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Molecular Weight and Degree of Acetylation of Ultrasonicated Chitosan

Shari Rene Baxter
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To the Graduate Council:

I am submitting herewith a thesis written by Shari Rene Baxter entitled "Molecular Weight and Degree of Acetylation of Ultrasonicated Chitosan." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

Svetlana Zivanovic, Major Professor

We have read this thesis and recommend its acceptance:

Jochen Weiss, John Mount

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Molecular Weight and Degree of Acetylation of Ultrasonicated Chitosan

A Thesis presented for the
Masters of Science degree
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Abstract

Chitosan is a glucosamine polymer produced by deacetylation of chitin from crustacean shells. The functional properties of chitosan, such as thickening, film-formation and antimicrobial activity, are related to its molecular weight and degree of acetylation (DA). High intensity ultrasonication has the potential to modify molecular weight of chitosan and thus alter or improve chitosan functional properties. The objective of this research was to determine the DA and molecular weight of chitosan molecules as a function of sonication intensity and treatment time.

High molecular weight shrimp chitosan was purified by alkaline precipitation and dialysis from aqueous solution. A 1 % (w/v) chitosan in 1 % (v/v) aqueous acetic acid was sonicated for 0, 1, 2, 10, 30, and 60 minutes at 25 °C. A Misonix 3000 ultrasonic homogenizer was used to sonicate 50 mL samples at power levels of 16.5, 28, and 35.2 W/cm^2 with pulsed output (1 s sonication, 1 s break). The DA was determined by high performance liquid chromatography with photodiode array detector (HPLC-PDA), monitoring acetyl groups released after complete hydrolysis and deacetylation of the samples and by Fourier Transform InfraRed Spectroscopy with Attenuated Total Reflection (FTIR-ATR). Molecular weight was determined by measuring the intrinsic viscosity of sonicated solutions.

The DA of purified chitosan was 21.5 %. Results indicated that neither power intensity nor sonication time deacetylated the chitosan molecules.

However, intrinsic viscosity of samples decreased exponentially with increasing sonication time. Reduction rates of intrinsic viscosity increased linearly with ultrasonic intensity. A first order kinetic reaction model of molecular weight decay as a function of sonication time was suggested and an Arrhenius-type relationship for the dependence of the reaction rate on the ultrasonic intensity was developed. Our results confirm the hypothesis that high intensity ultrasonication can be utilized to reduce molecular weight of chitosan while not reducing the degree of acetylation.

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1. Literature Review

1.1. Introduction

Chitin, an acetylated acetylglucosamine polymer, is the second most abundant polysaccharide in nature (Shahidi, Arachchi, & Jeon, 1999). Chitin is found in the exoskeleton of crustaceans, insects' cuticles, and fungal cell walls. Current procedures for chitin extraction involve harsh acid and base treatments to demineralize and deproteinize shrimp and crab shells. In order to produce chitosan, chitin is further deacetylated, usually with 10 N NaOH at 100 – 120 °C for several hours. However, the harsh treatments may influence the molecular weight and viscosity of the final chitosan product (Varum, Ottoy, & Smidsrod, 2001).

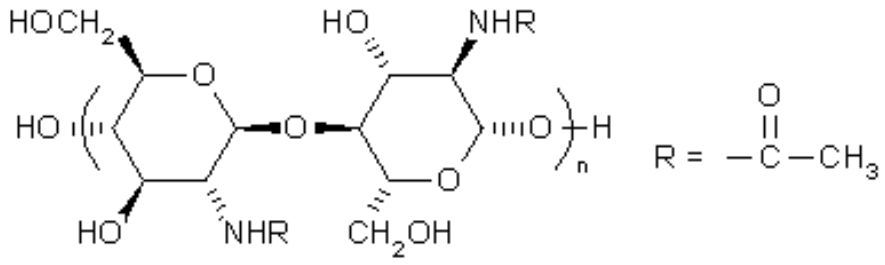
Chitin and chitosan are biodegradable, nontoxic compounds with multiple applications in the food, agricultural, pharmaceutical and chemistry industry. Current uses of chitin and chitosan include wastewater treatment, cosmetics, paper and textiles, biomedicine, seed treatment, antimicrobials, and formation of biodegradable films (Shahidi et al., 1999). The physical properties of the chitin and chitosan affect the potential uses. For instance, low molecular weight chitosan has low viscosity which limits its application. Also, oligomers of chitosan do not form films. Furthermore, the antimicrobial affect of chitosan is stronger if the molecular weight is greater than 100 kDa and has high degree of deacetylation (No, Park, Lee, & Meyers, 2002).

High intensity ultrasound is a novel technology that has the potential to assist in the extraction and production of chitosan. Through compressional and shear waves at large intensities and consequent cavitation of microscopic bubbles, ultrasound has the potential to be used in chitosan modifications, allowing more control over the product properties while creating a more environmentally friendly process.

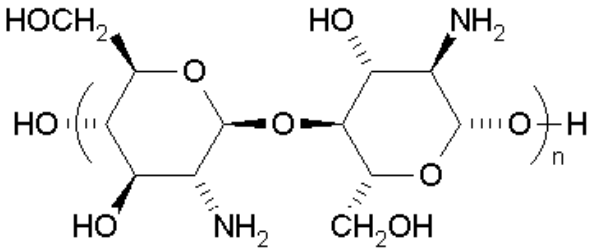
1.2. Molecular Properties of Chitosan

Chitosan has a chemical structure of 2-acetamido-2-deoxy-β-D-glucose monomers attached via β (1→4) linkages (Figure 1). The chemical characteristics of chitosan may be varied as required for a particular application; with the most important being the degree of acetylation (DA) or degree of deacetylation (DDA) and the molecular weight (Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003).

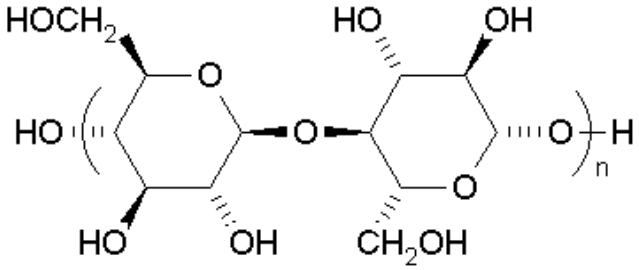
Dependent upon source, there are three main packing arrangements of chitin molecules: α-chitin (anti-parallel arrangement), β-chitin (parallel arrangement) and γ-chitin (mixed arrangement – two chains parallel for each chain anti-parallel). The most stable and most abundant form found in nature is α-chitin (Muzzarelli, 1977). The packing arrangement of chitin will affect the crystallinity of the produced chitosan and the degree of acetylation (Jaworska, Sakurai, Gaudon, & Guibal, 2003). Intensity of crystallization and the degree of acetylation in turn may have significant effects on chitosan functional properties,



Chitin



Chitosan



Cellulose

Figure 1: Chemical structure of chitin, chitosan and cellulose.

such as antimicrobial activity, viscosity, and gel and fiber formation (Jaworska et al., 2003).

Chitin and chitosan differ in the DA of the molecule. Generally, chitin has a DA of greater than 70 %. High levels of acetyl groups and extensive crystallization make chitin insoluble in water and common solvents. Most commercial chitosans have a DA of less than 30 % and are soluble in aqueous acidic solvents. Interestingly, molecules with equal fractions of acetylated and nonacetylated glucosamine monomers are easily soluble in water (Muzzarelli, 1977). Commercial chitosan typically has a maximum molecular weight in the range of 100 to 800 kDa. The chemical structure differences of chitin, chitosan and cellulose can be seen in Figure 1.

1.2.1. Sources of Chitosan

The biological origin of chitin that is deacetylated into chitosan strongly affects the molecular properties of the chitosan. The current main commercial sources of chitosan are shrimp and crab shells. Shrimp and crab shell waste has a production of approximately 10^9 – 10^{10} tons of waste per year worldwide (Peter, 1995). Methods of extracting chitin from fungal sources have the potential of commercial application (Muzzarelli, 1977).

1.2.2. Extraction of Chitin

Commercial production of chitin involves the use of harsh acids and bases at high temperatures for long periods of time. Shrimp and crab shells contain 17

– 32 % chitin, 17 – 42 % protein, 1 – 14 % pigments, and 41 – 46 % ash, mainly calcium (Shahidi and Synowiecki, 1991). The process begins with drying and grinding of the shells and is followed by two main steps: demineralization and deproteinization. Demineralization generally involves the use of acids including but not limited to: hydrochloric acid, nitric acid, sulfuric acid, acetic acid, and formic acid with hydrochloric acid being the preferred on a commercial scale. The typical concentration is between 0.275 and 2 M for 1 to 48 hours and temperatures ranging from 0 to 100 °C (Roberts, 1992). Deproteinization of chitin generally involves the use of an alkaline treatment. Demineralized material is treated with 1 M aqueous solutions of NaOH for 1 to 72 hours at temperatures ranging from 65 to 100 °C (Roberts, 1992).

Percot, Viton, and Domard (2003) optimized the extraction of chitin from shrimp shells, specifically, with the objective of creating a higher quality chitin with the highest molecular weight possible and the lowest amount of deacetylation. Acidic conditions applied for demineralization may cause depolymerization, whereas the deproteinization process with alkaline treatment can lead to a lower degree of acetylation. The authors optimized the demineralization process using 0.25 M HCl at a solid-to-solvent ratio of 1/40 (w/v) and a reaction time of 15 minutes which successfully removed acetyl groups and yielded higher molecular weight chitin. The use of 1 M NaOH with a solid-to-solvent ratio of 1/15 (w/v) at temperatures ranging from ambient temperature to 70 °C did not affect the degree of acetylation. However, when deproteinization

was conducted at temperatures above 70 °C, the rate of deacetylation of the chitin increased.

1.2.3. Methods of Deacetylation of Chitin to Chitosan

Deacetylation of chitosan can take place in one of two ways depending on the processing conditions. Homogeneous deacetylation creates a random distribution of acetyl groups along the polymer while heterogeneous deacetylation creates a block distribution of acetyl groups. Traditional means of deacetylation are heterogeneous and are carried out with 10 N or higher sodium or potassium hydroxide at 100 – 150 °C for several hours (Muzzarelli, 1977; No and Mayers, 1997). Under strong alkali conditions, the high temperatures lead to hydrolysis of glycosidic bonds. To avoid depolymerization, chitin is deacetylated at 30 – 60 °C for 20 to 144 hours while keeping the alkali concentration at 45 % (Alimuniar & Zainuddin, 1992).

Alternative methods of deacetylation have been investigated. Deacetylation of chitin by pressure of 15 psi in 45 % sodium hydroxide for 30 min resulted in chitosan with a degree of deacetylation of 90.4 % with a higher viscosity compared to conventional methods (No, Cho, Kim & Meyers, 2000).

Another alternative method was developed through homogeneous deacetylation (Nemtsev, Gamzazade, Rogozhim, Bykova, & Bykov, 2002). Dry or thawed chitin was mechanically disintegrated and suspended in a 13 – 24 % NaOH aqueous solution at a concentration of 1 – 10 %. The alkaline suspension of chitin was frozen in a cryostat and thawed at room temperature. Chitin

underwent pronounced swelling and formed an alkaline solution. For deacetylation, the alkaline chitosan solution was kept at room temperature or mildly heated. The solution lost its fluidity and formed a gel. This gel was mechanically disintegrated into 3 – 5 mm particles and washed with distilled water to remove alkali. Chitin was therefore converted to chitosan, which was dried at 50 – 55 °C. Deacetylation under homogenous conditions allowed for compounds with specific DA's while retaining high molecular weight characteristics and the ability to control the process through temperature and temporal factors (Nemtsev et al., 2002).

However, the common methods used for deacetylation cause limited hydrolysis of the chitosan molecule. A commercial chitosan with a DDA 75 % in powder form had lower molecular mass than that of the original chitin, indicating that depolymerization occurred to some extent during the manufacturing process for preparing chitosan (Hasegawa, Isogai, & Onabe, 1994).

Varum, Ottoy, and Smidsrod (2001) found that using concentrated sulfuric acid for hydrolysis, the rate of hydrolysis is more than 10 times higher than the rate of deacetylation. Furthermore, the extensively deacetylated chitosans were hydrolyzed at a lower rate by acid compared to the more acetylated chitosans (Varum et al., 2001).

1.3. Determination of Physicochemical Properties of Chitosan

Characteristics of commercially produced chitosan are highly variable with regard to physicochemical properties. The properties discussed here, degree of

acetylation and molecular weight, are dependent on the extraction and processing methods used in obtaining chitosan.

