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Depolymerization and decolorization of chitosan by ozone treatment

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DEPOLYMERIZATION AND DECOLORIZATION
OF CHITOSAN BY OZONE TREATMENT

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Food Science

by
Seung-wook Seo
B.S., Chung-Ang University, 2003
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ABSTRACT

Currently, depolymerization and decolorization of chitosan are achieved by chemical or enzymatic methods which are time consuming and expensive. Ozone has been shown to be able to degrade macromolecules and remove pigments due to its high oxidation potential.

In this study, the effects of ozone treatment on depolymerization and decolorization of chitosan were investigated. Crawfish chitosan was ozonated in water and acetic acid solution for 0, 5, 10, 15, and 20 minutes at room temperature with 12wt% gas. For the determination of viscosity–average Molecular weight of chitosan, an ubbelohde viscometer was used to measure the intrinsic viscosity, and the Mark-Houwink equation was used to calculate molecular weight. Color of ozone-treated chitosan was analyzed using a Minolta spectrophotometer. The degree of deacetylation was determined by a colloid titration method.

Molecular weight of ozone-treated chitosan in acetic acid solution decreased appreciably as the ozone treatment time increased. Ozonation for 20 minutes reduced the molecular weight of the chitosan by 92% (104 KDa) compared to the untreated chitosan (1333 KDa) with a decrease in viscosity of the chitosan solution. Ozonation for 5 min markedly increased the whiteness of chitosan; however, further ozonation resulted in development of yellowness. In case of the ozonation in water, there were no significant differences of the molecular weight and color between ozone-treated chitosans. However, results showed that ozone treatment of chitosan in both water and acetic acid solution was not effective in removing acetyl groups (deacetylation) in chitosan molecules.

This study showed that ozone can be used to modify molecular weight and remove pigments of chitosan without chemical use in a shorter time with less cost.

CHAPTER 1

INTRODUCTION

Chitosan is a natural polysaccharide comprising copolymer of glucosamine and N-acetylglucosamine, and can be obtained by the deacetylation of chitin from crustacean shells, the second most abundant natural polymer after cellulose (No and Meyers, 1989). Due to its biodegradability and biocompatibility, and low toxicity, chitosan has received increased attention as one of the promising renewable polymeric materials for their various applications in the pharmaceutical and biomedical industries for enzyme immobilization and purification, in chemical plants for wastewater treatment, and in food industries for food formulations as binding, gelling, thickening and stabilizing agent (Knorr, 1984).

Unlike other polysaccharides, chitosan contains amine groups at C-2 position. Amine groups (NH_2) of chitosan are protonated (NH_3^+) in acidic solution and polycationic properties of chitosan give rise to its unique functional properties (Knorr, 1984). The physicochemical characteristics of chitosan affect its functional properties, which differ with preparation methods.

Traditional isolation of chitin involves three steps including demineralization for removal of calcium carbonate/phosphate, deproteinization for protein removal, and decolorization for removal of pigments. Chitin obtained from the three steps can be converted to chitosan by deacetylation process. Controlling of degree of deacetylation, molecular weight and viscosity is very important because these properties reflect on the usefulness of chitosan for many applications. In the process of chitosan preparation, a large amount of harmful, highly concentrated chemical solutions are used. Currently, low molecular weight chitosan is produced by chemical or enzymatic hydrolysis. In chemical methods, hydrolysis of chitosan can be done with hydrochloric acid, nitrous acid, and phosphoric acid. Both chemical and enzymatic methods

are time consuming and expensive (No et al., 2003a). Decolorization is usually carried out by a bleaching treatment with strong chemicals such as acetone, chloroform, ethyl acetate, sodium hypochlorite and hydrogen peroxide solutions (No and Meyers, 1995). It causes an increase in the level of environmental pollution.

Ozone has been used as a replacement for chlorine-based chemicals (Kim et al., 2000). Previous studies have shown that ozone is able to degrade macromolecules and destroy pigments such as azo dyes due to the high oxidation potential of ozone. Ozone, a strong oxidant, does not remain in water for a long period of time, thus it may be used with no safety concerns about consumption of residual ozone in food products. Due to the high oxidation potential of ozone, we hypothesized that the use of ozone may be an alternative approach to achieving decolorization and depolymerization of chitosan simultaneously instead of using chemicals.

The objectives of this study were to determine the feasibility of ozone treatment to depolymerize and decolorize chitosan simultaneously; and to characterize some physicochemical and antibacterial properties of the resulting chitosans.

CHAPTER 2

LITERATURE REVIEW

2.1 Definition of Chitosan

Chitosan is a non-toxic and biodegradable cationic polymer derived by deacetylation of chitin, a homopolymer of β -(1-4)-linked N-acetyl-D-glucosamine. Chitin is a plentiful biomass, which is widely distributed in nature as the skeletal structure of crustaceans, insects, mushrooms, and cell wall of fungi (Knorr, 1984). However, commercial chitosan is only manufactured from crustaceans such as crab, krill and crawfish primarily because a large amount of the crustacean exoskeleton is available as a by-product of food processing (Methacanon et al., 2003).

Crustacean shells mainly consist of 30~40% protein, 30~50% calcium carbonate, and 20~30% chitin. These proportions vary with species and with season. Thus, the method of chitin/chitosan preparation can vary with different sources. The physical and chemical characteristics of chitin and chitosan accordingly differ with species and preparation methods (Brine and Austin, 1981). These variations in preparation methods are likely to result in differences in the degree of deacetylation, the distribution of acetyl groups, the chain length and the conformational structure of chitosan.

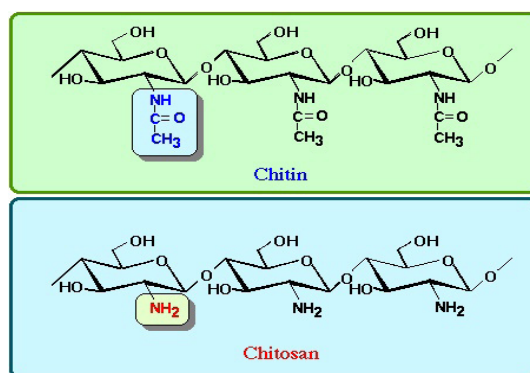


Figure 1. Chemical structure of Chitin and Chitosan
(Source: Dalwoo Corporation: <http://dalwoo.tripod.com/structure.htm>)

In terms of its chemical structure (Figure 1), chitin and chitosan have very similar chemical structure. Chitin exhibits structural similarity to cellulose and differs from it with the replacement of C-2 hydroxyl residues by acetamide groups (Kurita, 1998). Chitin can be transformed into chitosan that has free amino groups by removing acetyl groups ($\text{CH}_3\text{-CO}$) from chitin molecules. Chitosan (deacetylated chitin) is insoluble in water, alkali and organic solvents, but soluble in most diluted acids with pH less than 6. When chitosan is dissolved in an acid solution, it becomes a cationic polymer due to the protonation of free amino groups on the C-2 position of pyranose ring (Hsu et al., 2002). Its cationic properties in acidic solutions give it the ability to interact readily with negatively charged molecules such as fats, cholesterols, metal ions, and proteins (Li et al., 1992).

2.2 Physicochemical Characteristics of Chitosan

2.2.1 Degree of Deacetylation (DD)

Deacetylation process involves the removal of acetyl groups from chitin molecules. Degree of deacetylation determines the content of free amino groups ($-\text{NH}_2$) in the polysaccharides and can be used to differentiate between chitin and chitosan. Degree of deacetylation is one of the most important chemical characteristics that influence the physicochemical properties of chitosan and its appropriate applications (Muzzarelli, 1977; Li et al., 1992). In addition, the proportion of glucosamine residues in chitosan has a significant influence on chitosan's various properties including solubility, biodegradability, antimicrobial activity, and wound healing properties (Cho et al., 2000).

Degree of deacetylation ranges from 56% to 99%, depending on the species and the preparation methods (No and Meyers, 1995). Generally, chitin with a degree of deacetylation of 70% or above is known as chitosan (Li et al., 1992). According to No and Meyers (1995), there

are some factors affecting the extent of deacetylation such as concentration of the alkali, reaction temperature, time of reaction, particle size, and previous treatment of the chitin. For desired solubility, deacetylation of chitin has to be achieved by at least 85% (No and Meyers, 1995).

2.2.2 Molecular Weight (Mw)

Molecular weight of native chitin is usually larger than one million daltons while commercial chitosan product has molecular weight of 100,000~1,200,000 daltons, depending on the process and grade of the product (Li et al., 1992). Molecular weight of chitosan is one of the most important factors affecting antimicrobial activities of chitosan (Jeon et al., 2001; No et al., 2002). Molecular weight of chitosan varies with the raw material sources and the preparation methods. According to Galed et al. (2005), chitosan is susceptible to a variety of degradation mechanisms including free radical depolymerization and acid, alkaline and enzymatic-catalyzed hydrolysis. During chitosan preparation, degradation of chitosan polymer occurs by treatment with the concentrated acid and alkali. The molecular weight of chitosan is also affected by deproteinization conditions used for the isolation of the chitinous substrate (Synoweicki and Al-Khateeb, 2003).

2.2.3 Viscosity

Viscosity of chitosan solution is affected by many factors, such as the degree of deacetylation, molecular weight, concentration, ionic strength, pH, and temperature (Li et al., 1992). Viscosity is an important factor in determining chitosan's commercial applications. Furthermore, some studies have shown that viscosity of chitosan significantly affects its antimicrobial activities. The antimicrobial activity of chitosan against *E.coli* and *Bacillus sp.* increased with decreasing viscosity from 1000 to 10 cP (Cho et al., 1998).

Viscosity of chitosan is closely related to its molecular weight. High molecular weight

chitosan has higher viscosity than low molecular weight chitosan. Several studies have shown that physical and chemical treatments affect its viscosity. Viscosity of chitosan decreased with increasing treatment time of grinding, heating, autoclaving, ultrasonication and ozonation (No et al., 1999), and decreased from 248 to 32 cP with increasing deproteinization time from 0 to 30 min (No et al., 2003b).

