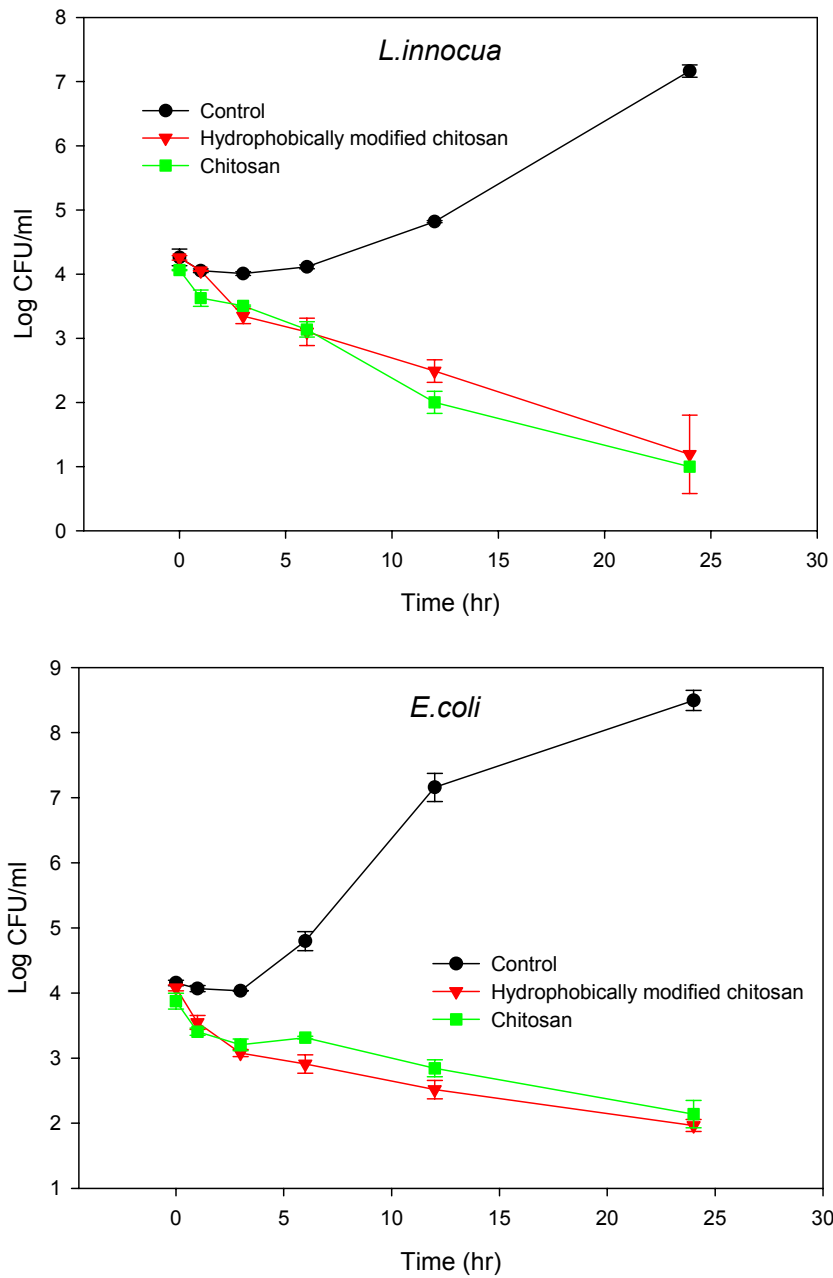


Figure 5-5: Antimicrobial activity of HM-chitosan and chitosan at concentration of 0.1% (pH 5.0).

* Values are represented as mean \pm standard deviation (n=3).

bacteria. The results showed that the hydrophobic modification of chitosan did not reduce its intrinsic antimicrobial activity. Our experiment also confirmed that gram- negative bacteria were more resistant to chitosan and HM-chitosan treatment, as evidenced by a 3 log reduction in the *L. innocu* and only a 2 log reduction in *E. coli* after 24 hours.

5-4.2 Physical, mechanical and antimicrobial properties of the blend film

5-4.2.1 Kinematic viscosity

Kinematic viscosity of chitosan and HM-chitosan blend solutions increased from 17.03 to 33.11, 58.83, 102.19, and 94.44 cSt for blend ratios of 0/100 (chitosan/HM-chitosan), 25/75, 50/50, 75/25 and 100/0, respectively (Figure 5-6). At blending ratio of 75/25, the measured viscosity 102.19 cSt was much higher than expected (around 75 cSt), which suggested a possible synergetic effect as a result of to the interaction of HM-chitosan and chitosan.

5-4.2.2 Film appearance, weight and thickness

Fresh chitosan films were shiny on both surfaces (chitosan-air top surface and chitosan-Petri-dish bottom surface) and were translucent, and homogeneous under the polarized microscope. With the addition of HM-chitosan, the blend films lost translucency, become gradually more opaque, and appeared more heterogeneous under the polarized microscope (Figure 5-7). It appeared that HM-chitosan self-assembled into micro-particles and these micro-particles were embedded inside the chitosan matrix. Additionally, all blend films had a shiny appearance at the bottom surface and a dull appearance at the top surface. Similar appearance on two sides of the film had been found on caseinate-beeswax film(Avena-Bustillos and Krochta, 1993).