1.3.1. Degree of Acetylation

Numerous methods have been proposed for determining the DA of chitin and chitosan. Published research has explored the use of HPLC-PDA (Niola, Basora, Chornet, & Vidal, 1993), IR spectroscopy (Duarte, Ferreira, Marvao, & Rocha, 2002; Neugebauer, 1989; Rathke & Hudson, 1993; Shigemasa, Matsura, Sashiwa, & Saimoto, 1996), conductimetric titration (Li, Revol, & Marchessault, 1997a), NMR (Kasaai, Charlet, & Arul, 2000a; Li et al., 1997a; Signini, Desbrieres, & Campana Filho, 2000), and UV spectroscopy (Pedroni, Gschaider, & Schulz, 2003). Each published method has presented advantages and disadvantages regarding the sample preparation, accuracy, and reproducibility. Generally, the biggest challenge in method development presents achieving uniform accuracy in the entire range of DA from 0 % being fully deacetylated chitosan and 100 % being fully acetylated chitin.

Acid hydrolysis of chitosan, *e.g.* with sulfuric and oxalic acid, liberates acetyl groups from the chitosan or chitin molecule. The acetic acid produced can then be determined through the use of high performance liquid chromatography (HPLC) with a spectrophotometric or photodiode array detector (PDA). The method proposed by Niola, Basora, Chornet and Vidal (1993) is based on the hydrolytic reaction. The method is advantageous because of its simplicity but shows little reproducibility and is not accurate for molecules with lower levels of

acetylation. Furthermore, the limited accessibility of acetyl groups present in highly crystallized chitin towards oxalic and sulfuric acid was assumed to be the cause of underestimation of the DA in chitin (Niola et al., 1993).

The most widely used method for the determination of the DA is based on Fourier Transformation InfraRed Spectroscopy (FTIR). Several papers have focused on optimization of the methods and peak areas used in the calculation. In the study by Duarte, Ferreira, Marvao, and Rocha (2002), FTIR was used to determine the DA of standards with a wide range of DA and the results were correlated with those obtained by Nuclear Magnetic Resonance Spectroscopy (NMR). Shigemasa, Matsura, Sashiwa, and Saimoto (1996) compared several published FTIR methods and determined that only few produce accurate values over the entire range of DA, from 0 to 100 %. Advantages of FTIR include simple sample preparation and recovery of sample after analysis, while variability due to impurities and environmental factors present the major disadvantages. Furthermore, commonly used as a reference, the peak at 3450 cm^{-1} varies in intensity due to the effect of adsorbed water (Domszy & Robers, 1985).

Near infrared spectroscopy (NIR) has also been investigated as a method for the determination of the DA (Rathke & Hudson, 1993). NIR has been found to be valid from 40 – 100 % *N*-deactylation (DDA) but had low accuracy for chitin samples (Rathke & Hudson, 1993).

The traditional method for determination of the degree of acetylation is the use of titration with picric acid. The method has been shown to be reliable for a large spectrum of substrates, relatively fast, simple and less expensive than

other methods available (Neugebauer, 1989). The advantage of the titration method is the simplicity but disadvantages are the lengthy process and high variability.

Nuclear Magnetic Resonance Spectroscopy provides the average amino group content of the sample which directly correlates to the DA (Li et al 1997a). Typically, NMR is used as the reference method to which other methods are compared. However, although it appears that NMR provides an accurate measurement of DA, high cost of equipment limits its use.

Pedroni, Gachaider, and Schulz (2003) successfully used ultraviolet (UV) spectroscopy to accurately determine the DA of chitosan. Measuring the spectra of prepared samples at 201 nm, UV spectroscopy provides a simple and rapid technique. Problems with the method are that both chitosan and *N*-acetylglucosamine show unique absorbance peaks close to that of acetic or hydrochloric acid, traditionally used as solvents (Pedroni et al., 2003).

It should be kept in mind that the variability of the data obtained by different authors may not be due to the method applied. As a biological polymer, chitosan is highly variable firstly because of the nature of its parent molecule, chitin, but also due to the applied extraction method and deacetylation process.

1.3.2. Molecular Weight

Molecular weight directly impacts the functionality of chitosan in all applications. Several methods have been employed to determine the molecular weight of both chitin and chitosan. Molecular weight is important in the solubility

of chitosan since longer chains are less soluble than shorter chains. Current published methods include size exclusion chromatography (Kasaai et al., 2000a; Mislovičová, Masárová, Bendžálová, Šoltés, & Machová, 2000), multiple angle light scattering (Chen & Tsaih, 1998; Kasaai et al., 2000a; Terbojevich, Carraro, & Cosani, 1988), intrinsic viscosity (Chen & Tsaih 1998; Kasaai et al., 2000a; Kasaai, Charlet, & Arul, 2000b), and membrane osmometry (Kasaai et al., 2000a).

One of the most common methods in determining molecular weight is size exclusion chromatography. Weight average degree of polymerization (dp) and number average dp can be calculated using a calibration curve obtained for pullulan standards, on the assumption that pullulan and chitin with equal dp have hydrodynamic equal volumes (Hasegawa et al., 1994). Chitin and chitosan molecular weights cannot be directly compared because no solvent systems can dissolve both chitin and chitosan (Hasegawa et al., 1994).

Light scattering is the use of multiple angles of light that are diffracted by the sample. This diffraction of light is measured and can be used to determine the molecular weight. Zimm plots are created from multiple measurements at multiple dilutions and the molecular weight is determined from the plot (Chen & Tsaih, 1998). Though accurate, methodology is complex and results are dependent on the purity of the sample. Samples at high concentrations can not be examined due to the high viscosity of the solutions. The low dn/dc values, used for the creating of the plots, cause a considerable error of ± 10 % in the determinations (Terbojevich et al., 1988).

Intrinsic viscosity is the viscosity of a solution with infinitely small amounts of solute. Intrinsic viscosity of a polymer solution is related to the polymer molecular weight according to the Mark-Houwink (MH) equation (Lapasin & Pricl 1999). The MH equation is $[\eta] = KM_v^a$ where $[\eta]$ is the intrinsic viscosity, M_v the viscosity-average molecular weight, and *K* and *a* are constants for the given solute-solvent system and temperature. The salt concentration can drastically influence the intrinsic viscosity of polyelectrolytes such as chitosan, particularly at low salt levels, therefore the solvent must be taken into consideration when determining molecular weight through the use of intrinsic viscosity (Signini et al., 2000). Kasaai, Charlet, and Arul (2000b) found that intrinsic viscosity or solution viscosity of chitosans can be estimated within reasonable error in the semi-dilute region using a master curve.

1.4. Current Application of Chitosan

The use of chitosan is limited because of its insolubility in water, high viscosity, and tendency to coagulate with proteins at high pH (Rabea et al., 2003). Even with limited use, chitosan has been applied as an antimicrobial agent, biodegradable film, waste recovery, waste water purification, additive to foods, nutritional additive, and medicinal purposes.

As an antimicrobial, chitosan has been found to be effective against yeasts, molds, and bacteria. The antimicrobial action of chitosan is influenced by intrinsic factors such as type of chitosan, the degree of chitosan polymerization,

the host, the natural nutrient constituency, the chemical or nutrient composition of the substrates or both, and the environmental conditions (Rabea et al., 2003).

Chitosan can also be used as an indicator of mold contamination in foods. Chitin is a main component of molds and the degree of fungal contamination in tomato process can be determined by a chemical assay for chitin (Bishop, Duncan, Evancho, & Young, 1982). The chemical assay has also been used to determine the fungal contamination in stored corn and soybean seeds (Donald & Mirocha, 1977).

Chitosan can form biodegradable films that good barriers to the permeation of oxygen, but with relatively low water vapor barrier characteristics (Butler, Vergano, Testin, Bunn, & Wiles, 1996). Mechanical properties are comparable to other medium strength commercial polymer films on the market (Butler et al., 1996). Only slight changes in mechanical or barrier characteristics of the films occur with storage time (Butler et al., 1996). Application of chitosan coating on cucumber and pepper fruits reduced transpiration losses and delayed the ripening (El Ghaouth, Arul, & Ponnampalam, 1991). Chitosan coatings have also been applied to extend the post-harvest shelf life of fruits and vegetables (Jiang & Li, 2001). For example, the application of chitosan coating delayed the change in eating quality, reduced respiration rate and weight loss, and partially inhibited the increase of polyphenoloxidase activity of the longan fruit (Jiang & Li, 2001). The delay of ripening implies that the chitosan coating may form a protective barrier on the surface of the fruit and reduce the supply of oxygen to the fruit (Jiang & Li, 2001).

Chitosan has also been applied to the recovery of waste in processing plants. A study conducted by Pinotti, Bevilacqua, and Zaritzky (1997) looked at the effect of sodium chloride concentration on the destabilization and flocculation of oil in oil in water emulsions. The longer the surfactant chain length, the greater the tendency toward polyelectrolyte association, therefore the greater the chitosan dose to reach zero change in an oil in water emulsion (Pinotti et al., 1997). To increase chitosan reactivity, agitation time was reduced resulting in lower initial charges and lower chitosan doses to reach flocculation (Pinotti et al., 1997). On a commercial scale, chitosan has been shown to be an effective coagulating agent for the reduction of suspended solids in vegetable processing waste water (Bough, 1975).

In water purification, chitosan acts as a chelating agent. The high nitrogen content of chitosan makes it a good chelating agent for the removal of metal ions (Rabea et al., 2003). The influence of chitosan chain packing and crystallinity is an important parameter in the ability of chitosan to sorb metal ions, therefore the properties of the chitosan must be considered (Jaworska et al., 2003). Tyrosinase containing chitosan gels have been used to remove phenols from process waste streams (Sun & Payne, 1996). These gels can potentially offer a non capital intensive means to selectively remove phenols from process streams for waste minimization (Sun & Payne, 1996).

Though not yet approved as a food additive in the United States, many studies have been conducted to look at the affect of chitosan in food systems. The addition of chitosan to tofu increased the shelf-life without affecting

microstructure or sensory (Kim & Han, 2002). Chitosan has also been used in cheese whey protein to remove lipids (Hwang & Damodaran, 1995). Addition of chitosan provided a cost effective method that required only a small amount of chitosan and created a high quality whey protein. Chitosans have a good affinity to phenolic compounds, which are the main components involved in the wine oxidation processes responsible for browning in white wines (Spagna, Pifferi, Rangoni, Mattivi, Nicolini, & Palmonari, 1996). The addition of chitosans to white wines did not adversely affect the sensory quality of the wine but appeared to give a better product than traditional means of removing phenolic compounds from the wine (Spagna et al., 1996).

Chitosan has been shown to reduce cholesterol levels in animals. In a study with rats, chitosan increased lipid excretion in the rat's feces (Deuchi, Kanauchi, Imasato, & Kobayashi, 1994). The mode of action in reducing cholesterol involves the chitosan dissolving in the stomach to form an emulsion with intragastric oil droplets that begin to precipitate in the small intestines at pH 6.0 – 6.5. As the numerous chains of polysaccharides start to aggregate, they would entrap fine oil droplets in their matrices, pass through the lumen and empty into the feces. These features imply that a suitable chitosan intake would be useful to control overnutrition and to prevent disease (Deuchi et al., 1994). In adding 2 % chitosan to chicken feed, an increase in total cholesterol and triacylglycerol values in chicken livers was suppressed. An increase in the values of cholesterol, triacylglycerol, and free fatty acid in hen's thigh muscles was also suppressed with 2 % chitosan feed indicating a possible production of

low-cholesterol meats (Hirano et al., 1990). Chitosan is safe and digestible in domestic animals. It can be useable as an ingredient at an appropriate dosage for domestic animal feeds, but the safety dosage varies with animal (Hirano et al., 1990).

Chitosan can be used as an indicator of lipid oxidation. When exposed to malonaldehyde, a product of lipid oxidation, chitosan forms fluorescence and can be used to detect lipid oxidation in foods using fluorescence spectrophotemetry (Weist & Karel, 1992).

In the medical field, chitosan has been evaluated for several applications. Chitin and chitosan have shown excellent wound healing in animals (Tanioka et al., 1993), but the degree of acetylation is an important factor affecting wound healing properties (Oksmoto et al., 1992). In drug delivery systems, chitosan is able to significantly enhance the immune response of nasally administered vaccines for influenza, pertussis, and diphtheria (Illum, Jabbal-Gill, Hinchcliffe, Fisher, and Davis, 2001).

1.5. High-intensity Ultrasound

1.5.1. Introduction and Definition of Power Ultrasound

Ultrasonic waves are similar to sound waves, but they have frequencies that are too high to be detected by the human ear, that is > 16 kHz. Ultrasonic waves are generated by the application of a sinusoidal force to the surface of a material. There are two classes of ultrasonic radiation: low intensity (< 1 W/cm^2) and high intensity (typically 10-1000 W/cm^2).