2.2.4 Color

The color of chitin and chitosan is associated with the carotenoid pigment. The main component of carotenoid fraction in crustacean exoskeleton is astaxanthin (No et al., 1989; Shahidi and Synowiecki, 1991; Chen and Meyers, 1982). The carotenoids are strongly bound to chitin molecule and associated with proteins in the epithelial layer of the exoskeleton. The carotenoid level in crustacean is very low and changes depending on dietary pigment availability, crustacean size, its maturation, and genetic differences (Synowiecki and Al-khateeb, 2003). For instance, the average values of pigment concentration determined in the shell waste from Louisiana crawfish (No et al., 1989), shrimp and crab (Shahidi and Synowiecki, 1991) were estimated as 108, 147 and 139 ppm, respectively.

2.2.5 Antimicrobial Properties

With its unique polycationic nature, chitosan has been used as an active material such as for antimicrobial activity. Several studies have shown that chitosan is effective in inhibiting the growth of bacteria. The antimicrobial activity of chitosan is reported to be dependent on its molecular weight, concentration, and the type of bacteria (Jeon et al., 2001; No et al., 2002; Zheng and Zhu, 2003). According to Cho et al. (1998) the antibacterial activity of chitosan against *E.coli* and *Bacillus sp.* increased with decreased viscosity from 1000 to 10 cP. Recent studies on the antimicrobial activity of chitosan and its oligomers have revealed that chitosan is

more effective in inhibiting growth of bacteria than chitosan oligomers (Jeon et al., 2001; No et al., 2002). Furthermore, No et al. (2002) found that 0.1% chitosan showed stronger bactericidal effect on gram-positive bacteria (*Listeria monocytogenes*, *Bacillus megaterium*, *B. cereus*, *Staphylococcus aureus*, *Lactobacillus plantarum*, *L. brevis*, and *L. bulgaris*) than on gram-negative bacteria (*E.coli*, *Pseudomonas fluorescens*, *Salmonella typhimurium*, and *Vibrio parahaemolyticus*). According to Jeon and Kim (2000), the molecular weight of chitooligosaccharides is critical for inhibition of bacterial growth and required higher than 10 KDa. Several studies discussing chitosan's antimicrobial activity have been reported in different conditions, with conflicting results. Where *E.coli* was used as the microorganism (Zheng and Zhu, 2003), the results showed that the greatest antimicrobial effects were observed in 0.25% chitosan with a molecular weight of less than 5 KDa. In another study, however, 0.1% chitosan of 746 KDa was shown to be most effective against *E.coli* (No et al., 2002). In addition, chitosan with a molecular weight of 40 KDa could inhibit 90% of *S.aureus* and *E.coli* at a concentration of 0.5% (Shin et al., 1997).

The minimum inhibitory concentration (MIC) of chitosans in 1% acetic acid range from 0.005 to 0.1% depending on the species of bacteria and molecular weight of chitosan (No et al., 2002). Uchida et al. (1989) reported the chitosan MIC for *E.coli* and *S. aureus* to be 0.025% and 0.05%, respectively. Jeon et al. (2001) reported that MIC values were less than 0.06% against Gram-negative bacteria and 0.06% against Gram-positive bacteria. According to Zheng and Zhu (2003), the antimicrobial effect was strengthened as the concentration of chitosan increased. Chitosan at 1.0% with a molecular weight of 305 KDa showed 100% inhibition against both *E.coli* and *S. aureus*.

The mechanism of antimicrobial activity of chitosan has not been fully elucidated yet,

but several hypotheses have been postulated. It has been suggested that a positive charge on the amine group of the glucosamine monomer at $\text{pH} < 6.3$ allows interactions with negatively charged microbial cell membranes that lead to the leakage of intracellular constituents (Helander et al., 2001). Other possible mechanisms mentioned in the literature are the formation of a polymer membrane of chitosan on the surface of the cell, which prevents nutrients from entering the cell (Zheng and Zhu, 2003), and the interaction of diffused hydrolysis products with microbial DNA, which leads to inhibition of mRNA and protein synthesis (Sudarshan et al., 1992).

2.3 Applications of Chitosan

Chitosan has been used in a variety of applications, such as in water treatment, agriculture, pulp, medicine, membranes, and food (Table 1). In the past, applications of chitosan were limited to specific fields; however, the present trend in industrial applications is toward producing high value products, such as cosmetics, drug carriers, semi-permeable membranes, and pharmaceuticals.

Chitosan has been approved as a food additive in Korea and Japan since 1995 and 1983, respectively, and thus considerable attention has been given to the use of chitosan as a natural preservative to improve the shelf-life of food (No et al., 2002). In the United States, the FDA has approved chitosan for fruit juice clarification, protein recovery from food process waste, edible coatings, and as an additive for animal feed (Davies et al., 1989; Hirano, 1997). In 2005, shrimp-derived chitosan was submitted to the U.S. FDA to be considered as GRAS based on the scientific procedures for use in foods, in general, including meat and poultry, for multiple technical effects. However, according to GRAS notice no. GRN 0001-70, at the notifier's request, the U.S. FDA ceased to evaluate the notice, effective October 31, 2005 (US FDA/CFSAN, 2006).

Table 1. Applications of chitosan

Application	Examples
Water treatment	Removal of metal iron Flocculant/Coagulant Filtration
Pulp and paper	Surface treatment Photographic paper Carbonless copy paper
Medical	Bandages, Sponges Artificial blood vessels Blood cholesterol control Tumor inhibition Membranes Dental plaque inhibition Skin burns/Artificial skin Contact lens Controlled release of drugs Bone disease treatment
Cosmetics	Make-up powder Nail polish Moisturizer Fixtures Bath lotion Face, hand and body creams Toothpaste Foam enhancing
Biotechnology	Enzyme immobilization Protein separation Chromatography Cell immobilization, Cell recovery Glucose electrode
Agriculture	Seed coating Leaf coating Hydroponic/Fertilizer Controlled agrochemical release
Food	Removal of dyes, solids, acids Preservatives Color stabilization Animal feed additives
Membranes	Reverse osmosis Permeability control Solvent separation

(Li et al., 1992)

2.4 Production of Chitin and Chitosan

A variety of procedures have been developed over the years for the preparation of chitin and chitosan. Main sources of the commercial chitosan come from crustaceans such as crab, krill and crawfish primarily because large amounts of the crustacean exoskeleton is available as a by-product of food processing (Methacanon et al., 2003).

Crustacean shells mainly contain about 20~30% chitin on a dry basis. This proportion varies with species and with season. Thus, the method of chitin/chitosan preparation can vary with different sources. Isolation of chitosan from crustacean shell wastes consists of four basic steps (Figure 2) including deproteinization (DP) for protein separation, demineralization (DM) for calcium carbonate separation, decoloration (DC) for pigments separation, and deacetylation (DA) for removal of acetyl groups. Chitin can be isolated from crustacean shell wastes by two basic steps; deproteinization and demineralization. These two steps can be conducted in a reverse order (No and Meyers, 1995).

2.4.1 Deproteinization (DP)

Crustacean shells contain about 30~40% protein on a dry basis. Crustacean shell waste is usually ground and treated with dilute sodium hydroxide solution (1-10%) at elevated temperature (65~100°C) to extract the proteins present. Reaction time usually ranges from 0.5 to 12 hr depending on preparation methods. Prolonged alkaline treatment under severe conditions causes depolymerization and deacetylation. Shahidi and Synowiecki (1991) conducted a study of extraction time of proteins from crab (2% KOH) and shrimp (1% KOH) shells at 90 °C with a solid to solution ratio of 1:20 (w/v). They found that a minimum period of 1hr was needed to extract over 90% of the proteins, and 2hr extraction time was required for removal of all proteins. To obtain uniformity in reaction, it is recommended to use relatively high ratios of solid to alkali

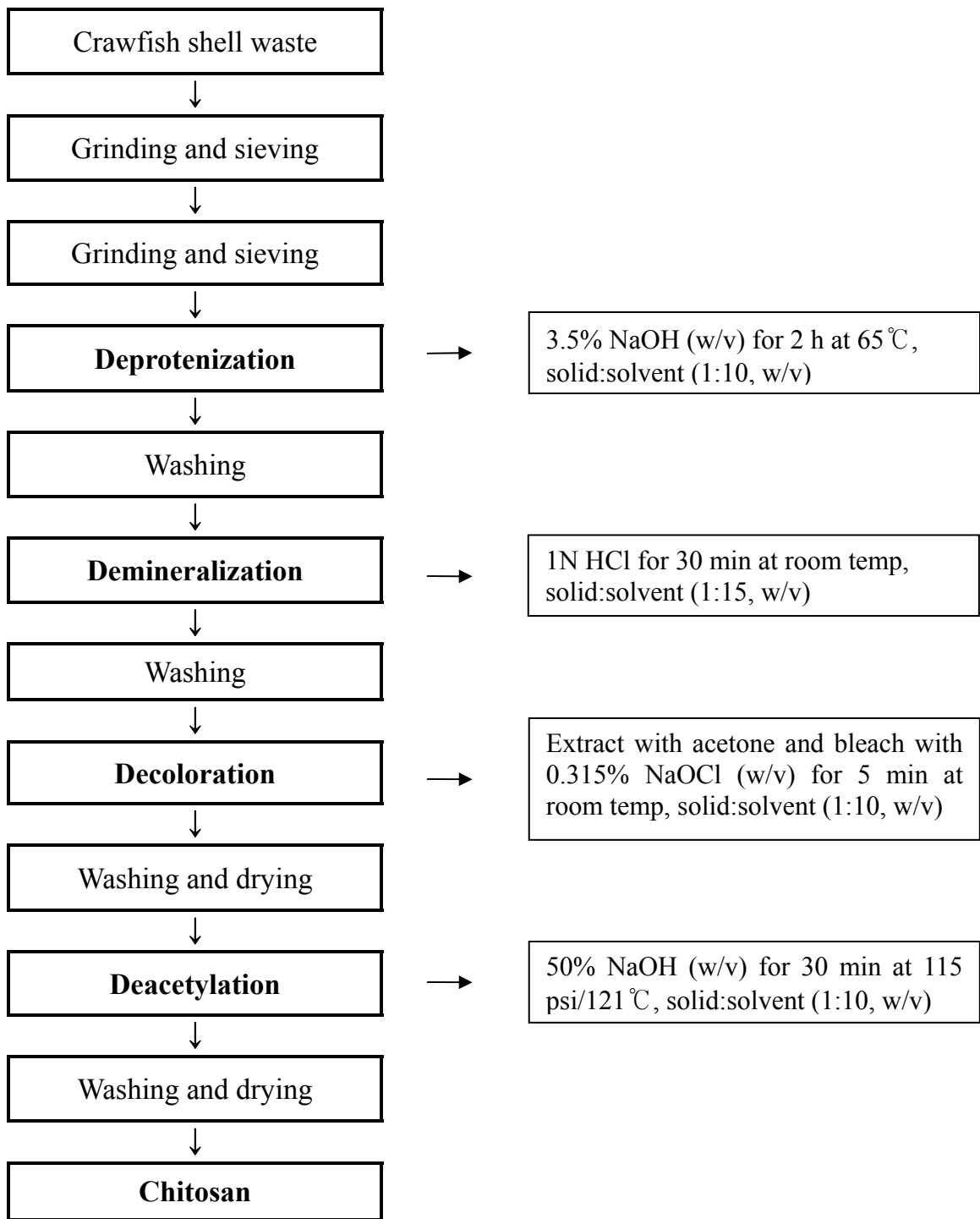


Figure 2. Traditional crawfish chitosan production (No and Meyers, 1995; No et al., 2000)