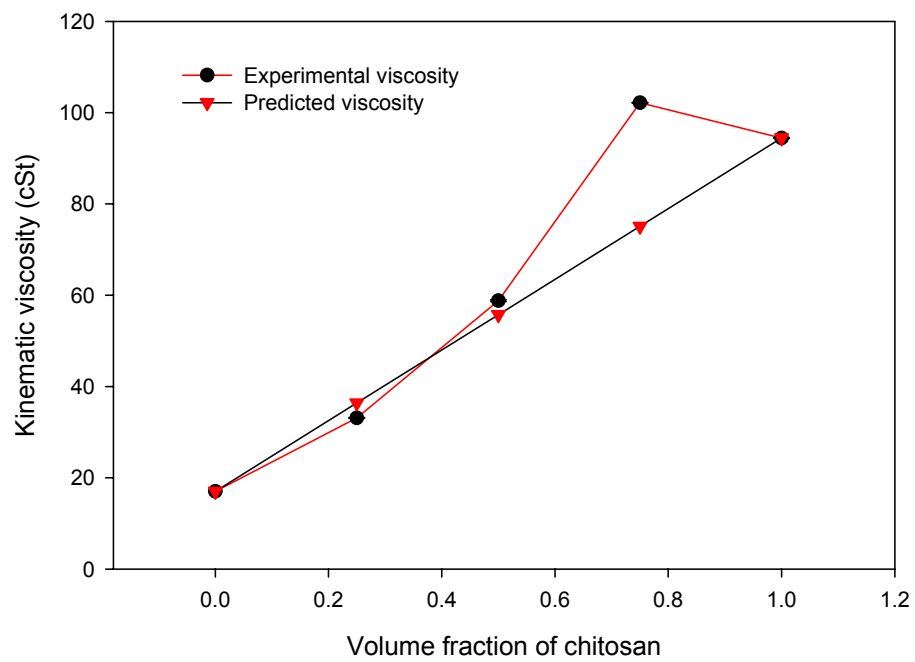


Figure 5-6: Kinematic viscosities of chitosan/HM chitosan blend solutions.

* Values are represented as mean \pm standard deviation (n=3)