Low-intensity ultrasound uses low power levels that are so small the ultrasonic wave causes no physical or chemical alterations in the properties of the material through which the wave passes, meaning it is non-destructive. The most common application of low-intensity ultrasound is as an analytical technique for providing information about the physicochemical properties of foods (McClements, 1995). Ultrasound waves with low intensities are primarily used for diagnostic purposes (Povey, 1998).

High-intensity ultrasounds apply such large forces they cause physical disruption of the material to which they are applied and can promote certain chemical reactions such as oxidation (Povey, 1998). When ultrasound of a frequency > 500 kHz is applied, radical reactions may become more pronounced (Portenlanger & Heusinger, 1997).

1.5.2. Physics of Ultrasounds

Ultrasound waves are of mechanical nature with frequencies between 16 kHz and 100 kHz (Cains, Martin, & Price, 1998; Mason & Cordmas, 1996; Mason, 1997). Ultrasound is similar to electromagnetic radiation because it obeys the general wave equation and travels at a velocity that depends upon the properties of the medium (Mason, 1992; Povey, 1998). As ultrasound travels through a mass medium, it compresses and shears the molecules in the medium (Price, White, & Clifton, 1995).

Propagation of compression and shear waves at large intensities create shock waves. During the process, the ultrasonic wave attains a “saw tooth”

shape at a finite distance from the ultrasonic transducer. At the edge of the "saw tooth" a decrease in pressure occurs and results in the spontaneous formation of microscopic bubbles. As these bubbles collapse, they produce highly turbulent flow conditions and extremely high pressures and temperatures. Temperatures of up to 5000 K and pressures up to 1200 bar have been calculated (Bernstein, Zakin, Flint, & Suslick, 1996). The effect of bubbles forming and collapsing is known as cavitation (Mason, 1992; Price, 1993; Leighton, 1995; Mason & Cordmas, 1996; Mason, 1997). The formation and collapse of bubbles occurs over a few microseconds (Hardcastle et al., 2000). The size of bubbles is inversely proportional to the frequency of the applied sound wave meaning that the larger the frequency the smaller the bubbles formed (Suslick, Casadonte, Green, & Thompson, 1987; Suslick & Price, 1999).

1.5.3. Sonochemistry of Carbohydrates

The application of high-intensity ultrasound can lead to the depolymerization of large macromolecules (> 100 kDa) due to mechanical effects associated with cavitation (Crum, 1995; Mason & Cordmas, 1996; Mason, 1997; Stephanis, Hatiris, & Mourmouras, 1997). In polysaccharides, high intensity sonication treatment has been proven as reproducible and convenient in obtaining lower molecular weight fragments with the same repeating unit as the parent molecule without loss of material (Szu, Zon, Schneerson, & Robbins, 1986).

The treatment of dextrans with high intensity ultrasounds resulted in a reduction and a narrowing of the molecular weight distribution of the depolymerized products (Szu et al., 1986). Cleavage of linkages in the dextran molecules has been shown to be nonselective, meaning that the cleavage does not occur due to a particular chemical bond. Therefore polysaccharides of diverse structures can be depolymerized by high intensity ultrasounds at a similar rate and to a similar finite size (Szu et al., 1986). The rate of depolymerization of the molecules can be monitored by measurement of the intrinsic viscosity of the reaction mixture (Szu et al., 1986). Also, since the mechanism of cleavage is related to the mechanical effects associated with cavitation, the rate of depolymerization is related to the viscosity of the solvent (Szu et al., 1986). In the case of dextrans, the immobilization of the molecule by the high viscosity solvent of glycerol enhances the effect of the high intensity sonication induced bending force (Szu et al., 1986).

Further research has been conducted with high intensity sonication treatments on agarose and carrageenan. Ultrasonic degradation of agarose and carrageenan during short periods follows first-order kinetics and is dependent of molecular size (Lii, Chen, Yeh, & Lai, 1999). It was also found that the inherent stability of the glycosidic linkages, concentration, conformation and viscosity of the polysaccharides may influence the degradation mechanism of agarose and carrageenan (Lii et al., 1999).

The effect of high intensity ultrasounds on chitin and chitin complexes has been studied. Sonication can be used to degrade the (1→4)-β-linkage and effect

the deacetylation of chitinous material (Mislovicová et al., 2000). Through the application of high intensity sonication on water-insoluble chitin-glucan, a cleavage of water-soluble fragments with high chitin content was achieved from the surface of swollen chitin-glucan particles. These fragments under further sonication formed aggregates of high molecular weight (approximately 600 kDa) which at higher concentrations can partially coagulate (Mislovicová et al., 2000). In carboxymethylated chitin-glucan extracted from *Aspergillus niger* the efficiency of the ultrasonic treatment was higher with less concentrated solutions (Machova, Kvapilova, Kogan, & Sandula, 1999). The efficiency was not only higher in lower concentrations but there was also a greater dp in ice-cooled samples in comparison with the un-cooled ones (Machova et al., 1999). Sonication of chitosan hydrochloride for up to 10 minutes showed that it was randomly degraded and that negligible changes in the molecular weight distribution occurred in the molecular weight after sonication (Signini et al., 2000). When synthetic long-chain polymer solutions were subjected to an ultrasonic treatment, the molecules underwent a controlled degradation with reduced molecular weight (Price, 1993).

1.5.4. Current Application of Ultrasound in the Food Industry

Both low and high intensity ultrasound treatments have been evaluated for use in the food industry. Low intensity sonication is used for analytical purposes while high intensity sonication is used to aid in fermentation, analysis of

polysaccharide content, extractions, deactivation of enzymes and degradation of food components (McClements, 1995).

The most common application of low-intensity ultrasound is as an analytical technique for providing information about the physicochemical properties of foods, such as composition, structure, physical state, and flow rate (McClements, 1995). The physicochemical properties of food materials can be determined through measurements of the adsorption and scattering of ultrasound. Information that can be determined includes concentration, viscosity, molecular relaxation and microstructure (McClements, 1995).

High intensity sonication can be used for multiple purposes in the food industry, one of which is aiding in the fermentation of milk. Sonicated fermentation is a promising process for manufacturing low-lactose fermented milk (Wang & Sakakibara, 1997). In this process, the degree of lactose hydrolysis directly corresponds to the amounts of β-galactosidase released (Wang & Sakakibara, 1997). In the case of fermentation of biomass, low level ultrasounds can increase the rate of fermentation, but the economic value is much less compared to the traditional technique (Schläfer, Onyeche, Bormann, Schrödet, & Sievers, 2002).

High intensity sonication is also being used in the determination of the total polysaccharide content of foods. The combination of high intensity ultrasounds with acid hydrolysis can be used to determine the total polysaccharide content in both environmental and food samples (Mecozzi, Acquistucci, Amici, & Cardarilli, 2002). The ultrasound and treatment has been

shown to be more accurate in the analysis of fruit samples because the partial degradation of fructose is avoided in the method (Mecozzi et al., 2002).

A sonication treatment has been shown to aid in the extraction of food components. The extractability of polysaccharides from sage was enhanced by an ultrasound treatment (Hromádková, Ebringerová, & Valachovič, 1999). High intensity ultrasound treatment has also been used to increase the extractability of corn bran hemicelluloses from *Zea mays*. L., a co-product generated by starch production (Ebringerová & Hromádková, 2002). Application of high intensity ultrasounds in combination with an alkaline medium has been used in the extraction of lignin (three-dimensional macromolecule with high molecular weight in the range of 100 kDa used in paper industry) from wheat straw. The application of ultrasounds led to an increased purity and yield making the treatment advantageous for commercial use (Sun & Tomkinson, 2002).

Sonication can be used in the deactivation of peroxidase in food. The action of ultrasounds in combination with a conventional heat treatment is quite effective in deactivating peroxidase. The efficiency of the treatment can be related to the ultrasound power density, the ultrasound power per unit area of tip of the probe and unit volume of liquid treated (De Gennaro, Cavella, Romano, & Masi, 1999).

The mechanical forces created during cavitation resulting from high-intensity sonication are the basis for using the treatment in the degradation of food components. Sonication treatment of xylan from corn cobs in an alkaline medium was shown to be more effective in the degradation of xylan than

traditional processes (Ebringerová, Hromádková, Hríbalová, & Mason, 1997). In the case of pectin, high intensity sonication had a negative impact on its rheological properties (Seshadri, Weiss, Hulbert, & Mount, 2003). With increased sonication time and intensity, the gel strength of pectin was reduced and the time of gelation was increased (Seshadri et al., 2003). A benefit of the sonication treatment on pectin was that optical properties were improved. Pectin solutions subjected to the ultrasonic treatment were less turbid making them more beneficial in a clear beverage application (Seshadri et al., 2003). High intensity sonication has been used to decrease the molecular weight of polyvinyl alcohol. The intrinsic viscosity of polyvinyl alcohol decreased with increasing sonication time. The constant value indicates that there is a limiting molecular weight, below which chain scission does not occur (Taghizadeh & Mehrdad, 2003). The rate constant of ultrasonic degradation of polyvinyl alcohol decreased with increasing solution concentration (Taghizadeh & Mehrdad, 2003). With increased solution concentration, the viscosity increased which reduces the shear gradient around the collapsing bubbles. Therefore, the degradation rate also decreases (Taghizadeh & Mehrdad, 2003).

1.6. Objective

The objective of the research was to determine the molecular weight and degree of acetylation of chitosan molecules as a function of sonication intensity and treatment time.

2. Materials and Methods

2.1. Materials

High molecular weight chitosan (crab shells; ~81 degree of deacetylation; viscosity 800 000 cps 1 % chitosan (wt/v) in 1 % acetic acid (v/v); average molecular weight 880kDa) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Acetic acids and sodium hydroxide were obtained from Fisher Scientific (Pittsburgh, PA). All solutions were prepared using distilled and deionized water. All other materials were of analytical grade and obtained from Fisher Scientific (Pittsburgh, PA).

2.2. Sample Preparation

2.2.1. Preparation of Chitosan Solutions

Chitosan solutions containing 1 % chitosan (wt/v) in 1 % (v/v) acetic acid were made using the following procedure. The chitosan was hydrated by heating 1 g of chitosan in 90 mL of water to 60 °C. The dispersion was cooled to room temperature while stirring and 10 mL of 10 % acetic acid was added to make 1 % acetic acid in the final solution. The solution was stirred overnight to ensure complete solubilization of the chitosan molecules. Once solubilized, the solution was filtered using Miracloth® (rayon-polyester; EMD Biosciences, San Diego, CA) to remove any impurities. Filtered solutions were immediately sonicated in aliquots of 50 mL.

2.2.2. Sonication Treatment

An ultrasonic processor (Model 550, Misonix Incorporated, Farmingdale, NY) with a 1.27 cm (1/2 inch) stainless steel probe was used to sonicate 50 mL chitosan solutions in 100 mL beakers that were immersed in a temperature-controlled water bath (T = 20 °C, Lauda RM6, Germany). Solutions were treated at power levels 16.5 (low power), 28.0 (medium power), and 35.2 W/cm^2 (high power) with pulsed output (1 second sonication, 1 second break) at 25 °C. At each power level, samples were sonicated for 1, 2, 10, 30, and 60 minutes. Duplicate samples were sonicated at each power level and treatment.

2.2.3. Power Determination

Ultrasonic wave intensities were determined calorimetrically by measuring the time-dependent increase in temperature of chitosan dispersions under adiabatic conditions (Bober, 1998). Ultrasonic intensity (I) was calculated from the slope of the initial rise in temperature (dT/dt_a), the slope of heat loss after turning off the sonicator (dT/dt_b), the sample mass (m), the heat capacity of the solvent (c_p), and the radius (r) of the ultrasonic probe. $I = \frac{mc_p}{\pi r^2}\left[\left(\frac{dT}{dt}\right)_a - \left(\frac{dT}{dt}\right)_b\right]$

where m = 50 g, c_p = 4.2 Jg^{-1}K^{-1} and r = 0.0065 m. The calculated intensities for power during the "on" phase were 16.5 (low power), 28.0 (medium power), and 35.2 W/cm^2 (high power), respectively.

2.2.4. Purification

Once sonicated, the chitosan was purified and freeze dried to be used for further analysis. Duplicate 50 mL sonicated samples were combined to create a 100 mL stock solution for each power and time treatment. The pH was adjusted to 10.0 using 1 M NaOH. Solutions were allowed to set for 8 hours at room temperature for complete precipitation of chitosan molecules. Preliminary work used a purification procedure involving centrifugation and the method can be found in Appendix A. Due to low yields, a second procedure was used. To remove sodium hydroxide and sodium acetate, the precipitated chitosan was dialyzed (Spectra/Por #2 molecular weight cutoff 12,000 – 14,000, Spectrum Rancho Dominguez, CA) at 4 °C against deionized water. After dialysis the chitosan was freeze dried and stored in a desiccator.