solution of 1:10 or 1:15-20 with proper agitation because a minimum ratio of 1:4 (w/v) of shell weight to KOH solution, had only a minor effect on the DP efficiency of shells (No and Meyers, 1995). Optimal conditions for deproteinization involve treatment of the crawfish shells with 3.5% (w/w) NaOH solution for 2 hr at 65°C with constant stirring and a solid to solvent ratio of 1:10 (w/v) (No et al., 1989).

2.4.2 Demineralization (DM)

Demineralization is conventionally accomplished by extraction with dilute hydrochloric acid (up to 10%) at room temperature with agitation to dissolve calcium carbonate as calcium chloride. A variety of the demineralization methods have been reported in the literature. The reaction time varies depending on the different species and preparation methods. The use of HCl at higher concentration and also 90% formic acid to achieve demineralization has been reported. Some drastic treatments with highly concentrated acids may result in modification such as depolymerization and deacetylation of the native chitin (No and Meyers, 1995). Optimum demineralization is achieved by constant stirring of the dried ground crawfish shell with 1N HCl for 30 min at ambient temperature and a solid to solvent ratio of 1:15 (w/v) (No et al., 1989). The ash content of the demineralized shell is an indicator of the effectiveness of the demineralization process.

2.4.3 Decolorization (DC)

Acid and alkali treatments alone produce a colored chitin product. When a bleached product is desired, pigments can be removed with reagents. The pigment, mainly carotenoid pigments, in the crustacean shells forms complexes with chitin residue (No et al., 1989). In earlier studies, Fox (1973) found one 4-keto-and three 4, 4'-diketo- β -carotene derivatives firmly bound to the exoskeletal chitin of red kelp crab. The level of association of chitin and pigments

varies from species to species among crustacean.

Several workers have used reagents to eliminate pigments from crustacean exoskeleton, usually from crab. However, with crawfish shell, the reagents alone were not as effective as the procedure developed currently. This suggests that carotenoids are more strongly bound to the crawfish shell matrix than are those reported for other crustacea (No et al., 1989). Hence, to obtain a white colored product the harsh treatment is required. No et al. (1989) prepared a white colored crawfish chitin by extraction with acetone, followed by bleaching with 0.315 % (v/v) sodium hypochloride solution (containing 5.25% available chlorine) for 5 min with a solid to solvent ratio of 1:10 (w/v), based on dry shell. Without prior acetone extraction, bleaching for more than 1 hr was needed to obtain a white product.

2.4.4 Deacetylation (DA)

Deacetylation is a process of removing acetyl groups from the chitin molecules and a process of conversion of chitin to chitosan. During deacetylation, conditions must sufficiently deacetylate the chitin to yield a final chitosan product that is soluble in dilute acid solution. But, it is difficult to prepare a chitosan with a degree of deacetylation higher than 90% without significant degradation of polysaccharide molecules. It is generally achieved by treatment with concentrated sodium hydroxide solution (40~50%) at 100°C or higher temperature and a solid/solvent ratio of 1:15 for 30 min or longer (No and Meyers, 1995). There are some critical factors that affect degree of deacetylation of chitosan such as concentration of alkali, temperature and time of reaction, and particle size (No and Meyers, 1995). Decreasing alkali concentration increased the time required to obtain soluble chitosans with a less viscous product, and prolonged time increased the percentage of deacetylation but reduced molecular size (Mima et al., 1983). No et al. (2000) conducted deacetylation of chitin with 40~50% sodium hydroxide

solution in a combination of elevated pressure and temperature conditions (15 psi/121 °C) and a solid/solvent ratio of 1:15. They demonstrated that a combination of elevated pressure and temperature was effective in obtaining acid-soluble chitosan in a relatively shorter reaction time (30 min) compared with conventional methods (No and Meyer, 1995).

2.5 Depolymerization

Chitosan and its derivatives have been used in a wide variety of applications such as thickening, film formation, metal binding and antimicrobial activity, but the effectiveness of these materials has been found to be dependent on their molecular weight and degree of deacetylation (No and Meyers, 1997). In addition, controlling depolymerization of chitosan is useful in order to adjust properties like viscosity, solubility and biological activity (Rege and Block, 1999). However, most native chitosans have quite large molecular weight (over one million daltons). Thus, it is necessary to establish a reproducible method for generating low molecular weight chitosan. Generally, low molecular weight chitosan can be prepared from high molecular weight chitosan by the depolymerization process.

2.5.1 Various Methods of Depolymerization

Depolymerization of chitosan can be achieved by chemical, enzymatic and physical methods (No et al., 2003a; Galed et al., 2005). In chemical depolymerization, various acids such as hydrochloric acid, nitrous acid, phosphoric acid and hydrogen fluoride have been used to obtain low molecular weight chitosan. Acidic hydrolysis is a common and fast method to produce a series of the chitosan oligomer, however, this method produces lower yields and a large amount of monomeric D-glucosamine (Jeon and Kim, 2000). In addition, this procedure has some drawbacks such as high cost and environmental pollution.

The enzymatic method, such as with chitosanase (Jeon et al., 2001) or protease (Li et al.,

2005) seems to be more preferable to chemical reactions because the reaction is under mild conditions and the hydrolysis course can be controlled easily. However, the expensive cost of enzymes inhibits their use in a commercial application.

Depolymerization of chitosan can also be achieved by oxidative degradation with oxidants such as ozone (Kabal'nova et al., 2001), sodium nitrite (Mao et al., 2004) and hydrogen peroxide (Tian et al., 2004; Chang et al., 2001).

Physical methods, such as ultrasonication and irradiation have been attempted to depolymerize chitosan by several workers. Baxter et al. (2005) studied the effect of ultrasonication on the degradation of chitosan. They found that intrinsic viscosity of samples decreased exponentially with increasing sonication time and rates of intrinsic viscosity decreased linearly with ultrasonic intensity. Choi et al. (2002) investigated the depolymerization of chitosan using irradiation with different doses (2~200 KGy) of gamma rays. Their results showed that viscosity of irradiated chitosan rapidly decreased with increasing irradiation dose.

2.5.2 Mechanism of Depolymerization

The mechanism of depolymerization of chitosan has been studied by several workers. According to Tian et al. (2004), the breakage of 1,4- β -D-glucoside bonds in the polysaccharide chain by hydrogen peroxide leads to depolymerization of chitosan. The nitrous acid depolymerization reaction mechanism has been found to be specific in the sense that HONO attacks the amino group of D-units, with the subsequent cleavage of the following glycosidic linkage (Allan and Peyron, 1995). Kabal'nova et al. (2001) found that oxidative destruction of β -D-glucoside bonds between units leads to depolymerization of polysaccharide.

During depolymerization, several chemical modifications in the chitosan molecule have been reported. Undesirable side reactions are mainly due to free radical species formed during

the reaction. The degradation of chitosan by hydrogen peroxide caused the formation of carboxyl group and loss of 15% of amino groups (Qin et al., 2002). Further degradation led to more ring-opening oxidation of degraded products and loss of more than 40% amino groups. The reduction of molecular weight and the increase of carboxyl group content in degraded chitosan were in close relation to deamination which results from H-abstraction at C-1 and C-2. Wang et al. (1999) reported that ozonolysis of polysaccharides in aqueous solution resulted in some side reaction such as acid formation. The formation of organic acids suggests that the reaction proceeded by a radical mechanism in which ozone attacked water molecules and formed hydroxyl radicals that led to degradation (Not specific for β -D-glycoside bonds). In the degradation by irradiation, brown color heavily developed for doses over 100 KGy (Choi et al., 2002).

2.6 Ozone

2.6.1 Chemical Properties of Ozone

Ozone (O_3) is an unstable gas having a pungent, characteristic odor. Ozone consists of three oxygen atoms connected in a bent line (with an O-O-O angle of $\sim 117^\circ$) and results from the rearrangement of atoms when oxygen molecules are subjected to high-voltage electric discharge. Ozone gas is sparingly soluble in water, about 13 times more soluble than oxygen, and the rate of decomposition in water is greatly affected by the purity of water and the cleanliness of glassware in which decomposition experiments are conducted (Hill and Rice, 1982). The half-life of ozone in distilled water at $20^\circ C$ is generally considered to be 20~30 min (Khadre et al., 2001). Excess ozone decomposes rapidly to produce oxygen, thus it leaves no residues in foods (Khadre et al., 2001). Because of no safety concerns about consumption of residual ozone in food, it has been used in a variety of fields in the food industry.