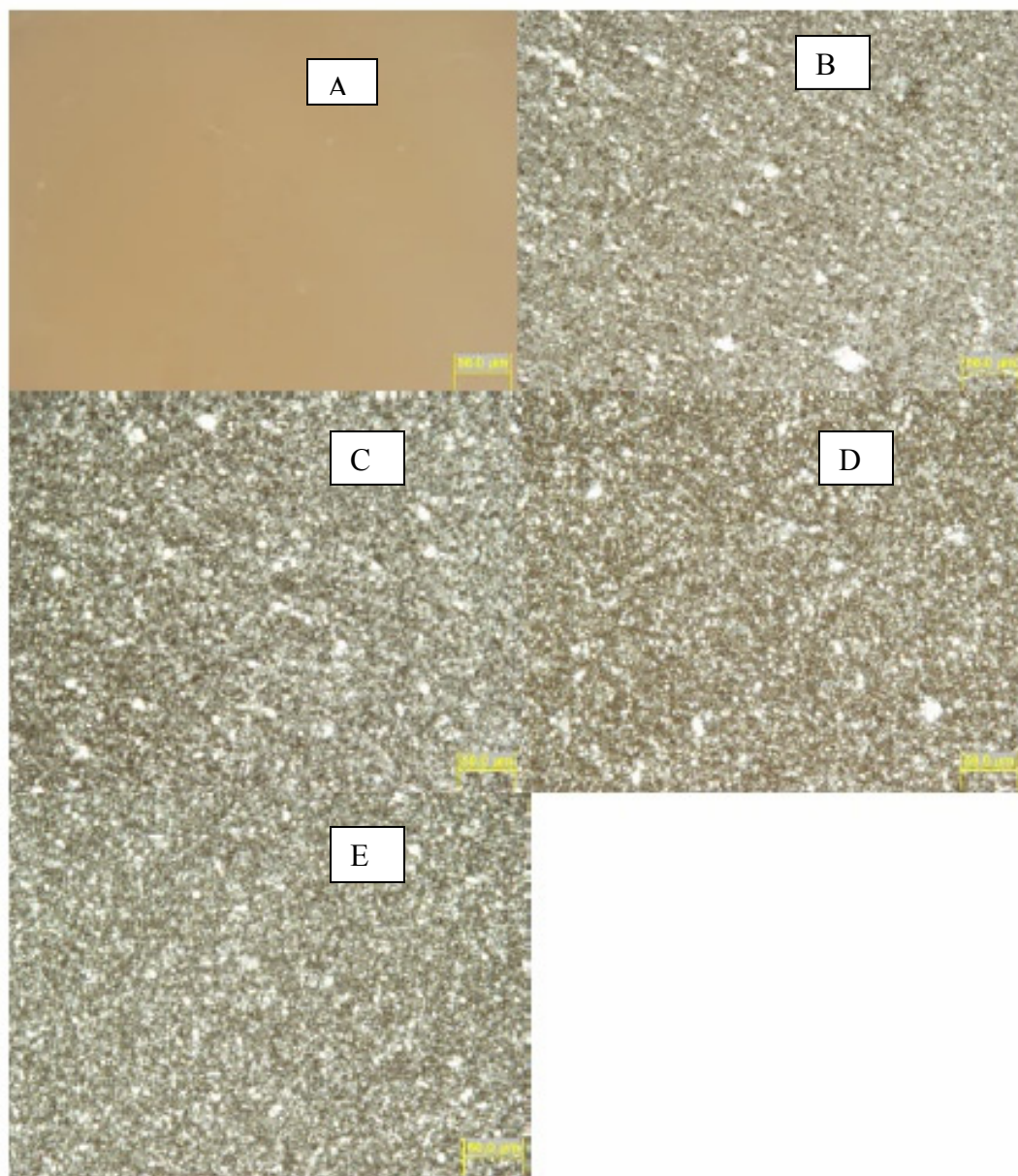


Figure 5-7: Representative images of chitosan blend films under polarized microscope (400X): (A) -100/0 (chitosan/HM-chitosan); (B)-75/25; (C) - 50/50; (D) - 25/75; (E) - 0/100.

The relative film weight (film mass/casting solution mass) gradually decreased from $1.30 \pm 0.02\%$ to $1.26 \pm 0.03\%$, $1.19 \pm 0.03\%$, $1.19 \pm 0.04\%$ and $1.16 \pm 0.02\%$ for 100/0, 75/25, 50/50, 25/75 and 0/100 blend films, respectively (Table 5-1). Since both initial concentrations of HM-chitosan and chitosan solution were fixed at 1%, decreased film weight indicated less amounts of solvent were trapped inside the films. The film thickness decreased from 0.067 ± 0.011 to 0.068 ± 0.015 , 0.061 ± 0.009 , 0.059 ± 0.012 , and 0.065 ± 0.013 mm for 100/0, 75/25, 50/50, 25/75 and 0/100 blend films, respectively (Table 5-1). The change of film thickness did not follow the same trend as that of film weight, which was probably due to the large standard deviation of thickness measurement.

5-4.2.3 Swelling of the films and release of acetic acid

The swelling extent of blend films was presented in Table 5-1. With the increased amount of HM-chitosan in the blend films, the swelling extent significantly decreased from 464.81% to 418.52%, 338.89%, 320.37% and 301.85% for 100/0, 75/25, 50/50, 25/75 and 0/100 blend films, respectively (Table 5-1). However, the release behaviors of acetic acid from 100% chitosan and 100% HM-chitosan films were actually similar (Figure 5-8). For both films, the trapped acetic acid was quickly released during the first 5 minutes. This was in agreement with a previous report that acids were always completely released from chitosan matrix in 5 to 10 minutes (Ouattara and others 2000). The total amount of acetic acid trapped inside the chitosan and HM-chitosan were significantly different, which were 16% and 10%, respectively. This result confirmed the previous conclusion from film weight analysis.

Table 5-1: Relative weight, thickness and extent of swelling of the blend films.

Volume fraction	Relative Film weight (%)	Thickness (mm)	Swelling (%)
100:0	1.30 ± 0.02 ^A	0.067 ± 0.011 ^A	464.81 ± 22.74 ^A
75:25	1.26 ± 0.03 ^B	0.068 ± 0.015 ^A	418.52 ± 28.19 ^B
50:50	1.19 ± 0.03 ^C	0.061 ± 0.009 ^{BC}	338.89 ± 8.33 ^C
25:75	1.19 ± 0.04 ^C	0.059 ± 0.012 ^C	320.37 ± 7.35 ^D
0:100	1.16 ± 0.02 ^D	0.065 ± 0.013 ^{BA}	301.85 ± 10.02 ^E

* Values are represented as mean ± standard deviation (n=9).

** Any two means in the same column followed by the same letter are not significantly different (P > 0.05) by Duncan's test.

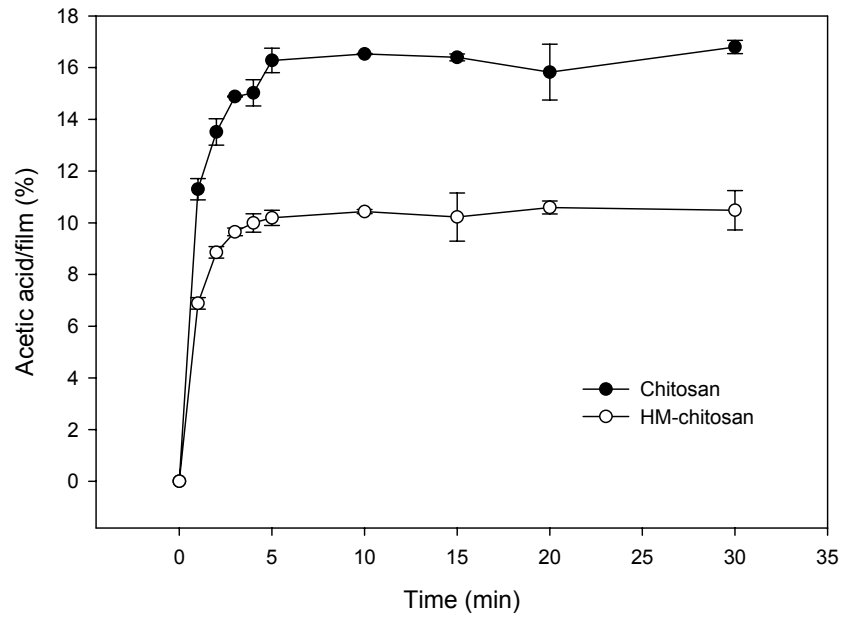


Figure 5-8: Release behaviors of acetic acid from chitosan and HM-chitosan films.