2.3. Rheology

2.3.1. Viscosity Measurements of Chitosan Solutions

Ultrasonicated chitosan solutions were prepared in acetic acid solution at 1 % biopolymer concentrations and subjected to rotational tests at controlled shear rates between 10^{-5} - 10^{3} 1/s. Shear stress (σ) of ultrasonically pretreated chitosan solutions were recorded as a function of shear rate ($\dot{\gamma}$) using a rotational rheometer (MCR 300, Parr Physica, NJ) with a double gap bob and cup apparatus (length = 40 mm, diameter = 26.66 mm, gap width = 0.225 mm). The temperature of the loaded sample was equilibrated to 20°C using a Peltier

system. Results were fitted to the power law model (Lapasin & Pricl, 1999) $\sigma = K(\dot{\gamma})^n$ where *K* is the consistency coefficient in Pas^n and *n* is the flow-behavior index. The flow behavior index *n* reflects the viscosity of the solution i.e. *n* = 1 if the solution behaves Newtonian and *n* ≠ 1 if the solution behaves non-Newtonian. Since viscosity of a polymer solution depends on the molecular weight and/or hydrodynamic radius of a biopolymer, the calculated *K* and *n* values at different sonication conditions can be used as a first indication for changes in the molecular properties of chitosan molecules.

2.3.2. Determination of Intrinsic Viscosity of Chitosan Solutions

Intrinsic viscosity of chitosan was determined following the ASTM standard practice for dilute solution viscosity of polymers (American Society for Testing and Materials, 2001). Viscosity of chitosan dispersions in acetic acid with known polymer solutions was measured and the reduced viscosity η_r was calculated by $\eta_r = \frac{\left(\frac{\eta}{\eta_0}\right) - 1}{c}$ where η is the viscosity of the chitosan solution at the polymer concentration *c* and η_0 is the solution viscosity; 1.002 mPas at 20 °C (Lide, 2004). Secondly, the inherent viscosity η_i was calculated as $\eta_i = \frac{\ln\left(\frac{\eta}{\eta_s}\right)}{c}$.

Intrinsic viscosity [η] of deacetylated chitosan in aqueous acetic acid solutions was determined from the intercept of both η_i and η_r where *c* was near zero (Pa & Yu, 2001; Berth & Dautzenbert, 2002).

2.4. Degree of Acetylation

2.4.1. HPLC-PDA

Acid hydrolysis was conducted on purified chitosan samples in vacuum hydrolysis tubes (5 mL volume) based on the method by Niola, Basora, Chornet, and Vidal (1993). A weighed amount of dried purified chitosan (10 ± 1 mg) was placed in a vacuum hydrolysis tube with 0.5 mL 12 M H_2SO_4 and 2 mL of the standard mixture (6.3 mg oxalic acid dehydrate and 0.5 mL of proprionic acid completed to 100 mL with HPLC grade water). The tube was sealed, air was evacuated and the tube was heated to 155 °C for 1 hour (Pierce Reacti-Therm III, Pierce, Rockford, IL), cooled in ice-water for 2 hours and then equilibriated to room temperature. The mixture was filtered (0.45 µm PVDF filters with polypropylene housing, Whatman, Clifton, NJ) and 20 µL was injected into the HPLC.

The HPLC system consisted of a Dionex GP50 gradient pump, LC20 chromatography enclosure, AS50 autosampler, and a PDA-100 photodiode array detector (Dionex, Sunnydale, CA). A 300 x 7.8 mm column HPX 87H (H^+) cation-exchange resin (Bio-Rad Laboratories, Mississauga, ON, Canada) was used for separation. The mobile phase used was 5 mM H_2SO_4 with an isocratic flow rate (0.6 mL min^{-1}) at 22 ± 2 °C. Detection was carried out at 210 nm. All data were acquired, stored and processed with Peak Net software (Dionex, Sunnydale, CA).

The total acetyl groups liberated from chitosan samples (m_x in mg) was calculated according the equation $m_x = K \times \frac{A_x}{A_{is}} \times m_{is}$ where K is the response factor, A_x and A_{is} are the areas of the acetic acid and proprionic acid (internal standard) peaks, respectively, and m_{is} (mg) is the amount of internal standard. The percentage of *N*-acetylation was calculated using the equation $DA(\%) = \frac{161 \times X}{43 - 42 \times X} \times 100$ where $X = m_x / M'$ and $M' = m - m_i$, (m = sample mass, m_i = mass of inorganic material); 161 is the molecular weight of a 2-amino-2-deoxy-D-glucose unit (g/mol); 43 is the molecular weight of an acetyl group (g/mol); and 42 is the molecular weight of a deprotonized acetyl group. The original equation (Niola et al., 1993) includes the mass of inorganic material (m_i) present in the chitosan. Since our chitosan samples were extensively purified, this factor was considered negligible and was not included in the calculation.

2.4.2. FTIR

Since determination of degree of acetylation by chromatography techniques requires extensive sample preparation and hydrolysis that can significantly affect reproducibility, the second method for DA determination was involved in the study. Fourier Transform Infrared Spectroscopy (FTIR) has been the most often used technique in determination of DA of chitosans having advantage in being accurate, quick, and nondestructive. The instrument used to record samples' spectra was a Nexus 670 FTIR spectrometer with attenuated

total reflection (ATR) accessory with Ge crystal (ThermoNicolet Co., Mountain View, CA). The spectra were collected between 4000 and 700 cm^{-1} with 64 scans and resolution of 4 cm^{-1}. Degree of acetylation (%) was calculated from absorption mode using OMINC 6.1 software (ThermoNicolet Co.). Based on the equation proposed by Brugnerotto, Lizardi, Goycoolea, Agguelles-Monal, Desbrieres, and Rinaudo (2001), the bands at 1420 cm^{-1} and 1320 cm^{-1} were selected as the reference and characteristic, respectively, and the DA was calculated as $DA(\%) = \frac{(A1320/A1420) - 0.3822}{0.03133}$.

2.5. Statistical Analysis

Data obtained from degree of acetylation analysis from the HPLC-PDA method were analyzed with a SAS statistical analysis program (SAS Institute, Inc; Cary, NC; version 9.1). Analysis of variance was done with mean separation using Tukey's test to determine if differences existed. Significance was established at $p \geq 0.05$. All SAS printouts are included in Appendix B.

3. Results and Discussion

3.1. Solution Viscosity of Ultrasonicated Chitosan

Shear stress of ultrasonically pretreated chitosan solutions at a concentration of 0.1 g/L were recorded as a function of shear rate. Figures 2 and 3 show flow curves of the 1 % (wt/v) chitosan solutions sonicated for up to 60 minutes at 16.5 and 35.2 W/cm^2, respectively. Shear stress at all shear rates decreased with increasing sonication time indicating a reduction in solution viscosity. For example, shear stress of solutions at a shear rate of 50 s^{-1} decreased from 11.2 Pa to 6.8 and 2.0 Pa after 10 and 60 minutes of sonication. At higher ultrasonic intensities the decrease in shear stress is more pronounced, *e.g.* the shear stress decreased to 2.0 and 0.8 Pa after 10 and 60 minutes of sonication.

The strong influence of both sonication time and ultrasonic intensity can also be seen from fits of the flow curve to the well-known power law model. Figures 4 and 5 show a plot of the power law indexes *K* and *n* of the 1 % (wt/v) chitosan solutions sonicated at the three different ultrasonic intensities as a function of sonication time. The value of *K* decreased from 0.267 to 0.037 at 16.5 and 28.0 W/cm^2 and to 0.01 at 35.2 W/cm^2 after 60 minutes of sonication while the power law index *n* increased from 0.0888 to 0.998 after 60 minutes of sonication. The increase of the power law index *n* indicates a shift towards a more Newtonian behavior, i.e. an ideal Newtonian fluid has a power law index of

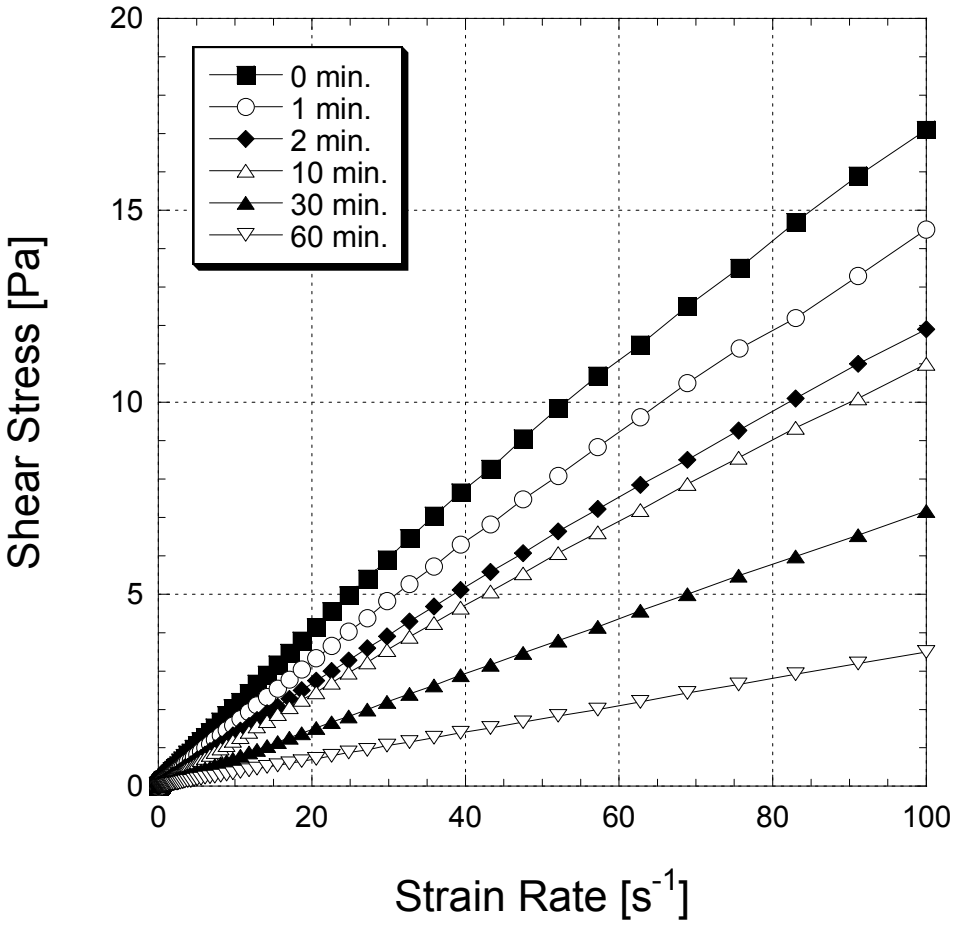


Figure 2: Shear stress (σ) versus shear rate ($\dot{\gamma}$) of 0, 1, 2, 10, 30 and 60 minute ultrasonicated high molecular weight chitosan solutions at ultrasonic intensities of 16.5 W/cm^2 (low power level).

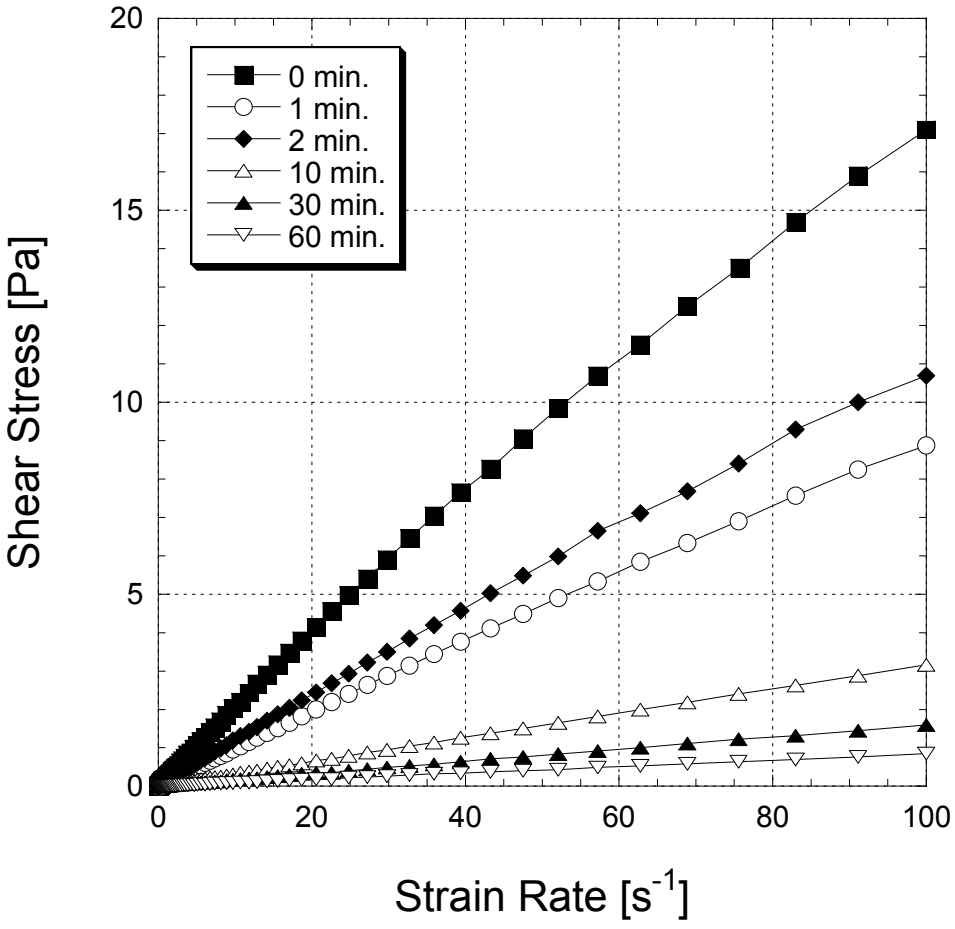


Figure 3: Shear stress (σ) versus shear rate ($\dot{\gamma}$) of 0, 1, 2, 10, 30 and 60 minute ultrasonicated high molecular weight chitosan solutions at ultrasonic intensities of 35.2 W/cm^2 (high power level).