2.6.2 Applications of Ozone

Ozone is being used as a replacement for chlorine-based chemicals for sanitation purposes in food processing, especially in the meat industry; and for water quality purposes, such as bacterial, odor, color, and toxic compound degradation. In the past, ozone has been used for the preservation of food and food ingredients such as milk, meat products, gelatin, casein, and albumin (de la Coux, 1904). Ozone also has been used as a selective disinfectant in brewing and cider manufacturing (Hill and Rice, 1982). However, ozone mainly has been used as a disinfectant for the treatment of drinking water, municipal and industrial waste waters.

The benefits of ozone are based on its ability to reduce microbial loads, oxidize toxic organic compounds, and decrease the biological oxygen demand in the environment (Henry et al., 2000).

In 1982, the U.S. FDA affirmed that ozone is generally recognized as safe (GRAS), with specific limitations, for use as a disinfectant in bottled water (FDA, 1982). In 1997, U.S. FDA affirmed ozone as a GRAS substance for broad food applications, thus ozone now can be used as a disinfectant or a sanitizer in food processing in the United States (Graham, 1997).

2.6.3 Reactivity of Ozone

Ozone is one of the strongest oxidizing agents and most potent sanitizers known (Khadre et al., 2001). Ozone is relatively unstable in aqueous solutions. It decomposes continuously to oxygen according to a pseudo first-order reaction (Tomiyasu et al., 1985). Similar to other disinfectants for water treatment, ozone undergoes reaction with some water components. However, the unique feature of ozone is its decomposition into OH radicals which are the strongest oxidant in water (Staehelin and Hoigne, 1985). The reactivity of ozone is attributed to the great oxidizing power of radical species, especially the hydroxyl radical.

The ozone molecules act as dipoles with electrophilic and nucleophilic properties. Ozone

is able to oxidize inorganic substances and organic compounds to their highest stable oxidation state (Park et al., 2004). According to Langlais et al. (1991), ozone is very useful for cleaving double, triple, and aromatic bonds, but shows very low reactivity to aliphatic compounds with single bonds. Organic and inorganic compounds in aqueous solutions react with ozone in one of two pathways (Staehelin and Hoigne, 1985); (a) Direct reaction of organic compound with molecular ozone, (b) Decomposition of ozone in water into reactive hydroxyl radicals which react with the compound.

2.6.4 Decolorization by Ozone Treatment

Ozone has been widely applied for water disinfection purposes for almost a century (Gunten, 2003). The application of ozone in wastewater treatment is ideal because ozone not only kills microorganisms, but it also helps improve color, odor and taste. The quantities of ozone necessary for color removal depend on a number of parameters, including the nature of the oxidizable organic matter; the manner in which ozone is introduced; the concentration of ozone in air or oxygen; and the required result (Richard and Brener, 1984). Sarasa et al. (1998) found that 62% of azo dyes were destroyed after treatment of the wastewater with a combination of ozone and calcium hydroxide. The color and odor of table olive debittering wastewater could be removed by ozonation with less than 0.5 g of ozone (Beltran et al., 1998). According to Selcuk (2005), ozonation was relatively effective in reducing color absorbances and toxic effects of textile effluents. Ozone treatment for 20 min removed almost complete color absorbances (over 98%).

Several studies have shown that ozone treatment resulted in decoloration of food products. Jiang et al. (1997) utilized ozone to improve its undesirable color of mackerel surimi by destroying myoglobin in the muscle tissue. The ozonation for 30 min increased L (lightness)

value and whiteness at pH 3 and 4. Increase of whiteness is due to the destruction of disulfide bonds of the protein or peptides and heme pigment by ozone treatment.

Ozone also changed the surface color of some fruits and vegetables. Sakaki and Kondo (1981) reported the destruction of chlorophyll and carotenoids in spinach leaves after ozone treatment. Carrots treated with ozone showed lighter color compared to untreated carrots (Liew and Prange, 1994). Oxidative degradation of carbon-carbon double bonds resulted in decoloration of carotenoids (Henry et al., 2000).

2.6.5 Depolymerization by Ozone Treatment

Ozonolysis method provides a convenient, inexpensive, and easily controllable means for producing small polysaccharides or large oligosaccharide fragments (Wang et al., 1999). Ozone has been shown to be able to degrade macromolecules. Previous studies have shown that ozone and hydrogen peroxide easily depolymerize structural analogs of chitosan-cellulose, and can easily be removed from a reaction medium (Demin et al., 1993). According to Kabal'nova et al. (2001), ozone reacts with chitosan molecules quickly and one ozone molecule was consumed approximately for eight elemental component units of chitosan under mild conditions in only 15 min; and the amount of ozone consumption was 1.5 times as much as that of polysaccharide. Ozonation of chitosan in dilute acid solution (0.33M CH₃COOH and 0.1M HCl) with ozone-oxygen (2% O₃) resulted in a remarkable decrease of molecular mass of polysaccharide in proportion to reaction time or amount of applied ozone. In addition, increase of reaction temperature accelerated the initial rate of destruction and decreased the degree of polymerization (Kabal'nova et al., 2001).

A decrease in molecular size, which is associated with a decrease in solution viscosity, can be critical for their proper applications. No et al. (1999) found that ozone treatment (0.5

ppm) considerably decreased the viscosity of 1% chitosan solution by 63% (206 cP), 85% (84 cP), and 93% (42 cP) by treatment for 10, 20, and 30 min, respectively, in comparison with that (556 cP) of the untreated chitosan solution.

Several studies have shown the depolymerization mechanisms of macromolecules by ozone treatment. Wang et al. (1999) investigated the depolymerization of polysaccharide by ozone treatment. According to their research, carbohydrates are degraded by ozone in aqueous solution by several mechanisms: ozonolytic degradation of β -D-glycosidic linkages, oxidative degradation by radical species (hydroxyl radicals), and acid hydrolysis. Ozonolytic oxidation leads to selective depolymerization of polysaccharides, whereas radical reactions and pH-induced acid hydrolyses result in non-selective degradation pathways.

Kabal'nova et al. (2001) and their previous studies showed that the basic mechanism of depolymerization of chitosan during ozonation is the rupture of 1, 4- β -D-glycoside bonds in macromolecules. According to Demin et al. (1993), the initial stage of the interaction of ozone with polysaccharide is its electrophilic attack on C(1)-H bond with the formation of labile hydrotrioxides, destruction of which results in depolymerization of polysaccharide (Figure 3).

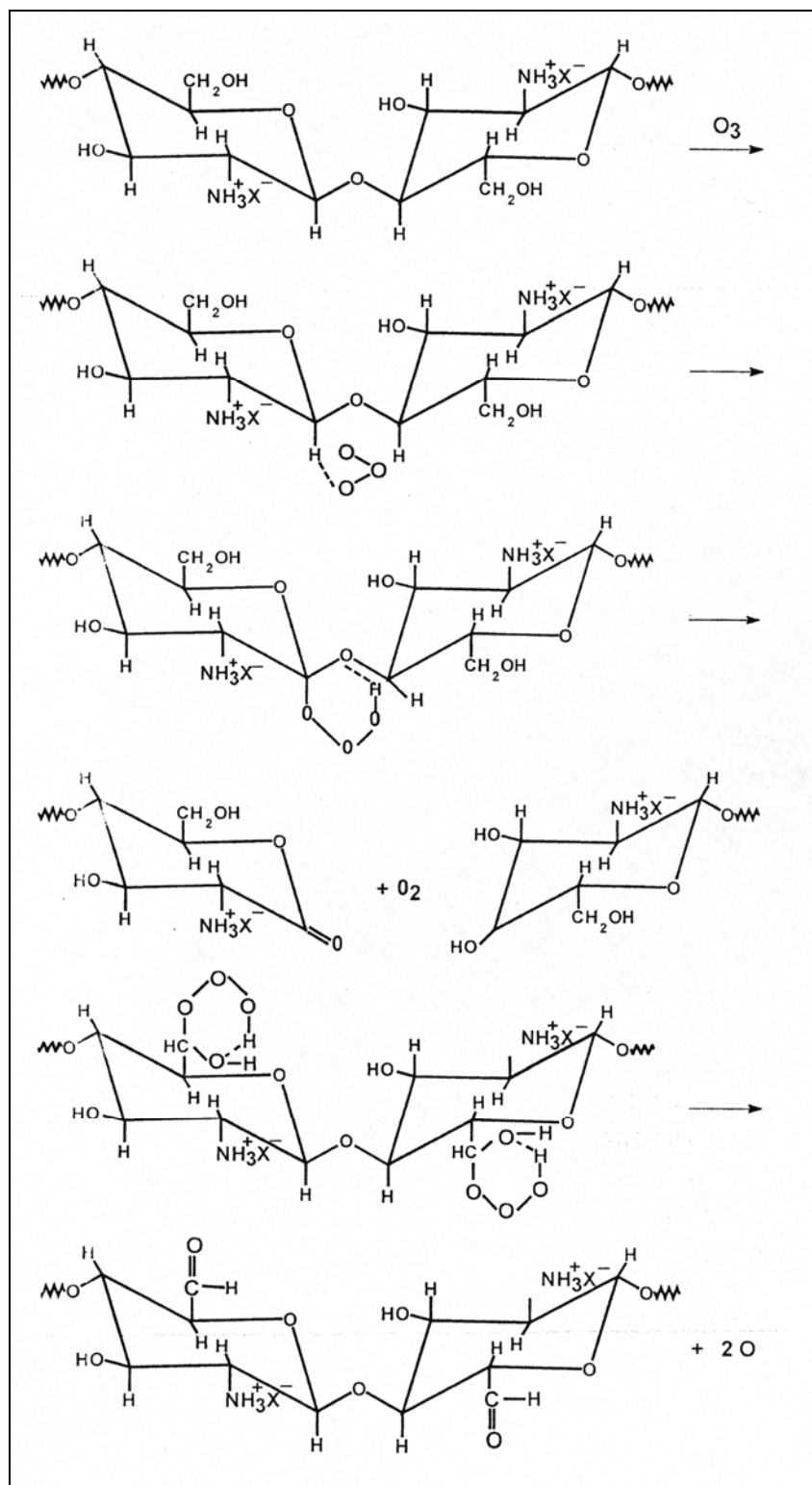


Figure 3. Mechanism of oxidative destruction under the action of ozone
 (Adopted from Kabal'nova et al., 2001)

CHAPTER 3

MATERIALS AND METHODS

3.1 Crawfish Chitosan Production

3.1.1 Raw Material

Undersized cooked crawfish shell waste was obtained from a commercial crawfish processor (Catahoula Crawfish Inc, St Martinville, Louisiana). Upon receipt, tail shells and the head were separated, and placed separately into double black polyethylene bags and kept in carton boxes. These materials were then stored at -20°C until utilized. Preceding preparation of crawfish chitosan, the frozen tail shells were thawed at ambient temperature, washed under running warm tap water to remove soluble organics, adherent proteins and other impurities. The tail shells were then dried in the oven (Model # E32-Bakbar Turbofan oven-Moffat Limited, Christchurch, New Zealand) at 70°C for a period of 24 hrs or longer until completely dried shells were obtained. To obtain a uniform size product, the dried shell was ground through a centrifugal grinding mill (Model # DR64857-Retsch / Brinkmann ZM-1, Westbury, NY) and shifted with 20-(0.841 mm) and 40-mesh (0.425 mm) sieves. Dried ground shell was placed in opaque plastic bottles and stored at ambient temperature until used.