* Values are represented as mean \pm standard deviation (n=3).

5-4.2.4 Surface characteristic of blend films were investigated by FTIR-ATR and AFM

The infrared spectra of blend films at the top surface (chitosan-air surface) and bottom surface (chitosan-Petri dish surface) were scanned and compared in Figure 5-9. Pure chitosan film had equivalent infrared spectra fingerprint at both top and bottom surfaces (Figure 5-9-A), whereas for blend films, all bottom surfaces showed higher infrared absorbance than top surface (Figure 5-9 B-E).

AFM showed that pure chitosan films had similar features on both surfaces (Figure 5-10 A-B), whereas HM-chitosan had different features on the two surfaces (Figure 5-10 C-E). Pure chitosan films were rather smooth and had similar roughness, average height and maximum height for both surfaces (Table 5-2). The presence of many tiny bumps was observed on both surfaces (Figure 5-10 A-B). HM-chitosan films were apparently rougher and had significantly different surface features at the bottom and top surfaces (Table 5-2). In a small scan area ($1\ \mu\text{m} \times 1\ \mu\text{m}$), the bottom surface still had many tiny bumps (Figure 5-10-C), while the bump size was larger than that observed in pure chitosan surface. However, very large bumps presented at the top surface (Figure 5-10-D). On a large scan area ($10\ \mu\text{m} \times 10\ \mu\text{m}$), much larger bumps were observed (Figure 5-10-E). The film roughness, average height and maximum height further increased to 200.24 nm, 750.87 and 1722.92 nm, respectively (Table 5-2). It appeared that HM-chitosan particles aggregated into large particles on the top surface. Size dependent aggregation of latex particle during film preparation was investigated by several studies (Vorobyova and Winnik, 2001; Rakers and others 1997). During film drying, the

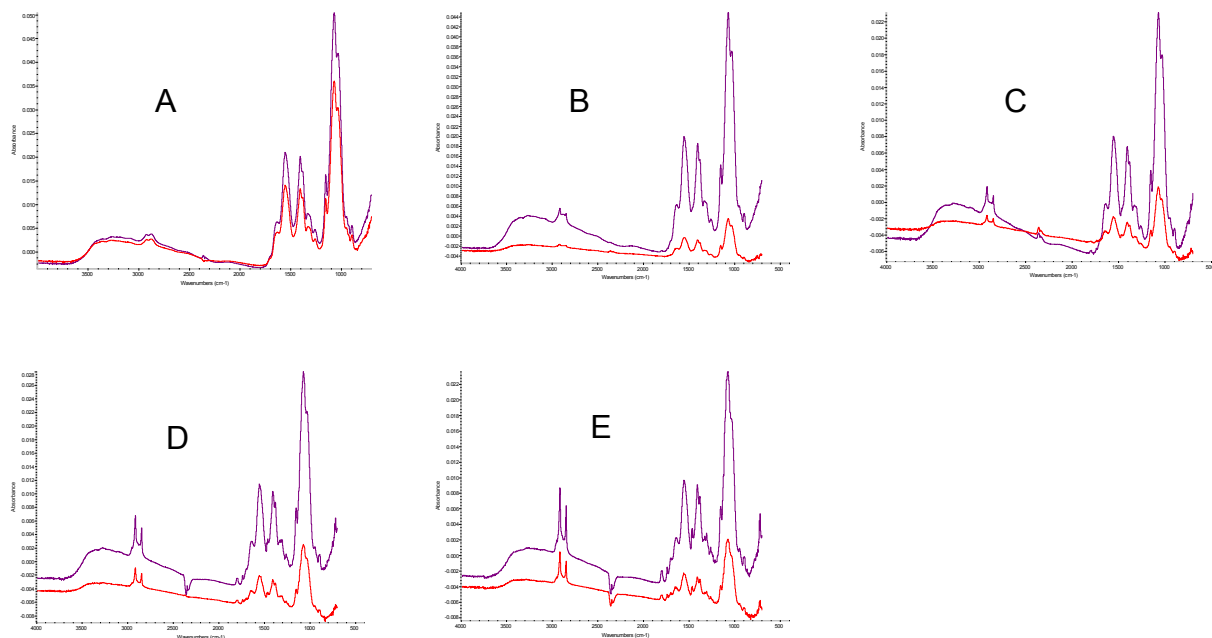


Figure 5-9: FTIR spectra of blend films: (A) -100/0 (chitosan/HM-chitosan); (B) – 75/25; (C)– 50/50; (D) – 25/75; (E)– 0/100; solid line-top surface; dotted line – bottom surface.