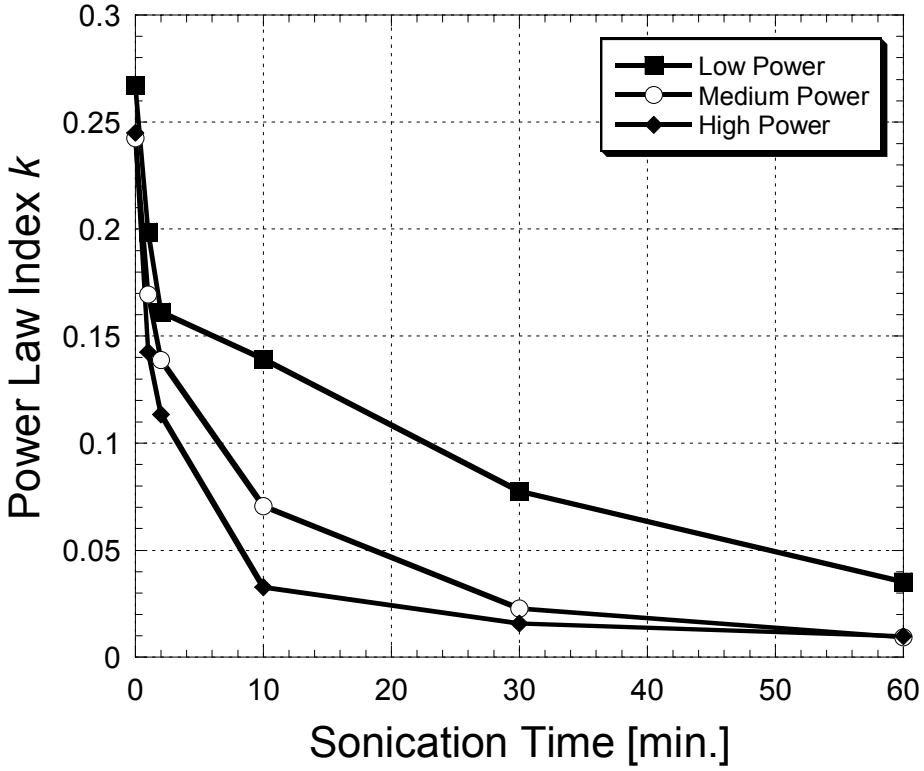


Figure 4: Power law index *K* obtained from non-linear curve fits of measured shear stress versus shear rate data of chitosan solutions treated with high intensity ultrasound 16.5 (low power), 28.0 (medium power) and 35.2 (high power) W/cm^2 for 0, 1, 2, 10, 30 and 60 minutes.

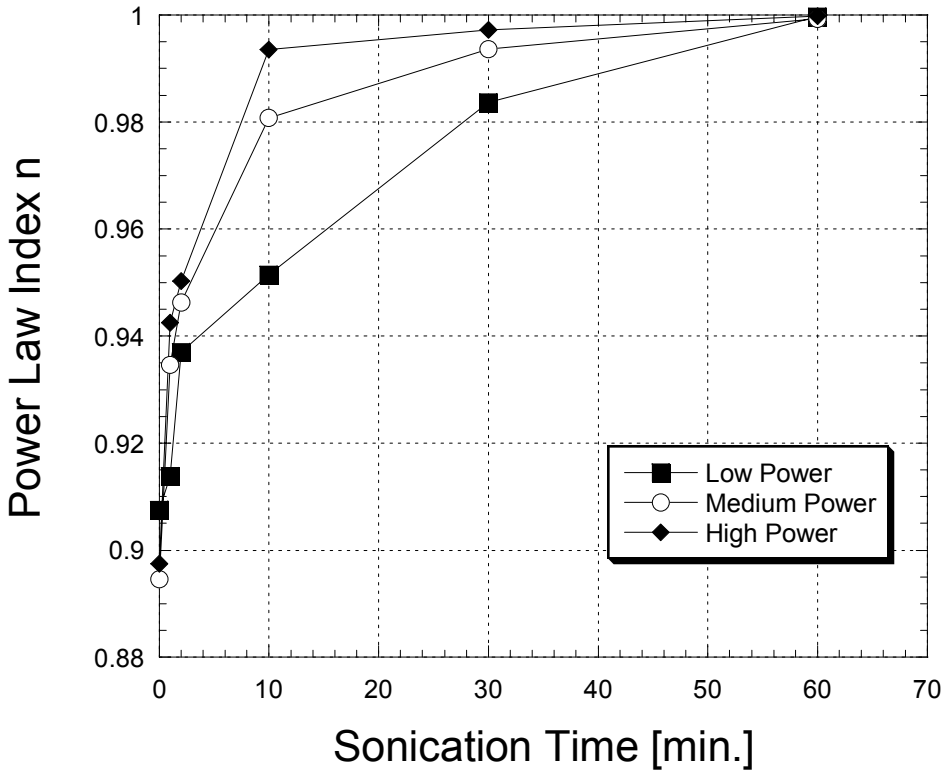


Figure 5: Power law index *n* obtained from non-linear curve fits of measured shear stress versus shear rate data of chitosan solutions treated with high intensity ultrasound 16.5 (low power), 28.0 (medium power) and 35.2 (high power) W/cm^2 for 0, 1, 2, 10, 30 and 60 minutes.

n = 1. Polymer dispersions on the other hand may exhibit shear thinning or thickening behavior with results in $n \neq 1$. The extent of shear thinning or thickening depends on a number of intrinsic and extrinsic parameters that include polymer properties such as size, shape and concentration of macromolecules in solution, solvent type, presence of ions and temperature. These factors govern the extent of entanglement and intermolecular interactions between polymer molecules. Since ions had been previously removed via dialysis and temperature, solvent type and polymer concentration were kept constant throughout all experiments, the results suggest that the intrinsic properties of the polymer that is polymer size and shape were altered by the application of high-intensity ultrasound.

3.2. Intrinsic Viscosity and Molecular Weight of Ultrasonicated Chitosan Solution

The intrinsic viscosity of chitosan samples sonicated for 0, 1, 2, 10, 30, and 60 minutes at ultrasonic intensities of 16.5 (low power), 28.0 (medium power), and 35.2 W/cm^2 (high power) was determined (Figure 6). The intrinsic viscosity of all chitosan solutions decreased exponentially as the sonication time increased from 0 to 60 minutes. Intrinsic viscosity of chitosan sonicated at lowest intensity for 60 minutes decreased from 3.85 to 1.6 dL/g. The extent of decrease of intrinsic viscosity was strongly influenced by the applied ultrasonic intensity,

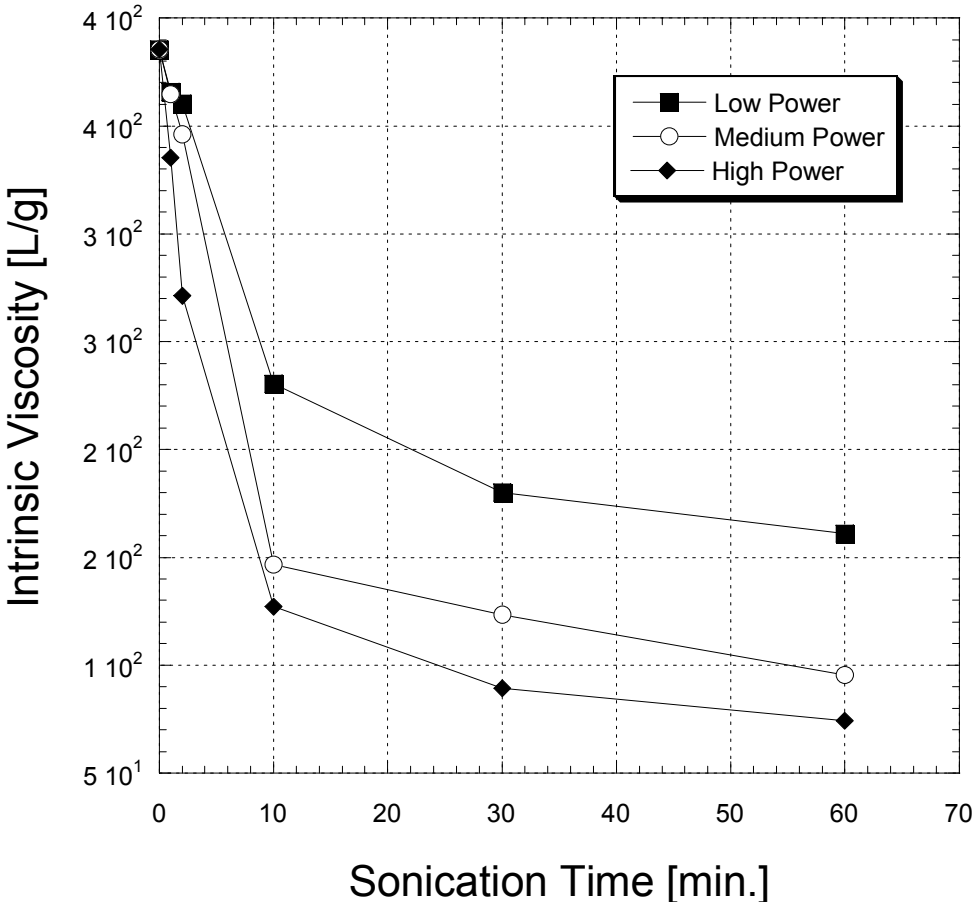


Figure 6: Intrinsic viscosity of chitosan solutions as a function of sonication time for ultrasonic intensities of 16.5 (low power), 28.0 (medium power) and 35.2 (high power) W/cm^2.

e.g. the intrinsic viscosity of chitosan sonicated at the highest intensity level of 60 minutes decreased to 0.76 dL/g.

Average molecular weights of chitosan were calculated from measured intrinsic viscosities shown in Figure 6 using the classical Mark-Houwink relationship $[\eta] = K_m M_w^a$. K_m and *a* are the so-called Mark-Houwink parameters. For chitosan, the Mark-Houwink parameters depend on the degree of acetylation, temperature, and solvent type. For example, *a* has been reported to decrease from 1.12 to 0.81 with K_m increased from 0.1 to 16 x 10^{-5} (dL/g) as the degree of deacetylation increased from 69 to 100%. In this study, K_m = 2 x 10^{-5} (dL/g) and *a* = 0.89 was used based on available light scattering data and literature data of chitosans with initial molecular weights and degree of acetylations close to that of our sample (M_w ≈ 880 kDa; DA ≈ 20%) (Wang, Shuqin, Li & Qin, 1991; Chen 1998). Calculated molecular weights for the untreated samples were 867 kDa (Table 1), which is in fair agreement with the manufacturer's data. Upon 60 minutes of sonication, the molecular weight of chitosan samples decreased to 325 kDa, 181 kDa, and 140 kDa at ultrasonic intensities of 16.5 (low power), 28.0 (medium power), and 35.2 W/cm^2 (high power), respectively (Table 1). The data also indicates that with increasing sonication time, the molecular weight of the solutions approaches a limiting final value M_e, that is $M_e = \lim_{t \to \infty} M_t$. Extrapolation of molecular weight versus time data using a simple exponential decay function predicts that the molecular weight changes less than 5 % after a

Table 1: Average molecular weight of chitosan dispersions ultrasonicated for 0, 1, 2, 10, 30 and 60 minutes at intensities of 16.5, 28 and 35.2 W/cm^2 calculated from intrinsic viscosity using the Mark-Houwink parameters *a* = 0.79 and *K* = 2.14 x 10^{-3}.