3.1.2 Isolation of Chitosan

Crawfish chitosans were produced by the methods of No and Meyers (1995) and No et al. (2000) (Fig. 2). In this study, however, deproteinization and decolorization steps were eliminated. According to No et al. (2003), elimination of the deproteinization step yields a chitosan with a lower degree of deacetylation, but higher molecular weight and viscosity than those of deproteinized chitosan. However, elimination of the deproteinization step did not significantly affect other functional properties, such as binding capacities and antimicrobial

activities. Residual proteins in the shells can be removed during the deacetylation step that involves harsh alkali treatment. The decolorization step was eliminated to investigate the decolorizing effects of ozone treatment.

1) Demineralization (DM)

Crawfish shells, 800 g (400g×2), were demineralized with 1N HCl for 30 min at ambient temperature with a solid to solvent ratio of 1:15 (w/v) (No et al., 1989), and then filtered under vacuum. The filtrate was washed to neutralize for 30 min with tap water, rinsed with distilled water, and filtered under vacuum to remove excess moisture. The shells were dried in a forced-air oven at 60°C for 24 hrs.

2) Deacetylation (DA)

Removal of acetyl groups from the demineralized shell (300 g) was achieved by autoclaving at a pressure of 15 psi for 30 min at 121°C using 50% concentrated NaOH solution with a solid to solvent ratio of 1:10 (w/v) according to No et al. (2000). The resulting chitosans were washed to neutrality in running tap water, rinsed with distilled water, filtered, and dried at 60°C for 24 hr in a forced air oven.

3.2 Ozone Treatment

3.2.1 Ozone Treatment of Chitosan in Water

Chitosan suspension (4 g) in distilled water with a solid to solvent ratio of 1:100 (w/v) was prepared in erlenmeyer flasks. Ozone gas (12 Wt%) produced by an ozone generator (Lynntech Inc., Lynntech, TX) was purged into the chitosan sample flasks with a flow rate of approximate 140 scc/m (standard cubic centimeter per minute) for 0 (control), 5, 10, 15 and 20 min. at room temperature with constant agitation using a magnetic stirrer. Ozone concentration was measured by using a spectrophotometer at 254 nm and the weight percent of ozone was

calculated with the obtained value (absorbance) using the following equation:

$$\text{Wt \% O}_3 = \frac{\text{Absorbance} \times 254 \times 30}{24.313 + \text{Absorbance}} \times 100 \quad (\text{Lynntech, Inc. 2001})$$

Ozone treated chitosan was transferred to a buchner funnel, rinsed with one liter of distilled water, and filtered with Whatman No.4 filter paper under vacuum to remove excess moisture. The residue was dried at 60°C for 24 hr in a forced air oven. Three separate batches of ozone-treated chitosan were prepared.

3.2.2 Ozone Treatment of Chitosan in Acetic Acid Solution

Chitosan (4 g) was dissolved in 1% acetic acid solution (400 ml) to prepare 1% chitosan solution in erlenmeyer flasks. Ozone gas (12 Wt%) produced by ozone generator (Lynntech Inc., Lynntech, TX) was purged into the chitosan sample flasks with a flow rate of approximate 140 sccm (standard cubic centimeter per minute) for 0 (control), 5, 10, 15 and 20 min. at room temperature with constant agitation using a magnetic stirrer. After ozonation, ozone treated chitosan solutions were immediately precipitated with 1N NaOH by increasing the pH to 10.0 (Baxter et al., 2005). For complete precipitation, chitosan solutions were then allowed to stand for 8 hours. After that, precipitated chitosans were transferred to a buchner funnel and washed with distilled water until neutral pH 7.0 was reached. Collected chitosans were then centrifuged at 7500 rpm for 15 min, and freeze dried. Three separate batches of ozone-treated chitosan were prepared.

3.3 Measurement of Physicochemical Properties

3.3.1 Determination of Degree of Deacetylation

Degree of deacetylation of chitosan was determined by a colloid titration method (Toei and Kohara, 1976). Chitosan (0.5 g) was dissolved in aqueous 5% (v/v) formic acid solution (99.5 g). One gram of chitosan/formic acid solution was diluted to 30 ml distilled/demineralized

water in an erlenmeyer flask. After adding 2 to 3 drops of 0.1% toluidine blue indicator (w/v), the solution was titrated with n/400 potassium polyvinyl sulfate solution (PVSK; factor = 1.01; Wako chemical, Japan). A single molecule of PVSK reacts with each deacetylated amino group in the chitosan molecule. The degree of deacetylation was calculated from the molar ratio of deacetylated amino groups in the chitosan molecule, which was estimated from the volume of PVSK solution consumed. Measurements were made in duplicate on each sample, and degree of deacetylation was calculated using a following formula.

$$DD(\%) = \left[\frac{X / 161}{X / 161 + Y / 203} \right] \times 100$$

Where, X (Amount of glucosamine in molecule) = $1/400 \times 1/1000 \times f \times 161 \times V$

Y (Amount of N-acetylglucosamine in molecule) = $0.5 \times 1/100 - X$

V: Titrated volume (ml) of n/400 PVSK; f: Factor of PVSK solution = 1.01

3.3.2 Determination of Molecular Weight

Five different concentration (0.015625~1.0%) solutions of chitosan in 0.1M acetic acid – 0.2M NaCl (1:1, v/v) were prepared. The solution was passed through a filter (Whatman # 4) to remove insoluble materials. The ubbeloohde-type capillary viscometer (Canon-Fenske, No. OB) was used to measure the passage time of the solutions flowing through the capillary in a constant-temperature water bath at 25°C. Three measurements were made on each sample. The running times of the solution and solvent were recorded as seconds (Sec) and used to calculate intrinsic viscosity $[\eta]$.

$$\eta_{rel} \text{ (Relative viscosity)} = \frac{t_{solution} \text{ (efflux time of solution)}}{t_{solvent} \text{ (efflux time of solvent)}}$$

$$\eta_{sp} \text{ (Specific viscosity)} = \eta_{rel} - 1$$

$$\eta_{inh} \text{ (Inherent viscosity)} = \frac{(\ln \eta_{rel})}{C}$$

$$\eta_{red} \text{ (Reduced viscosity)} = \frac{\eta_{sp}}{C}$$

$$[\eta] = \lim_{c \rightarrow 0} \frac{\eta_{sp}}{c} \equiv \lim_{c \rightarrow 0} c^{-1} \ln \eta_{rel}$$

Where, C: Concentration of chitosan solution (g/dL, %)

Both η_{inh} and η_{red} were plotted on a same graph. The common intercept of the two plots on the ordinate at C=0 gives intrinsic viscosity $[\eta]$ (dL/g). The intrinsic viscosity was obtained by extrapolating reduced viscosity vs. concentration data to zero concentration.

The viscosity-average molecular weight of chitosan solutions was calculated using the Mark Houwink equation which provides the relationship between intrinsic viscosity and molecular weight.

$$[\eta] = K(Mw)^a \text{ (Mark Houwink equation)}$$

Where K and a are constants for given solute-solvent system and temperature. Values of 'K' and 'a' were 1.81×10^{-5} and 0.93, respectively (No et al., 2003b). Mw decrease (%) = $(Mw_i - Mw_t)/Mw_i * 100$, where Mw_i is the initial Mw (Molecular weight) and Mw_t is the Mw after t time of ozone treatment.

3.3.3 Color Measurement

The chitosan sample was put in a transparent petri dish (35×10 mm). Color of chitosan was measured using a Minolta spectrophotometer (Model CM-508d) which was standardized with a calibration white plate (X= 86.95, Y= 91.82, Z= 98.93; L*= 96.75, a*= -0.18, b*= -0.24).

The color of ozone-treated chitosan was expressed in L*, a*, b*, chroma (c), and hue angle (h) values. L*, a*, b* values indicate lightness, redness (negative a* value: greenness), and yellowness (negative b*: blueness), respectively. Chroma $[(a^{*2}+b^{*2})^{1/2}]$ is a measure of saturation, and represents the distance from the neutral axis. Hue angle $[\tan^{-1}(b^*/a^*)]$ is

represented as an angle ranging from 0° to 360°. Angles that range from 0° to 90° are reds, oranges, and yellows; 90° to 180° are yellows, yellow-greens, and greens. Whiteness of the sample was calculated using the following formula.

$$\text{Whiteness} = 100 - [(100-L^*)^2 + a^{*2} + b^{*2}]^{1/2}$$

Each measurement was recorded as an average value of five readings. Measurements were made in triplicate on each sample.

3.3.4 Determination of Viscosity

Chitosan solution was prepared in 1% (v/v) acetic acid at 1% concentration on a moisture-free basis. The chitosan solution was then filtered using Miracloth (rayon polyester; EMD Biosciences, CA) to remove insoluble materials. After that, the solution was allowed to stand for several hours to remove air bubbles. Viscosity of chitosans was determined with a Brookfield viscometer, model RVDV-II + (Brookfield Engineering Laboratories, Stoughton, MA). Measurements were made in triplicate on each sample using a RV No.2 spindle at 25°C. Values were reported in centipoise units (cP). The percentage of viscosity decrease was calculated as follows: viscosity decrease (%) = $(V_i - V_t) / V_i * 100$, where V_i is initial viscosity and V_t is the viscosity after t , time of ozone treatment.