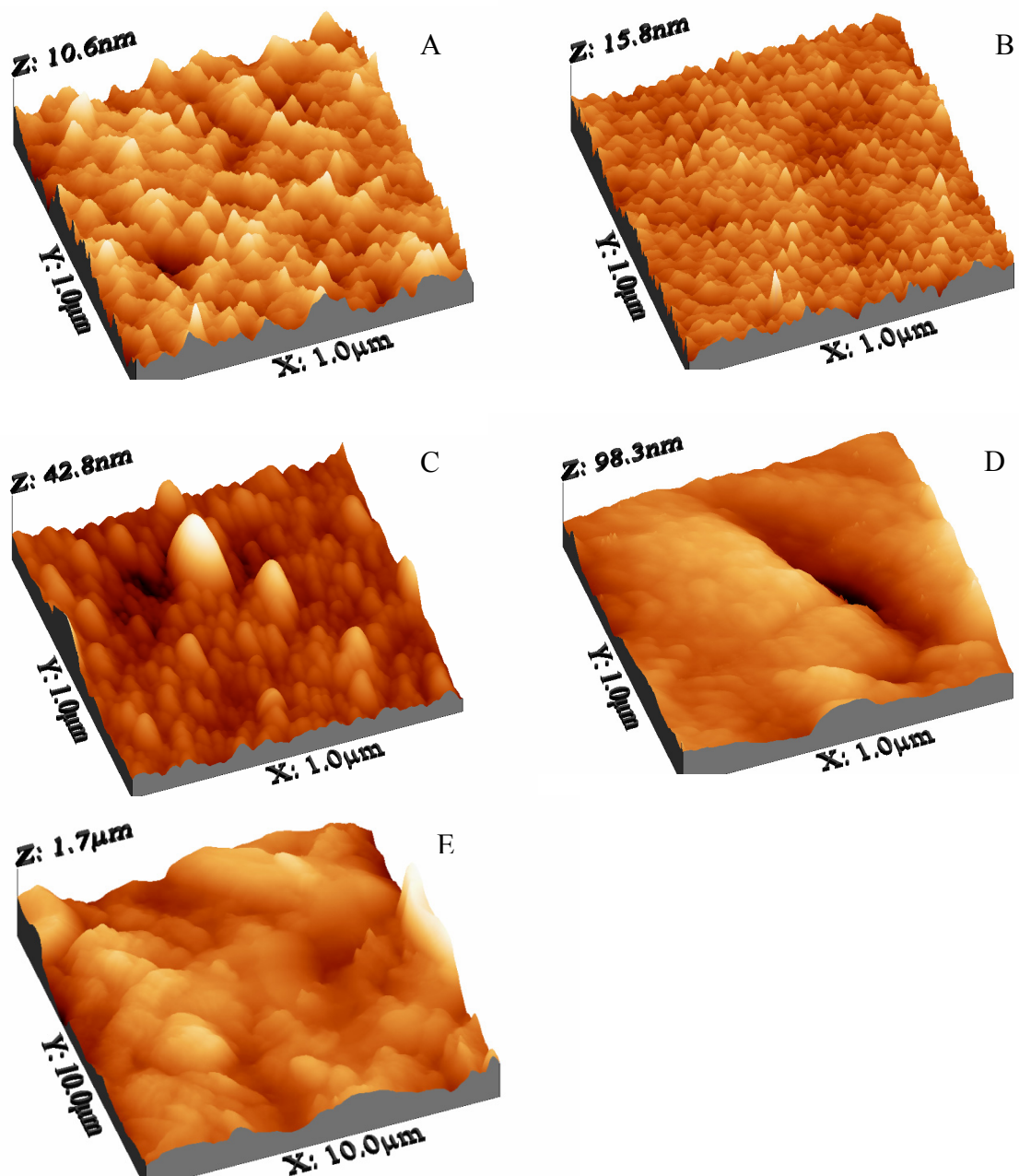


Figure 5-10: Representative AFM images of pure chitosan films: (A) - bottom surface; (B) – top surface and HM-chitosan films: (C) - bottom surface; (D) – top surface-1; (E)-top surface-2. (Z axis height represents the maximum height of the surface feature)

Table 5-2: Surface roughness, average height and maximum height of chitosan films imaged by atomic force microscopy (AFM)

Volume fraction		Surface Roughness (Xie et al.)	Average Height (Xie et al.)	Maximum Height (Xie et al.)
100/0	bottom	1.35 ± 0.07	6.13 ± 0.61	12.96 ± 2.50
	top	1.52 ± 0.25	6.56 ± 2.37	13.77 ± 2.51
0/100	bottom	5.24 ± 2.09	14.39 ± 2.89	42.69 ± 9.55
	top	9.25 ± 3.29	31.55 ± 11.05	80.33 ± 16.14

* Values are represented as mean ± standard deviation (n=3).

lateral capillary forces acts on the largest particles to form aggregates, which behaviors as nuclei for further aggregation. During further drying, the smaller particles starts to move toward the already existing aggregates to form even larger aggregates, or starts to form new aggregates(Rakers et al., 1997). This aggregation phenomenon was not distinguished on the bottom surface, which was probably because that the particle movement at film-Petri dish surface was hindered.