Sonication Time	Low Power 16.5 Wcm^{-2}		Medium Power 28.0 Wcm^{-2}		High Power 35.2 Wcm^{-2}	
	M_w	ΔM_w	M_w	ΔM_w	M_w	ΔM_w
0	867191	61117	867191	61117	867191	61117
1	817339	69561	815117	55220	741614	62921
2	803932	79496	768425	35806	584547	65037
10	486764	39679	360799	11698	249640	12057
30	368853	15437	241220	26696	167566	29344
60	325469	9364	181141	22189	140983	8589

sonication time longer than 60 minutes, a fact that has also been reported by other investigators using synthetic polymers. For example, Madras, Kumar & Chattapadhay (2000) found that ratio ultrasonicated to initial molecular weight $X_{Mn} = M_t / M_0$ of both polystyrene (M_w = 157 kDa; PD = 1.2) and poly (vinyl acetate) (M_w = 270 kDa; PD = 1.1) decreased from $X_{Mn} = 1$ at t = 0 to $X_{Mn} \approx 0.25$ at t > 60 minutes but then remain constant. The presence of a limiting final molecular weight is typical for the degradation of large molecules by high-intensity ultrasound. Similarily, Xiuyang, Yuefang, Bailin & Xi (2001) using hydroxyethyl cellulose with an initial molecular weight of 70 kDa found that after 60 minutes of sonication the molecular weight approached a final molecular weight of ~ 18 kDa.

3.3. Ultrasonically Driven Depolymerization Kinetics of Chitosan

The presence of a final molecular weight has been attributed to the fact that the sensitivity of linear stiff rod macromolecules to high-intensity ultrasonically generated shear and normal stresses decreases with decreasing molecular weigh (Schmid, 1940). The remaining molecule while strongly reduced in length still retains a considerable degree of polymerization. Interestingly, initial models suggested that the decrease in the reduction of molecular weight with increasing sonication time was not due to the production of a molecule that can no longer be depolymerized but that instead with increasing

disruption of intramolecular bonds in the macromolecules the number of total molecules in the solution increased. If simultaneously the number of bonds that can be broken within a given time interval remains constant but the number of available molecules it would consequently lead to a decrease in the depolymerization kinetics because less bonds can be broken per available molecule. However, reaction models based on, for example, simple mid-chain splits, *e.g.* P(x) → 2 P(x/2), that lead to simple first order kinetics without the introduction of a rate limiting factor such as a final molecular weight have not been suitable to describe experimentally obtained results. Interestingly, the introduction of the dependence of the rate on a limiting molecular weight such as $k(M) = k(M - M_e)$ has lead to the development of a model with a quasi first order reaction kinetics in the from of (Madras, et al., 2000; Madras & Chattopadhyay, 2001) $\ln H = \ln\left(\frac{(M_e - M_0)}{(M_e - M_t)}\right) = kM_e t$. That shows good agreement with experimental data obtained with polypropylene and polybutadiene degraded in various solvents. Unfortunately, the model did not provide a good fit with our experimental data, that is polts of *ln H* versus the time exhibited strong non-linearity (data not shown).

We therefore interpreted our data in terms of as early degradation model developed by Schmid (1940), where $\frac{1}{N_L}\frac{dx}{dt} = k(P - P_e)$. Combining the previous three equations followed by integration from t = 0 with M_0 to t with M_t yields

$-\frac{M_e}{M_t} - \ln\left(1 - \frac{M_e}{M_t}\right) = \frac{k}{c} P_e^2 t + C$, where P_e is the final degree of polymerization given by $P_e = M_e / M_{monomer}$. Thus if the last equation holds, then a plot of the so-called Schmid declination factor (right-hand side of the equation) versus time should yield a straight line. Figure 7 shows a poly of the Schmid declination factor calculated with the molecular weight data of our chitosan solutions sonicated at the three power levels as a function of sonication time *t* using a constant final molecular weight of 130 kDa. Generally, regression factors of $R^2 > 0.98$ were obtained indicating a good agreement with the theory. Finally, the rate constant *k* was calculated from the slope of the Schmid declination factors versus time $k = \frac{c}{P_e^2} m$, using P_e of 390 based on an assumed average molecular weight of the monomeric unit of 333 g/mol. Table 2 shows the ultrasonic degradation rate *k* as a function of ultrasonic intensity. The rate constant increased with increasing ultrasonic intensity. A plot of the three rate constants and a hypothetical rate constant of zero if the molecular weight remains unchanged suggests an exponential dependence of the rate constant on the ultrasonic power level similar to the Arrhenius law that predicts an exponential increase in the chemical reaction rates with temperature. However, the number of investigated power levels is too low to develop a conclusive model and confirm this hypothesis. Additional experiments will be needed to conclusively answer this question.

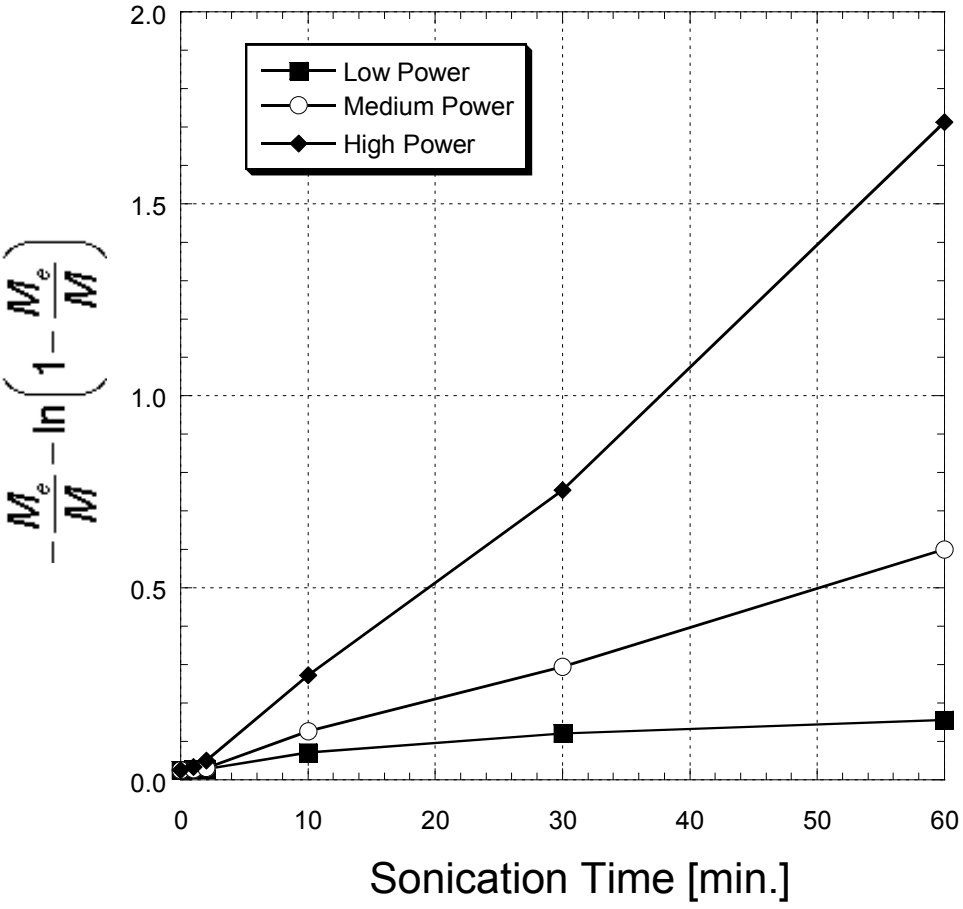


Figure 7: Schmid declination factor as a function of treatment time for chitosan solution ultrasonicated at 16.5 (low power), 28.0 (medium power), and 35.2 W/cm^2 (high power) (Schmid, 1940)

Table 2: Depolymerization rates *k* calculated from slopes *m* of Schmid plots for 1 % (wt/v) chitosan solutions sonicated at three different intensities: 16.5, 28.0, and 35.2 W/cm^2 (Schmid, 1940)

Power	**Intensity (W/cm^2)**	***m* (min^{-1})**	**Δ*m***	***k* (Mol min^{-1} L^{-1} 10^{12})**	**Δ*k***
Low	16.5	0.0034	0.0006	0.26	0.0454
Medium	28.0	0.0177	0.0004	1.34	0.0285
High	35.2	0.550	0.0017	4.23	0.1352

Alternatively, rate constants could be calculated using different final molecular weights per ultrasonic intensity levels, *e.g.* 300 kDa, 170 kDa and 130 kDa at 16.5 (low power), 28.0 (medium power), and 35.2 W/cm^2(high power), respectively. In this case, a single reaction rate is obtained (k = 4.2 ± 0.36 mol/min L x 10^{12}). In this case, the dependence of the degradation reaction on the ultrasonic intensity emerges through the variation in the final molecular weight. A plot of the final molecular weight M_e versus the ultrasonic intensity reveals a similar exponential dependence, that is the final molecular weight decrease exponentially as the ultrasonic power increases. Thus the proposed model by Schmid that is not based on mid-chain splitting kinetics appears to be suitable to describe the results obtained in this study. Generally, the question of where precisely the chain scission occurs is difficult to answer and requires additional experiments. The situation is also complicated by the fact that the stress distribution within the system during sonication cannot be assumed to be homogeneous since the ultrasonic energy experienced by the chitosan macromolecules is a function of location within the sonication vessel. For example, in the case of probe sonicators, the ultrasonic intensity decreases exponentially with increasing distance from the tip of the ultrasonic probe.

3.4. Degree of Acetylation

High pressure liquid chromatography with photodiode array detector (HPLC-PDA) and Fourier Transform Infrared Spectroscopy with attenuated total

reflection accessory (FTIR-ATR) were used to determine the degree of acetylation (DA) of sonicated and nonsonicated chitosan samples. Average DA of untreated samples was 21.5 %, which is in good agreement with the manufacturer's specifications for this lot (~19 %). Mean values and standard deviations of DA of chitosan solutions sonicated for up to 60 minutes at all three intensities are shown in Figure 8 and ranged from 15.8 to 32.3 %. Statistical analysis based on Tukey's mean separation showed no significant difference between samples, regardless of power levels or times of sonication. The results are in agreement with those found in literature. Signini, Desbrieres, and Campana Filho (2000) found that the average DA of the commercial chitosan hydrochloride and samples prepared by ultrasound depolymerization were similar and concluded that ultrasound treatment provoked no changes in the degree of acetylation. Tang, Huang, and Lim (2003) sonicated chitosan nanoparticles for 10 minutes at the power levels from 14 to 99 W/cm^2 at room temperature and found that the FTIR spectra and the DA were not affected either by ultrasound intensity or by time. Similarly, Kasaai, Arul, Chin, and Charlet (1999) applied intense femtosecond laser pulses to depolymerize dissolved chitosan and reported that no significant change in DA occurred in the fragmented products. These results confirm stability of acetylated glucosamine residues and show promise in application of ultrasound treatments for depolymerization of chitin and chitosan molecules with no alteration in degree of acetylation.