3.3.5 Determination of Nitrogen Content

Nitrogen content of chitosan was determined using a Perkin Elmer 2410 Series II Nitrogen Analyzer (Perkin Elmer Instrument; Shelton, CT). Measurements were made in triplicate on each sample, and the results were displayed as percent nitrogen.

3.4 Antimicrobial Test of Chitosan

Four bacteria were tested for the antimicrobial activity of the ozone treated chitosan (0, 5, 10, 15 and 20 min.) using the spot-on-lawn method. These include two Gram-positive bacteria

(*Listeria monocytogenes* and *Staphylococcus aureus*) and two Gram-negative bacteria (*Escherichia coli* O157:H7 and *Pseudomonas fluorescens*). Three different concentrations (0.1, 0.5 and 1.0 %) of ozone-treated chitosan solutions (pH 5.5) were prepared in 1% (v/v) acetic acid. Blank sample (without chitosan) was also prepared for the comparison. The chitosan solutions were filtered with a No. 4 Whatman filter paper to remove insoluble impurities.

The bacterial cultures were grown in brain heart infusion (BHI; Difco Laboratories, Detroit, MI) broth for 16 h at 37°C. After incubation 10µl of the bacterial cultures were inoculated into 10 ml of melted BHI soft agar (tempered to 47°C). Next the soft agars with the indicators were poured over the surface of BHI agar plates and allowed to solidify. For the spot-on-lawn method 10 µl of the ozone treated chitosan and non-treated chitosan were inoculated onto the agar overlay plates and the plates were incubated at 37°C for 24 hour. After incubation, the plates were examined for zones of inhibition and zones greater than 2 mm were measured.

3.5 Statistical Analysis

Ozone treatment of chitosan was carried out in triplicate, and analyses were made in duplicate or triplicate on each sample. The data analysis was done using the Statistical Analysis System (SAS) software, version 9.1 (SAS Institute, Inc. Cary, NC). Average values (means) and standard deviations were reported. Mean separations were analyzed using the ANOVA and Tukey's studentized range tests (HSD) at $\alpha = 0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Crawfish Chitosan Production

Crawfish chitosans were produced from crawfish shell wastes according to the traditional chitosan production method (Fig. 2). However, deproteinization and decolorization steps were eliminated. Molecular weight and degree of deacetylation of resulting chitosans were approximately 1300 KDa and 76%, respectively. The molecular weight of natural chitin is usually larger than 1000 KDa, while that of commercial chitosan products are between 100 KDa and 1200 KDa. The harsh deacetylation processes used on chitin result in the degradation of chitin and chitosan molecules. Relatively high molecular weight chitosans were produced in this study because of the removal of deproteinization step. According to No et al. (2003b), elimination of the deproteinization step produced higher molecular weight and viscosity than those of deproteinized chitosan. However, elimination of deproteinization step did not significantly affect other functional properties, such as binding capacities and antimicrobial activities. Chitosan exhibited pale pink color and the yield of chitosan from starting materials (dried crawfish shells) was approximately 22% on a dry basis.

4.2 Effect of Ozonation on Molecular Weight of Chitosan

Molecular weight is one of the key factors governing the functional properties of chitosan. In general, the intrinsic viscosity of linear macromolecular substances is related to the molecular weight or degree of polymerization (Chen and Tsaih, 1998). Average molecular weights of ozone-treated chitosan were calculated from measured intrinsic viscosities shown in Fig. 4 using the Mark-Houwink equation. As shown in Fig.4, intrinsic viscosity of ozone-treated chitosan varied with different reaction conditions. Intrinsic viscosity of ozone-treated chitosan in

acetic acid solution decreased as the ozone treatment time increased from 0 to 10 min. Intrinsic viscosity of ozone-treated chitosan in acetic acid solution decreased from 8.99 to 0.85 (dL/g) in 20 min. Rapid decrease of the intrinsic viscosity was observed in the initial 10 min ozone treatment and then the intrinsic viscosity remained constant with increasing ozone dose. However, in the case of ozone-treated chitosan in water, intrinsic viscosity was not changed by ozone treatment probably due to the high density of hydrogen bonds between chitosan polymers in the solid state.

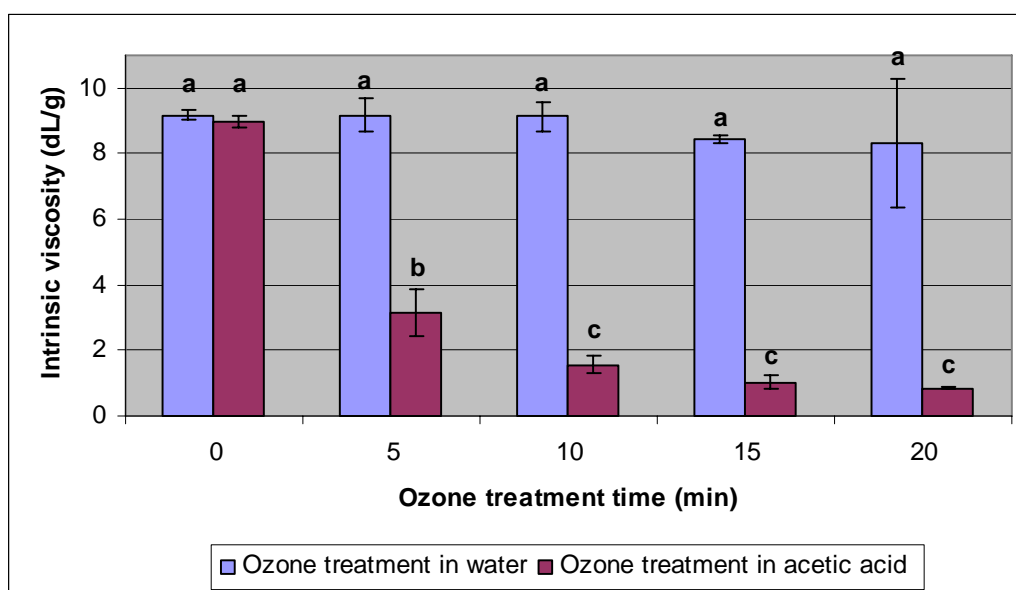


Figure 4. Intrinsic viscosity of ozone-treated chitosan

Mean values with different letters indicate significant differences ($p < 0.05$) between times for each solvent type.

Table 2 shows the molecular weight changes of ozone-treated chitosan. Results showed similar patterns with intrinsic viscosity. Molecular weight of ozone-treated chitosan in acetic acid solution decreased appreciably as the ozone treatment increased. Chitosans with molecular weight of 432, 201, 131, and 104 KDa were obtained by ozone treatment for 5, 10, 15, and 20

min, respectively. The rate of degradation of chitosan was apparently not proportional to the ozone treatment time. The molecular weight of chitosan was significantly reduced by 68% (432 KDa) in 5 min ozone treatment, and further ozonation for 20 min reduced the molecular weight of chitosan up to 92% (104 KDa) compared to the untreated chitosan (1333 KDa). On the other hand, ozonation in water did not affect the molecular weight of chitosan. A slight decrease in the molecular weight was observed after 15 min ozone treatment. However, statistical analyses showed that there were no significant differences of the molecular weight between ozone-treated chitosans in water.

Table 2. Molecular weight of ozone-treated chitosan

Ozone treatment time (min)	Molecular weight (KDa)	
	Ozone treatment in water	Ozone treatment in acetic acid
0	1362 ± 22.50 ^a (0)	1333 ± 29.69 ^a (0)
5	1363 ± 82.03 ^a (-0.07)	432 ± 105.98 ^b (67.59)
10	1356 ± 69.64 ^a (0.44)	201 ± 38.28 ^c (84.92)
15	1245 ± 17.90 ^a (8.59)	131 ± 24.76 ^c (90.17)
20	1229 ± 304.95 ^a (9.77)	104 ± 2.31 ^c (92.20)

Means ± standard deviation. Means with different letters within a column indicate significant differences ($p < 0.05$). Numbers in parentheses are % molecular weight decrease.

Previous studies have shown the mechanism of oxidative depolymerization of chitosan by oxidants such as ozone (Kabal'nova et al., 2001), hydrogen peroxide (Chang et al., 2001), and nitrous acid (Allan and Peyron, 1995). According to Kabal'nova et al. (2001), depolymerization of a macromolecule is mainly due to the oxidative destruction of β -D-glucoside bonds between units by the electrophilic attack on C(1)-H bond by ozone molecules. Another study conducted

by Liang et al. (2001) has shown that free radicals (hydroxyl and other free radicals) formed during the decomposition of hydrogen peroxide subsequently broke the β -1, 4 D-glucosidic linkages and decreased the molecular weight of chitosan. Therefore, it is believed that depolymerization of chitosan is mainly due to the oxidative degradation of polymer chains by ozone molecules and the free radicals formed during the decomposition of ozone molecule in aqueous solution.

Chitosan exhibits semi-crystalline polymer and the degree of crystallization is a function of the degree of deacetylation. Because of its stable crystalline structure and its large molecular weight, chitosan is insoluble in aqueous solution above pH 7. In addition, due to strong intermolecular hydrogen bonding, chitosan is insoluble in water. However, in dilute acids, the free amino groups are protonated and it becomes soluble below pH 6 (Ho et al., 2005). The protonation of amino groups in chitosan molecules in acidic condition leads to chain repulsion and swelling (Yao et al., 1994). Decrease in pH of aqueous solution makes the hydrogen bonds more flexible and promotes the solubility of chitosan in acidic solution (Hahn and Nam, 2004). Therefore, ineffectiveness of ozonation on depolymerization in water is probably due to the strong intermolecular hydrogen bonding and rigid molecular structure of chitosan in water.