Combining the AFM results and obtained FTIR fingerprints, we proposed a hypothesis to explain the relationship between film appearance, infrared absorbance intensity and surface morphology. A germanium crystal was used in our FTIR-ATR instrument and the penetration depth of IR evanescent wave was about 0.4-0.664 μm (manufacturer data). The aggregation of HM-chitosan particles forms large bumps with average height of 750.87 nm and void space would exist in the path of IR evanescent wave. Since the infrared absorbance A also obeys Beer-Lambert law $A = \epsilon \times b \times c$, where ϵ is the molar extinction coefficient; b is the path length of the sample and c is the concentration of the compound, more void space at top surface consequently resulted in a lower infrared absorbance intensity and also resulted in the dull appearance.

5-4.2.5 Water vapor permeability (WVP)

The water vapor permeance provides an objective value to compare barrier properties of different films, whereas the WVP of hydrophilic films depends on the measurement condition and thickness(McHugh and others 1993; Avena-Bustillos and Krochta, 1993). The comparison of water vapor permeance or WVP of films with bottom or top surface facing the high RH environment showed that film orientation actually had no effect on the measured water vapor permeance and WVP (Figure 5-11). A previous

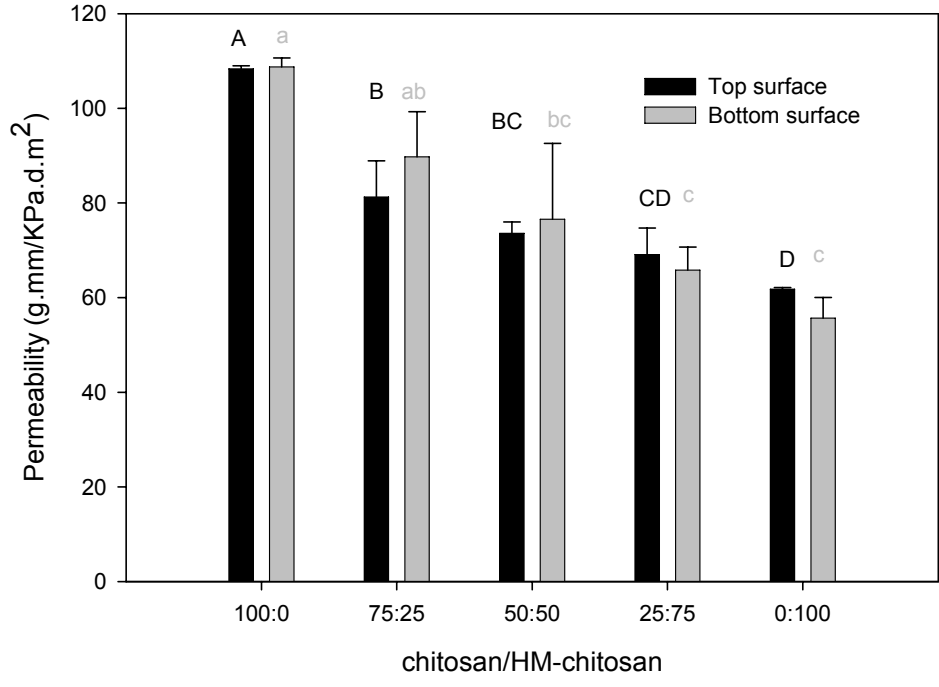
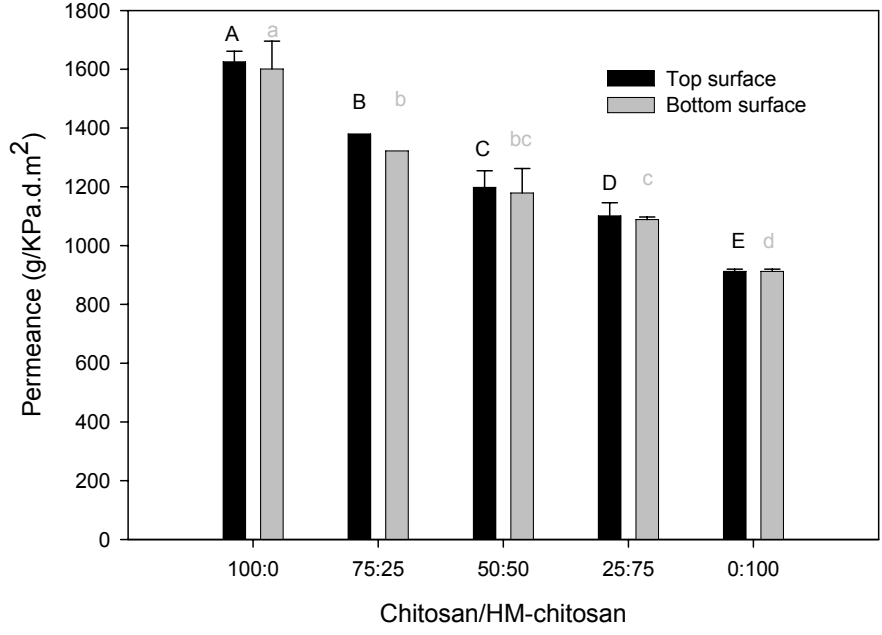


Figure 5-11: Water vapor permeance and permeability of blend films.