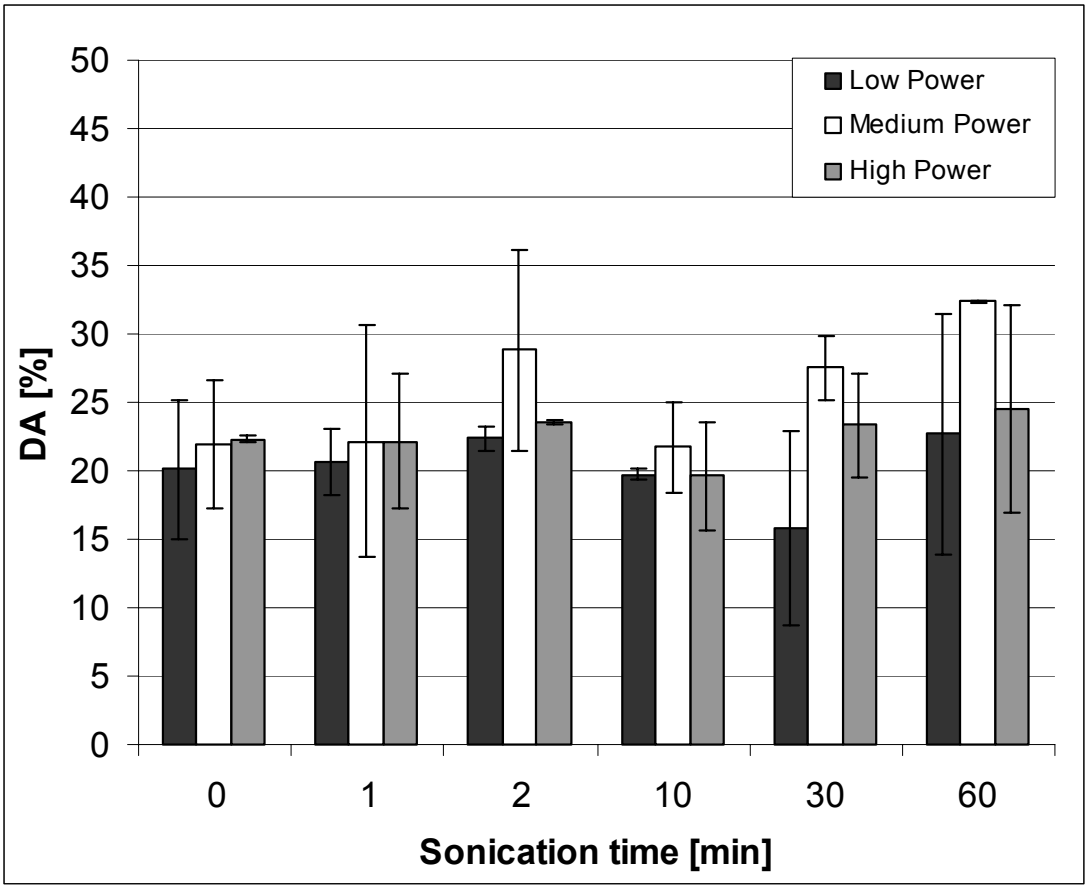


Figure 8: Average degree of acetylation of purified chitosan based on the HPLC-PDA method. Samples were sonicated at powers 16.5 W/cm^2 (low power), 28 W/cm^2 (medium power), and 35.2 W/cm^2 (high power) for 0, 1, 2, 10, 30, and 60 minutes.

The relatively wide range of DA values obtained by HPLC can be attributed to the applied methodology. It has been recognized that some techniques used for determination of DA in chitinous materials, including liquid and gas chromatography, have drawbacks in length of sample preparation and low accuracy (Muzzarelli, Rocchetti, Stanic, & Weckx, 1997; Roberts, 1992). The applied method requires hydrolysis of the chitosan samples in order to liberate acetic acid from acetylglucosamine residues. Niola, Basora, Chornet, and Vidal (1993), who established this analysis, detected significant carbonization of sugar molecules when hydrolysis lasted longer than 60 minutes. Additionally, they recognized a possibility of degradation of oxalic and propionic acid used as a reagent and internal standard, respectively, as well as glucosamine and acetylglucosamine, and formation of additional quantities of acetic acid as a product of degradation reactions. Although the authors suggested that no degradation products were formed when hydrolysis lasted up to 60 minutes, we did observe development of brownish coloration in some of the samples after only 60 minute-hydrolysis. We speculate that the coloration may be the consequence of formation of Schiff's base, furfural, and hydroximethyl furfural, and the sign of sugar degradation that, in turn, caused inconsistency in detected acetic acid quantities.

Another potential reason for observed variations is in the possibility of a presence of residual acetate ions in the samples. During the experiments, the sonicated chitosan was precipitated from solutions with alkali, dialyzed to remove excess of sodium hydroxide and sodium acetate, and freeze-dried. To evaluate a

possible presence of the residual acetate ions, the chitosan samples were analyzed without hydrolysis. The values for degree of acetylation calculated based on the difference between hydrolyzed and non-hydrolyzed samples ranged from 8.73 to 21.44 % (data presented in Appendix D), but the standard deviation between replications was not reduced.

The second method applied for the DA determination was FTIR-ATR. The characteristic FTIR absorbance spectra are shown in Figure 9. The DA values of sonicated chitosan ranged from 4.61 to 11.27 % (Table 3). The FTIR is most often used for determination of degree of acetylation in chitins and chitosans (Brugnerotto et al., 2001; Duarte et al., 2002; Shigemasa et al., 1996). The particular advantage of this technique is in direct analysis of powders and films with no need for sample preparation. However, disagreement exists regarding which peaks give the most accurate estimation of DA values. Two factors, the presence of absorbed water and level of acetylation, are of major importance in selecting reference and characteristic peaks. The common reference bands include 3450 cm^{-1} (OH stretching; Domard & Rinaudo 1983; Duarte et al., 2002), 2877 cm^{-1} (stretching of CH from $-CH_2OH$ and $-CH_3$ groups; Duarte et al., 2002), and 1159, 1074, and 1025 cm^{-1} (stretching of CO from COH, COC, CH_2OH groups; Duarte et al., 2002). The characteristic bands are usually chosen at 1655 cm^{-1}, 1630 cm^{-1} (amide I), and 1560 (amide II) from acetylated residues (Domard & Rinando, 1983; Duarte et al., 2002; Rueda, Secall, & Bayer, 1999).

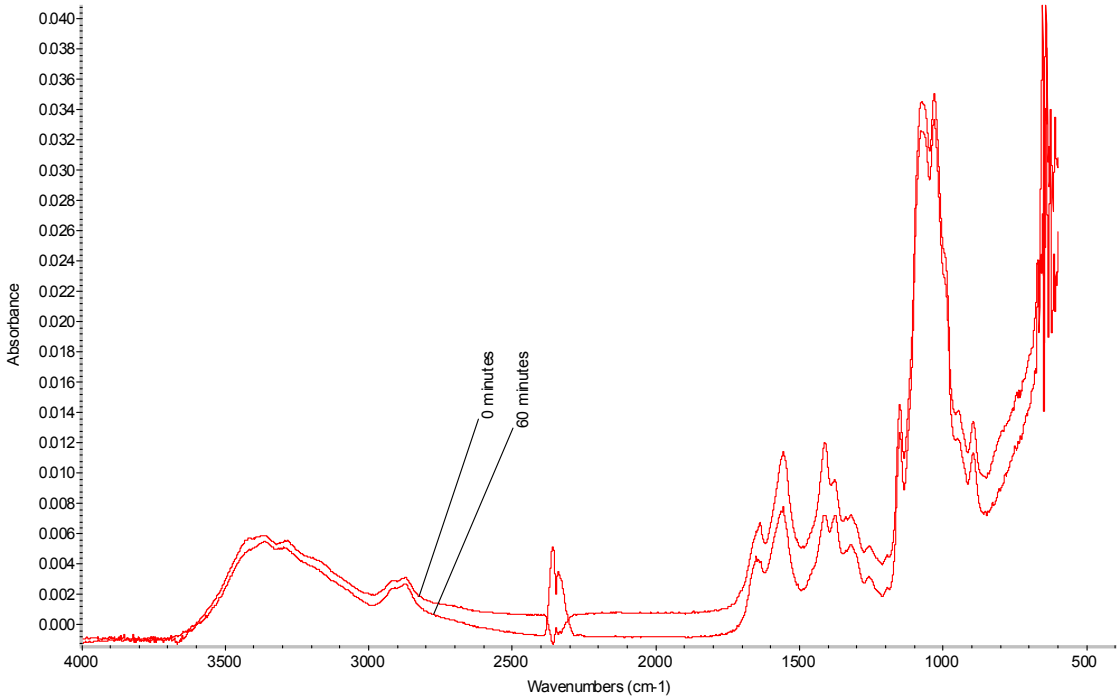


Figure 9: Characteristic FTIR-ATR spectra of sonicated chitosan samples at 35.2 W/cm2 (high power) for 0 and 60 minutes sonication time.

Table 3: Average degree of acetylation of purified chitosan based on the FTIR-ATR method. Samples were sonicated at room temperature at powers 16.5, 28, and 35.2 W/cm^2 for 0, 1, 2, 10, 30, and 60 minutes.

Time (min)	Sonication Power (W/cm^2)		
	16.5	**28.0**	**35.2**
0	10.75 ± 4.8	10.02 ± 3.5	9.35 ± 2.5
1	8.85 ± 4.3	10.86 ± 3.0	10.77 ± 1.7
2	8.20 ± 3.4	7.45 ± 4.5	9.32 ± 4.8
10	8.04 ± 4.3	6.98 ± 2.5	9.76 ± 2.6
30	7.80 ± 3.2	7.43 ± 3.7	11.27 ± 2.2
60	6.19 ± 2.2	4.61 ± 3.3	4.89 ± 3.0

* The DA values were calculated using 1420 and 1320 cm^{-1} as reference and characteristic peaks, respectively (Brugherotto et al. 2001)

However, the presence of water sharply increases the band at 1640 cm^{-1} (Shigemosa et al. 1996) which may interfere with the amide I bands. Brugnerotto, Lizardi, Goycoolea, Agguelles-Monal, Desbrieres, and Rinaudo (2001) suggested 1420 cm^{-1} as a reference band since they did not observe any changes in its intensity in the wide range of DA. The band at 1320 cm^{-1} showed the best fit (r = 0.99) with the results obtained with liquid ^{1}H NMR and solid state CP/MAS ^{13}C NMR in the whole range of DA (from 0.5 to 97.9 %). This was the first time that 1320 cm^{-1} was used as a characteristic band and the authors annotated it as "characteristic to –OH, -NH2, and –CO groups". It has to be pointed out that in calibration and optimization studies, such as of Brugnerotto, Lizardi, Goycoolea, Agguelles-Monal, Desbrieres, and Rinaudo (2002), Shigemosa, Matsura, Sashiwa, and Saimoto (1996), and Duarte, Ferreira, Marvao, and Rocha (2002), good fitting was achieved only when samples with the full range of DA values (from < 5 % to > 95 %) were used. In our study, one chitosan sample was sonicated at different power levels for different times, and DA apparently did not change. Consequently, the degree of acetylations of all the samples were in a narrow range (~ 10 – 20 %) and variations of the values were inevitable.

4. Conclusions

Ultrasonic treatment in the medium to low power range has the potential to replace time consuming chemical or enzymatic methods that are currently used to modify the molecular weight of chitosan. In the presence of an acidic solvent, the degree of acetylation remains unchanged by the application of ultrasound, which is generally desirable for its biological activity. High intensity ultrasound offers a convenient and easily controllable methodology to tailor this important functional carbohydrate. Future studies will concentrate on the specific chemical modifications that are caused by the application of ultrasound and relate it to chitosan functional properties such as antimicrobial activity and metal binding.

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Appendices

Appendix A

Purification of chitosan through centrifugation

Chitosan solutions were prepared as explained in the section 2.1.1. After filtering with Miracloth®, the chitosan solutions are vacuumed filtered using Whatman No. 1 filter paper. The pH is adjusted to 10.0 using 1 N sodium hydroxide to allow the chitosan to precipitate. To allow for complete precipitation, the solution is stored at room temperature overnight. Once completely precipitated, the solution is centrifuged at 4°C at approximately 72,000 g's for 30 minutes per cycle. During centrifugation, the pH is adjusted to neutral through washing of the chitosan. The isolated chitosan is freeze dried and stored in desicators.