Kabal'nova et al. (2001) found that ozonation of water suspension of chitosan resulted in oxidation of amino groups accompanied by deamination and formation of insoluble gel fractions in acidic solution. However, ozonation in dilute acid solution did not change the elemental composition of chitosan. When chitosan is dissolved in acidic solution, most amino groups in the chitosan molecule are protonated. It has been shown that the stability of amino groups to ozone increases considerably under their protonation with acids (Pryor et al., 1984). Fig. 5 shows the nitrogen contents of ozone-treated chitosan. The results agree with those of Kabal'nova et al.

(2001). Ozonation in acetic acid solution did not affect the nitrogen contents of chitosan, whereas ozonation in water resulted in a slight decrease in nitrogen contents of chitosan.

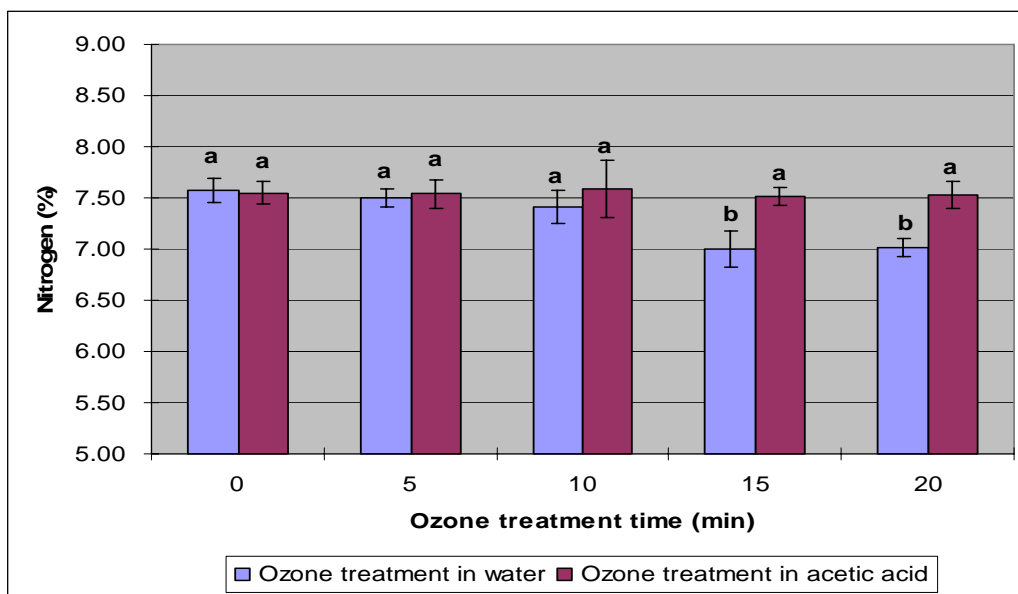


Figure 5. Nitrogen contents of ozone-treated chitosan
Mean values with different letters indicate significant differences ($p < 0.05$) between times for each solvent type.

4.3 Effect of Ozonation on Degree of Deacetylation of Chitosan

The effects of ozone treatment on the degree of deacetylation are shown in Fig. 6. The degree of deacetylation of the samples ranged from 73.58 to 76.39%. Statistically, there were no significant differences between ozone-treated chitosans in acetic acid solution. However, ozone-treated chitosan in water for 15~20 min showed a lower degree of deacetylation than untreated chitosan. Based on the degree of deacetylation calculation, a decrease in average volume of potassium polyvinyl sulfate (PVSK) solution consumed indicates a decrease in X value which is the amount of glucosamine in the chitosan molecule. According to Choi et al. (2004), lower nitrogen contents are mainly due to the hydrolytic deamination. As shown in Fig. 5, ozone-

treated chitosan in water for 15~20 min showed lower nitrogen contents compared to the untreated chitosans. Therefore, deamination during ozonation in water resulted in a decrease in the degree of deacetylation. However, according to Pryor et al. (1984), the stability of amino groups to ozone increases considerably under their protonation with acidic condition. Therefore, ozonation in acetic acid solution did not affect the degree of deacetylation. This study showed that ozone treatment was not effective in removing acetyl groups from the chitosan molecule. Similarly, Baxter et al. (2005) utilized ultrasonication in acetic acid solution to reduce the molecular weight of chitosan and reported that no significant differences were observed between samples for degree of deacetylation, regardless of sonication treatment time.

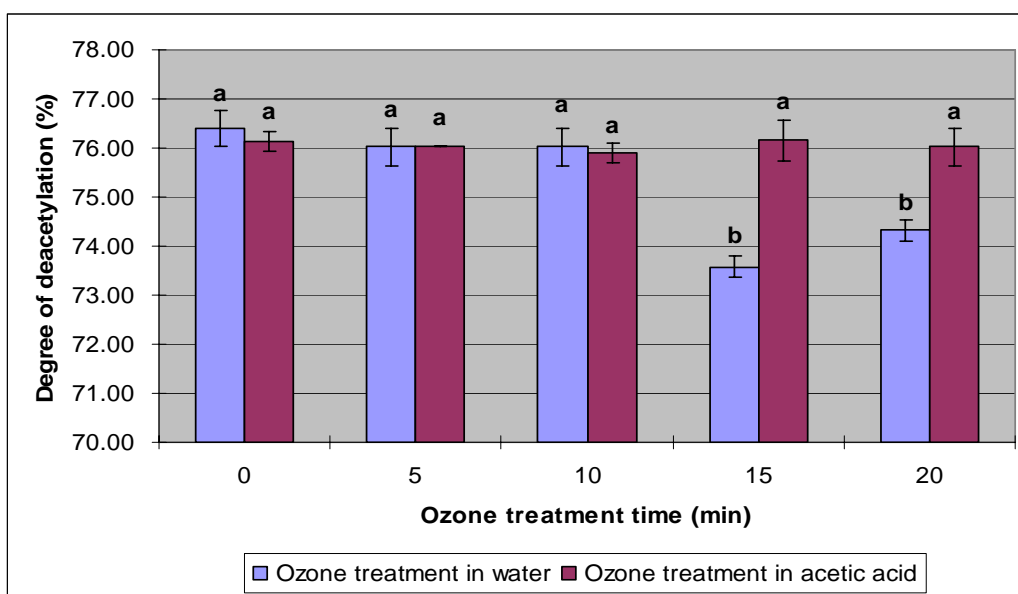


Figure 6. Degree of deacetylation of ozone-treated chitosan
Mean values with different letters indicate significant differences ($p < 0.05$) between times for each solvent type.

4.4 Effect of Ozonation on Viscosity of Chitosan

It is known that reduction of molecular mass of polymers is closely related to the

reduction of polymer solution viscosity. Fig. 7 shows the changes in viscosity of chitosan solution with ozone treatment in different conditions. The viscosity rapidly decreased by 91% from 331 to 29 cP in the initial 5 min and then slowly decreased to 14, 12, and 10 cP in 10, 15, and 20 min, respectively. Decrease in viscosity of chitosan solution with ozone treatment also was reported by No et al. (1999). Their study showed that ozone treatment (0.5 ppm) considerably decreased the viscosity by 63% (206 cP) for 10 min in comparison with untreated chitosan solution (556 cP). The differences of viscosity decrease rate were probably due to the different ozone concentration, flow rate of introduced ozone gas, and the initial viscosity of chitosan solution. In case of the ozone treatment in water, ozone-treated chitosan for 5 min showed a significant decrease in viscosity, however, after 5 min, there were no significant differences in viscosity between samples. Viscosity changes of chitosan solution also can be contributed to the degradation of chitosan molecule by the high oxidation potential of ozone.

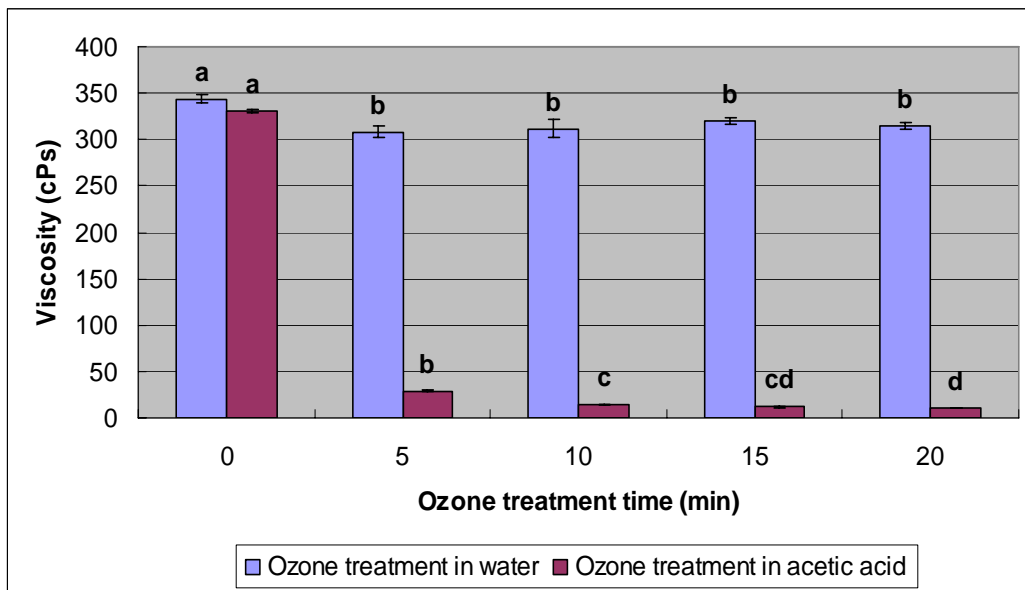


Figure 7. Viscosity of ozone-treated chitosan

Mean values with different letters indicate significant differences ($p < 0.05$) between times for each solvent type.

4.5 Effect of Ozonation on Color of Chitosan

Table 3 and 4 show the changes in color of chitosan with ozone treatment in water and acetic acid solution, respectively. As seen in Table 3, a^* values slightly decreased and b^* and H values increased as the ozone treatment time increased. However, no significant changes of L^* and C were observed among ozone-treated samples in water.

Increase in b^* and H values indicates the development of yellowness during ozonation. These changes, however, did not contribute to noticeable color changes of chitosan. There were also no significant differences in whiteness of ozone-treated chitosans in water (Fig. 8).