* Values are represented as mean ± standard deviation (n=3).

** Any two means in the same surface followed by the same letter are not significantly different (P > 0.05) by Duncan's test.

study of caseinate/beewax film showed around 50% reduction in WVP was obtained with the shiny surface facing the high RH environment(Avena-Bustillos and Krochta, 1993). It was hypothesized that lipid and caseinate were phase separated and concentrated in the dull and shiny side of the film, respectively(Avena-Bustillos and Krochta, 1993). This difference in constituent orientation resulted in a swelling difference that could affect the barrier properties of the film(Avena-Bustillos and Krochta, 1993). In our case, the dull and shiny appearance was probably caused by the aggregation of HM-particles, not the phase separation and thus no difference in WVP was observed on films based on orientation of the films during analysis. Therefore, the rest of the comparison was made using WVP data of films with bottom surface facing the high RH environment. As can be seen from Figure 5-11, with the increased amount of HM-chitosan in the blend films, the water vapor permeance decreased from the initial 1651 to 1380, 1237, 1132 and 917 $\text{g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}\cdot\text{KPa}^{-1}$ for 25, 50, 75 and 100% HM-chitosan, respectively and the maximum reduction was 44%, obtained at 100% HM-chitosan film. Thus blending with HM-chitosan effectively reduced the water vapor permeability of chitosan films, even the hydrophobic chain was palmitoyl group. The improved barrier properties could be attributed to the presence of large amount of HM-barrier particles embedded inside the films resulting in a slow mass transfer process.

The WVP value of pure chitosan film was measured as 108.7 $\text{g}\cdot\text{mm} / \text{m}^{-2}\cdot\text{d}^{-1}\cdot\text{KPa}^{-1}$, which was in the range from 48.2 to 456 $\text{g}\cdot\text{mm} / \text{m}^{-2}\cdot\text{d}^{-1}\cdot\text{KPa}^{-1}$ reported by several other researchers under similar measurement conditions (Zivanovic and others 2007; Park and others 2005; Sebti and others 2005; Kim and others 2006). With the increase amount of HM-chitosan, the value decreased by 17.4, 29.5, 39.4 and 48.8% compared to the original

value for films with addition of 25, 50, 75 and 100% HM-chitosan. The WVP value ranged from 17.3 to 86.4, 71.1, 43.2 and 189.6 to 309.6 $\text{g}\cdot\text{mm} / \text{m}^2\cdot\text{d}^{-1}\cdot\text{KPa}^{-1}$ for high amylose corn starch film(Bertuzzi and others 2007), soy protein film(Cho and Rhee, 2004), β -casein film(Mauer and others 2000) and caseinate film(Banerjee and Chen, 1995), respectively. Therefore, 100% HM-chitosan film can be still considered as hydrophilic with WVP value comparable to those common polysaccharide and protein films.

5-4.2.6 Mechanical properties

Results of the mechanical tests, for puncture strength (PS), tensile strength and elongation at break (%E) for films with different chitosan/HM chitosan ratios are shown in Table 5-3. With the addition of HM-chitosan, the PS, TS and %E of blends films decreased in a non-linear fashion. Even at the ratio of 75/25, where synergetic effect between chitosan and HM-chitosan was observed in the solution, all measured mechanical properties did not show improvement. It is possible that interaction between chitosan and HM-chitosan was actually destroyed during film drying process and resulted in decrease of the mechanical properties.

5-4.2.7 Antimicrobial activity of the films

As shown in Table 5-4, the HM-chitosan and chitosan films had comparable antimicrobial activity against *E. coli* K12. This result was similar with the previous test results with HM-chitosan and chitosan solution. However, most of the HM-chitosan and chitosan films dissolved in the buffer and no firm conclusions could be drawn on the effects of film structure and morphology on the antimicrobial activity.

Table 5-3: Puncture strength (PS), tensile strength (TS) and elongation at break (%E) of the chitosan/HM-chitosan films with volume ratio from 100:0 to 0:100.

Volume fraction	PS(N/mm)	TS (MPa)	%E
100:0	468.25 ± 44.93 ^a	113.19 ± 10.34 ^a	7.29 ± 0.93 ^a
75:25	361.03 ± 50.88 ^b	98.135 ± 3.836 ^b	7.40 ± 0.83 ^a
50:50	219.14 ± 23.90 ^c	65.023 ± 11.69 ^c	4.41 ± 1.09 ^b
25:75	165.37 ± 8.33 ^{cd}	65.018 ± 7.037 ^c	4.83 ± 0.76 ^b
0:100	135.33 ± 26.95 ^d	48.657 ± 10.94 ^d	3.61 ± 2.27 ^b

* Values are represented as mean ± standard deviation (n=3).

** Any two means in the same column followed by the same letter are not significantly different (P > 0.05) by Duncan's test.