Appendix B

SAS Program

```
data one; input power time hydrolysis difference;
datalines;
1      1      25.75 16.62
1      1      18.97 7.60
1      1      15.71 6.30
1      2      .     32.22
1      2      22.39 17.25
1      2      18.92 14.35
1      3      .     37.34
1      3      23.05 12.23
1      3      21.73 10.27
1      4      .     24.10
1      4      20.00 10.60
1      4      19.42 11.65
1      5      .     41.03
1      5      10.76 1.70
1      5      20.82 15.75
1      6      31.74 24.41
1      6      14.14 3.92
1      6      22.16 10.13
2      1      17.99 10.81
2      1      20.74 14.78
2      1      27.10 23.19
2      2      31.88 27.23
2      2      18.33 14.58
2      2      16.26 11.30
2      3      .     30.08
2      3      23.56 13.52
2      3      .     26.37
2      4      21.65 16.88
2      4      18.45 14.88
2      4      25.07 20.96
2      5      26.38 17.54
2      5      25.86 17.67
2      5      30.32 22.07
2      6      .     60.70
2      6      32.25 16.87
2      6      32.45 26.00
3      1      22.11 18.85
3      1      .     27.54
3      1      22.54 17.20
3      2      27.45 20.86
3      2      21.32 15.86
3      2      17.73 12.83
3      3      23.73 17.26
3      3      23.42 18.96
3      3      23.54 17.14
3      4      16.67 11.60
3      4      24.17 18.54
```

```
3       4       17.96 13.61
3       5       21.89 10.63
3       5       20.47 13.69
3       5       27.60 19.88
3       6       24.95 15.60
3       6       31.79 22.88
3       6       16.64 5.62
;
proc glm; class power time; model hydrolysis=power time power*time ;
lsmeans power time power*time/pdiff; run;
means power time power*time /tukey;run;
```

SAS Results

The GLM Procedure

Class Level Information

Class	Levels	Values
power	3	1 2 3
time	6	1 2 3 4 5 6

Number of Observations Read	54
Number of Observations Used	46

The GLM Procedure

Dependent Variable: hydrolysis

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	445.454693	26.203217	1.03	0.4574
Error	28	711.096300	25.396296		
Corrected Total	45	1156.550993			

R-Square	Coeff Var	Root MSE	hydrolysis Mean
0.385158	22.33659	5.039474	22.56152

Source	DF	Type I SS	Mean Square	F Value	Pr > F
power	2	125.0644615	62.5322307	2.46	0.1035
time	5	169.6178264	33.9235653	1.34	0.2782
power*time	10	150.7724056	15.0772406	0.59	0.8053

Source	DF	Type III SS	Mean Square	F Value	Pr > F
power	2	141.4613597	70.7306799	2.79	0.0789
time	5	175.5361739	35.1072348	1.38	0.2609
power*time	10	150.7724056	15.0772406	0.59	0.8053

The GLM Procedure
Least Squares Means

power	hydrolysis LSMEAN	LSMEAN Number
1	20.2280556	1
2	24.8755556	2
3	22.5725000	3

Least Squares Means for effect power
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: hydrolysis

i/j	1	2	3
1		0.0255	0.2147
2	0.0255		0.2303
3	0.2147	0.2303	

time	hydrolysis LSMEAN	LSMEAN Number
1	21.4705556	1
2	21.6594444	2
3	23.1711111	3
4	20.3444444	4
5	22.2100000	5
6	26.4966667	6

Least Squares Means for effect time
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: hydrolysis

i/j	1	2	3	4	5	6
1		0.9418	0.5636	0.6641	0.7753	0.0602
2	0.9418		0.6075	0.6123	0.8317	0.0698
3	0.5636	0.6075		0.3396	0.7436	0.2627
4	0.6641	0.6123	0.3396		0.4732	0.0234
5	0.7753	0.8317	0.7436	0.4732		0.1059
6	0.0602	0.0698	0.2627	0.0234	0.1059	

The GLM Procedure
Least Squares Means

power	time	hydrolysis LSMEAN	LSMEAN Number
1	1	20.1433333	1
1	2	20.6550000	2
1	3	22.3900000	3
1	4	19.7100000	4
1	5	15.7900000	5
1	6	22.6800000	6
2	1	21.9433333	7
2	2	22.1566667	8
2	3	23.5600000	9
2	4	21.7233333	10
2	5	27.5200000	11
2	6	32.3500000	12
3	1	22.3250000	13
3	2	22.1666667	14
3	3	23.5633333	15
3	4	19.6000000	16
3	5	23.3200000	17
3	6	24.4600000	18

Least Squares Means for effect power*time
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: hydrolysis

i/j	1	2	3	4	5	6	7	8	9
1		0.9122	0.6291	0.9256	0.3521	0.5426	0.6651	0.6284	0.5618
2	0.9122		0.7332	0.8526	0.3426	0.6632	0.7815	0.7465	0.6415
3	0.6291	0.7332		0.5991	0.2010	0.9502	0.9233	0.9599	0.8510
4	0.9256	0.8526	0.5991		0.4432	0.5238	0.6311	0.5990	0.5378
5	0.3521	0.3426	0.2010	0.4432		0.1454	0.1918	0.1773	0.2185
6	0.5426	0.6632	0.9502	0.5238	0.1454		0.8592	0.8997	0.8809
7	0.6651	0.7815	0.9233	0.6311	0.1918	0.8592		0.9590	0.7832
8	0.6284	0.7465	0.9599	0.5990	0.1773	0.8997	0.9590		0.8112
9	0.5618	0.6415	0.8510	0.5378	0.2185	0.8809	0.7832	0.8112	
10	0.7039	0.8181	0.8858	0.6650	0.2077	0.8178	0.9577	0.9169	0.7546
11	0.0838	0.1468	0.2743	0.1007	0.0165	0.2494	0.1862	0.2030	0.5018
12	0.0130	0.0278	0.0580	0.0182	0.0027	0.0447	0.0316	0.0350	0.1655
13	0.6390	0.7428	0.9898	0.6079	0.2053	0.9390	0.9345	0.9711	0.8429
14	0.6267	0.7449	0.9616	0.5975	0.1767	0.9016	0.9571	0.9981	0.8125
15	0.4129	0.5324	0.8005	0.4093	0.1022	0.8316	0.6968	0.7350	0.9995
16	0.8959	0.8203	0.5491	0.9811	0.4146	0.4604	0.5736	0.5394	0.5018
17	0.4466	0.5670	0.8413	0.4392	0.1129	0.8775	0.7404	0.7795	0.9674
18	0.3031	0.4152	0.6562	0.3107	0.0699	0.6686	0.5457	0.5801	0.8782

Least Squares Means for effect power*time
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: hydrolysis

i/j	10	11	12	13	14	15	16	17	18
1	0.7039	0.0838	0.0130	0.6390	0.6267	0.4129	0.8959	0.4466	0.3031
2	0.8181	0.1468	0.0278	0.7428	0.7449	0.5324	0.8203	0.5670	0.4152
3	0.8858	0.2743	0.0580	0.9898	0.9616	0.8005	0.5491	0.8413	0.6562
4	0.6650	0.1007	0.0182	0.6079	0.5975	0.4093	0.9811	0.4392	0.3107
5	0.2077	0.0165	0.0027	0.2053	0.1767	0.1022	0.4146	0.1129	0.0699
6	0.8178	0.2494	0.0447	0.9390	0.9016	0.8316	0.4604	0.8775	0.6686
7	0.9577	0.1862	0.0316	0.9345	0.9571	0.6968	0.5736	0.7404	0.5457
8	0.9169	0.2030	0.0350	0.9711	0.9981	0.7350	0.5394	0.7795	0.5801
9	0.7546	0.5018	0.1655	0.8429	0.8125	0.9995	0.5018	0.9674	0.8782
10		0.1699	0.0285	0.8969	0.9150	0.6582	0.6099	0.7009	0.5114
11	0.1699		0.3027	0.2684	0.2039	0.3445	0.0645	0.3161	0.4633
12	0.0285	0.3027		0.0565	0.0352	0.0664	0.0098	0.0597	0.0974
13	0.8969	0.2684	0.0565		0.9728	0.7898	0.5584	0.8303	0.6462
14	0.9150	0.2039	0.0352	0.9728		0.7368	0.5378	0.7813	0.5817
15	0.6582	0.3445	0.0664	0.7898	0.7368		0.3437	0.9533	0.8291
16	0.6099	0.0645	0.0098	0.5584	0.5378	0.3437		0.3737	0.2475
17	0.7009	0.3161	0.0597	0.8303	0.7813	0.9533	0.3737		0.7838
18	0.5114	0.4633	0.0974	0.6462	0.5817	0.8291	0.2475	0.7838	

The GLM Procedure

Tukey's Studentized Range (HSD) Test for hydrolysis

NOTE: This test controls the Type I experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	28
Error Mean Square	25.3963
Critical Value of Studentized Range	3.49918

Comparisons significant at the 0.05 level are indicated by ***.

power Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
2 - 3	1.966	-2.452	6.383
2 - 1	4.156	-0.478	8.789
3 - 2	-1.966	-6.383	2.452
3 - 1	2.190	-2.310	6.690
1 - 2	-4.156	-8.789	0.478
1 - 3	-2.190	-6.690	2.310

The GLM Procedure

Tukey's Studentized Range (HSD) Test for hydrolysis

NOTE: This test controls the Type I experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	28
Error Mean Square	25.3963
Critical Value of Studentized Range	4.32167

Comparisons significant at the 0.05 level are indicated by ***.

time Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
6 - 3	2.593	-5.724	10.910
6 - 5	2.753	-4.948	10.453
6 - 2	3.980	-3.720	11.680
6 - 1	4.401	-3.299	12.101
6 - 4	5.341	-2.359	13.041
3 - 6	-2.593	-10.910	5.724
3 - 5	0.159	-8.158	8.476

3 - 2	1.387	-6.930	9.704
3 - 1	1.808	-6.509	10.125
3 - 4	2.748	-5.569	11.065
5 - 6	-2.753	-10.453	4.948
5 - 3	-0.159	-8.476	8.158
5 - 2	1.227	-6.473	8.928
5 - 1	1.649	-6.051	9.349
5 - 4	2.589	-5.111	10.289
2 - 6	-3.980	-11.680	3.720
2 - 3	-1.387	-9.704	6.930
2 - 5	-1.227	-8.928	6.473
2 - 1	0.421	-7.279	8.121
2 - 4	1.361	-6.339	9.061
1 - 6	-4.401	-12.101	3.299
1 - 3	-1.808	-10.125	6.509
1 - 5	-1.649	-9.349	6.051
1 - 2	-0.421	-8.121	7.279
1 - 4	0.940	-6.760	8.640
4 - 6	-5.341	-13.041	2.359
4 - 3	-2.748	-11.065	5.569
4 - 5	-2.589	-10.289	5.111
4 - 2	-1.361	-9.061	6.339
4 - 1	-0.940	-8.640	6.760

The GLM Procedure

Level of power	Level of time	N	hydrolysis Mean	hydrolysis Std Dev
1	1	3	20.1433333	5.12180958
1	2	2	20.6550000	2.45366053
1	3	2	22.3900000	0.93338095
1	4	2	19.7100000	0.41012193
1	5	2	15.7900000	7.11349422
1	6	3	22.6800000	8.81151519
2	1	3	21.9433333	4.67269016
2	2	3	22.1566667	8.48402224
2	3	1	23.5600000	.
2	4	3	21.7233333	3.31060921
2	5	3	27.5200000	2.43877018
2	6	2	32.3500000	0.14142136
3	1	2	22.3250000	0.30405592
3	2	3	22.1666667	4.91500085
3	3	3	23.5633333	0.15631165
3	4	3	19.6000000	4.00995012
3	5	3	23.3200000	3.77397668
3	6	3	24.4600000	7.58687683

Appendix C

HPLC-PDA Degree of Acetylation Determination for Second Dialysis Treatment

Table 4: Average degree of acetylation of purified chitosan from replicated dialysis treatment based on the HPLC-PDA method. Samples were sonicated at powers 16.5 (low power), 28 (medium power), and 35.2 W/cm^2 (high power) for 0, 1, 2, 10, 30, and 60 minutes.

Sonication Time	Sonication Power (W/cm^2)		
	16.5	28.0	35.2
0	34.8 ± 8.2	35.5 ± 12.2	26.4 ± 5.8
1	30.3 ± 20.3	46.6 ± 15.5	42.1 ± 6.9
2	40.1 ± 11.1	40.7 ± 21.2	18.9 ± 5.9
10	16.3 ± 2.6	52.6 ± 22.9	45.7 ± 11.2
30	29.0 ± 7.7	37.7 ± 7.1	66.1 ± 10.5
60	32.2 ± 13.7	32.0 ± 19.0	53.1 ± 12.2

Appendix D

Degree of Acetylation Considering Residual Acetic Acid Values

Table 5: Average degree of acetylation (%) of sonicated and unsonicated samples at 16.5 (low power), 28 (medium power), and 35.2 W/cm^2 (high power) for both dialysis treatment one and the replicate dialysis treatment two as a difference of hydrolyzed and nonhydrolyzed chitosan samples determined by method of Niola et al (1993)

Sonication Time	Low Power 16.5 W/cm^2		Medium Power 28 W/cm^2		High Power 35.2 W/cm^2	
	Dialysis 1	Dialysis 2	Dialysis 1	Dialysis 2	Dialysis 1	Dialysis 2
0	12.1	24.8	16.3	29.0	18.0	21.8
1	15.8	23.7	17.7	42.1	16.5	36.4
2	11.2	24.5	19.9	33.3	17.8	13.1
10	11.1	8.7	17.6	35.7	14.6	40.7
30	8.7	21.9	19.1	29.2	14.7	57.5
60	12.8	22.4	21.4	17.2	14.7	43.4

Vita

Shari Rene' Baxter was born on January 28, 1980 in Defiance, Ohio. In 1993 Shari and her family moved from Defiance to Hickory, North Carolina where she graduated from St. Stephens High school in 1998. Shari attended North Carolina State University and graduated with a Bachelor's degree in Food Science in May 2002. In August 2002, Shari decided to continue her education by joining the Department of Food Science and Technology at the University of Tennessee to pursue a Master's Degree.

During her studies at the University of Tennessee, Shari has presented posters at the 2003 World Congress of Food Science and Technology and the 2004 Annual Meeting of the Institute of Food Technologists. After graduation, Shari plans to pursue her PhD. in Food Science at the University of Maine, Orono.