Table 5-4: Reduction of *E. coli* K12 (log CFU/ml) by 0.2% chitosan films

	Control	Chitosan film	HM-chitosan film
0 hour	4.28 ± 0.04	4.23 ± 0.01	4.08 ± 0.11
24 hour	6.70 ± 0.09	<1	<1
Reduction		>3.23	>3.08

* Values are represented as mean ± standard deviation (n=3)

5-5 Conclusions

The hydrophobic modification altered the solubility and thermal properties of chitosan, but had no effects on the antimicrobial activity against tested organisms. New functionalities, such as self aggregation and thermal responsive were introduced. The addition of HM-chitosan into chitosan films resulted in a significant change of film appearance, internal structure and surface morphology. The water vapor permeability was successfully reduced, but no improvement in mechanical properties was achieved.

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6 Overall Conclusions

The current 1st derivative UV method for degree of acetylation (DA) determination was successfully improved in the first part of this research. Using the conc. (85%) phosphoric acid as a solvent, applying heat treatment at 60°C for 40 min to enhance solubilization, and incubation of diluted solutions at 60°C for 2 hrs, the DA of both chitin and chitosan can be analyzed by a single method in a routine analytical lab in less than 3 hrs without the use of expensive equipment. The method improvement is based on the thorough exploitation of the chemical reactions associated with the use of concentrated phosphoric acid as a solvent for highly acetylated chitin. Compared to previous methods, this method is quick, easy to perform and can be easily accepted as a low cost industrial standard method for routine analysis of DA.

Results from the second and third parts of this research indicate that the high intensity ultrasound treatment can be used in the degradation process of chitosan, rather than the deacetylation process of chitin. Our study showed that application of short high intensity ultrasound (HIU) pretreatment (<30 min) did not change the DA values of sonicated chitin and resulting chitosan. At the same time, reduction of chitin particle size from form of flakes to powder effectively increased the efficiency of deacetylation due to the increased surface area, but decreased the yield. Our study also showed that the molecular weight, radius of gyration and polydispersity of chitosan were efficiently reduced by ultrasound treatment while molecular conformation and degree of deacetylation did not change after sonication. The degradation of chitosan by ultrasound is primarily driven by mechanical forces and degradation mechanism can be described by a random scission model. The degradation rate is proportional to M_w^3 and the degradation

rate coefficients are significantly influenced by ultrasound intensity, solution temperature, polymer concentration and ionic strength while acid concentration has little effect. Additionally, the degradation rate coefficients are affected by the degree of acetylation of chitosan and independent of the initial molecular weight. The research findings provide important guideline for the industrial scale up of chitosan ultrasonic degradation process. Future work in this area may be directed to develop a continuous process for the ultrasonic degradation food industrial important polysaccharides. In addition, with the precise control of chitosan molecular weight by the ultrasonic process, fundamental studies may be performed to investigate or re-examine the relationship between chitosan molecular weight and its functional properties.

In the four part of this research, we developed a new way of introducing desirable functionality to chitosan, which can greatly boost the application of chitosan in the food industry as antimicrobial agent and active packaging component. As shown in our study, the hydrophobic modification altered the solubility and thermal properties of chitosan, but had no effects on the antimicrobial activity against tested organisms. New functionalities, such as self aggregation and thermal responsive properties were introduced by the modification. The addition of HM-chitosan into chitosan films resulted in a significant change of film appearance, internal structure and surface morphology. The water vapor permeability was successfully reduced, but no improvement in mechanical properties was achieved. Future studies can be directed to increase the mechanical properties of HM-chitosan films, while maintaining the desired water vapor permeability.

Due to the thermal-responsive property, if hydrophobically modified chitosan (HM-chitosan) is used alone or incorporated into packaging films, it could be expected that the structure and permeability of such films would undergo a significant change above a specific temperature, which may be controlled by the type and number of grafted groups. Therefore, such packaging films could be used as temperature switchable breathable membranes for fresh produce. Similar product, such as BreatheWay membrane has already been found in the market. The introduced thermal responsive property may also be very useful in creating new controlled release system for active food ingredient triggered by temperature change. Without this property, the active compound can only be released from those self assembled nanoparticle formed by HM-chitosan through molecular diffusion.

The thermal-responsive and self-assembled properties of hydrophobically modified chitosan may find applications in the areas of temperature sensitive packaging and encapsulation of food additive. The decreased water vapor permeability of HM-chitosan film may find niche application in the shelf life extension for high moisture food products.

Vita

Tao Wu was born in Wuhan, Hubei, China on January 15, 1975. He went to Huazhong Agricultural University in September of 1994. There he received a bachelor's of Agronomy degree in Plant Protection in June 1998.

After graduation from Huazhong Agricultural University, he had been working as an analytical chemist assistant in Hubei Entry – Exit Inspection and Quarantine Bureau for four years.

In August of 2002, he entered University of Tennessee, Knoxville to pursue advanced degrees in Food Science and Technology. Tao received his Master of Science degree in August 2004 working on a project entitled production and characterization of fungal chitin and chitosan under the direction of Dr. Svetlana Zivanovic. In August 2004, he began to work on his Ph.D. project sonochemical and hydrophobic modification of chitin and chitosan under the direction of Dr. Svetlana Zivanovic. Upon the completion of his degree, Tao Wu is going to work as a post-doctoral research associate at University of Tennessee and hopes to accept a faculty position in a leading research and teaching university in future.