However, ozone treatment of chitosan in acetic acid solution resulted in significant color changes of the chitosan (Table 4). Except for a^* values, all color values appreciably increased as the ozone treatment period increased with the 5 min ozone-treated sample showing the highest L^* value (82.15) among samples. Further ozone treatment showed no significant differences in L^* values. However, there were no statistical differences between samples. a^* values rapidly decreased from 2.80 to -2.15 after 15 min of ozone treatment, whereas a slight increase of a^* value was observed during further ozone treatment. In b^* analysis, ozone treatment for 5 min showed the lowest b^* value (13.20); however, further ozone treatment resulted in a rapid increase in b^* value to 28.86. Ozone treatment of 1% acetic acid solution for 20 min did not result in color changes. The highest L^* and the lowest b^* value of 5 min ozone-treated chitosan contributed to the highest whiteness among the ozone-treated chitosans (Fig. 8). A decrease in a^* value indicates the loss of red pigments in chitosan. Ozonation for 0~15 min produced chitosan with higher L^* and whiteness compared to untreated chitosan. These are probably due to the decrease in a^* value. Increase of b^* value indicates the development of yellowness and resulted in the decrease of whiteness. As can be seen in Fig.7, the highest whiteness was observed in 5

Table 3. Color analysis of ozone-treated chitosan in water

Ozone treatment time (min)	Color value				
	L*	a*	b*	C	H
0	72.69 ± 0.06 ^a	2.82 ± 0.02 ^a	16.45 ± 0.02 ^c	16.69 ± 0.03 ^a	80.32 ± 0.05 ^c
5	72.63 ± 0.08 ^a	2.78 ± 0.03 ^{ab}	16.47 ± 0.00 ^c	16.63 ± 0.14 ^a	80.43 ± 0.09 ^{bc}
10	72.66 ± 0.07 ^a	2.76 ± 0.03 ^b	16.53 ± 0.02 ^{bc}	16.69 ± 0.12 ^a	80.53 ± 0.09 ^{ab}
15	72.77 ± 0.08 ^a	2.75 ± 0.02 ^b	16.62 ± 0.06 ^{ab}	16.77 ± 0.07 ^a	80.62 ± 0.05 ^{ab}
20	72.75 ± 0.02 ^a	2.73 ± 0.02 ^b	16.67 ± 0.04 ^a	16.82 ± 0.1 ^a	80.70 ± 0.09 ^a

Means ± standard deviation. Means with different letters within a column indicate significant differences ($p < 0.05$). L* = Lightness, a* = Redness (-a* = Greenness), b* = Yellowness (-b* = Blueness), C = Chroma, and H = Hue angle.

Table 4. Color analysis of ozone-treated chitosan in acetic acid solution

Ozone treatment time (min)	Color value				
	L*	a*	b*	C	H
0	72.96 ± 0.10 ^b	2.80 ± 0.00 ^a	16.42 ± 0.07 ^{bc}	16.65 ± 0.07 ^b	80.33 ± 0.03 ^b
5	82.15 ± 0.52 ^a	-1.07 ± 0.11 ^b	13.20 ± 0.14 ^c	13.24 ± 0.15 ^c	94.64 ± 0.41 ^a
10	81.04 ± 2.34 ^a	-0.87 ± 1.52 ^b	18.59 ± 1.10 ^{bc}	18.65 ± 1.07 ^b	92.84 ± 4.66 ^a
15	81.59 ± 0.24 ^a	-2.10 ± 0.16 ^b	23.10 ± 0.68 ^{ab}	23.19 ± 0.69 ^a	95.18 ± 0.28 ^a
20	80.91 ± 1.45 ^a	-1.82 ± 0.62 ^b	28.86 ± 7.91 ^a	25.60 ± 2.23 ^a	94.17 ± 1.72 ^a

Means ± standard deviation. Means with different letters within a column indicate significant differences ($p < 0.05$). L* = Lightness, a* = Redness (-a* = Greenness), b* = Yellowness (-b* = Blueness), C = Chroma, and H = Hue angle.

min ozone-treated chitosan. Further ozonation for 5~15 min, however, resulted in gradual decrease in whiteness of chitosan, and there were no significant differences of whiteness between ozone-treated chitosan for 20 min and untreated chitosan.

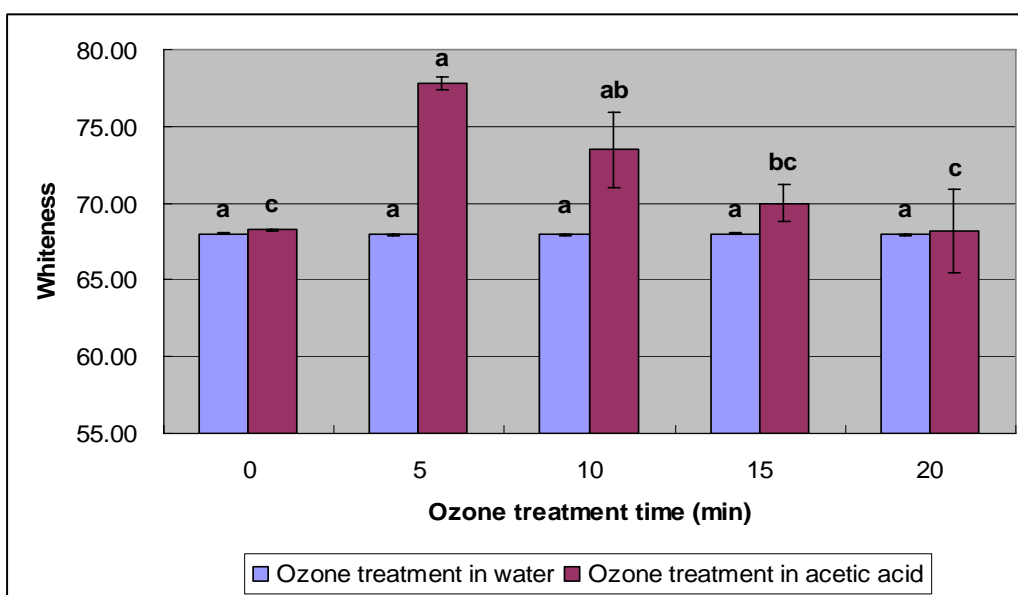


Figure 8. Whiteness of ozone-treated chitosan

Mean values with different letters indicate significant differences ($p < 0.05$) between times for each solvent type.

The color of chitosan is associated with the carotenoid pigment. The main component of carotenoid fraction in crustacean exoskeleton is astaxanthin, and the carotenoids are strongly bound to chitin molecule and associated with proteins in the epithelial layer of exoskeleton. (No et al., 1989; Shahidi and Synowiecki, 1991). According to Henry et al. (2000), oxidative degradation of carbon-carbon double bonds resulted in decoloration of carotenoids. Therefore, decolorization of chitosan probably resulted from the oxidative destruction of double bonds present in carotenoids by ozone treatment. The reason that prolonged ozone treatment creates the yellowing chitosan has not been investigated yet. The development of yellow pigments, however,

is probably due to the side reactions that occurred during ozonation. In another study, Choi et al. (2002) utilized irradiation to produce low molecular weight chitosan oligomers. Similarly, intense brown color was produced with increasing irradiation dose (over 100 KGy). According to Nagasawa et al. (2000), the browning during irradiation was due to double bond formation by chain scission.

4.6 Antimicrobial Activity of Chitosan

Results were reported in both diameter of inhibition zone (mm) and degree of inhibition within the zones because some inhibition zones showed different intensity of inhibition even though they had the same inhibition diameter. Results were shown in Tables 5 to 10.

Results showed that the antimicrobial activity of chitosan was dependent on its molecular weight, concentration, and the type of bacteria. Tables 5 to 7 show the antimicrobial activity of different molecular weights and concentrations of chitosan against *Listeria monocytogenes* and *Staphylococcus aureus* (Gram-positive bacteria). At low concentration (0.1%), there were no antimicrobial activities against the *Listeria monocytogenes* and *Staphylococcus aureus* regardless of size of molecular weight (Table 5). For *Listeria monocytogenes*, 0.5% chitosan with molecular weight of 102~106 KDa showed slight inhibition, however, chitosan with molecular weight of 170~1333 KDa did not inhibit the growth of the bacteria (Table 6). All of the 1.0% chitosan samples showed antimicrobial activity against *Listeria monocytogenes* (Table 7). However, the chitosan with molecular weight of 102~244 KDa greatly inhibited the growth of *Listeria monocytogenes* among the samples. For *Staphylococcus aureus*, 0.5% chitosan with molecular weight of 102~244 KDa showed the inhibition of bacterial growth with greater inhibition zones, but several colonies were observed in the zones (Table 6). Higher concentration of chitosan (1.0%) with molecular weight of 102~244

KDa markedly inhibited growth of the bacteria with clear inhibition zones (Table 7).

Antimicrobial activities of chitosan with different molecular weights and at different concentrations against *Escherichia coli* O157:H7 and *Pseudomonas fluorescens* are shown in Tables 8 to 10. Chitosan at 0.1% with molecular weight of 112~549 KDa slightly inhibited the growth of *Escherichia coli*, but chitosan with molecular weight below 112 KDa did not inhibit the growth (Table 8). All 0.5% chitosan samples had slight inhibition against *Escherichia coli*, but there were no significant differences in degree of inhibition between the samples (Table 9). However, with 1.0% chitosan, high molecular weight chitosan (342~1333 KDa) showed a greater inhibition than low molecular weight chitosan (102~244 KDa) (Table 10). In contrast, for *Pseudomonas fluorescens*, low molecular weight chitosan showed stronger antimicrobial activities than high molecular weight. Chitosan of 342~1333 KDa did not inhibit the growth of *Pseudomonas fluorescens*, whereas chitosan of 102~159 KDa greatly inhibited the growth of this bacteria with clear inhibition zones even if the concentration was quite small (0.1%) (Table 8). Both 0.5 and 1.0% chitosan showed similar patterns of inhibitions against *Pseudomonas fluorescens*, however, 1.0% chitosan of 122~159 KDa exhibited the greatest inhibition among samples.

Molecular weight relationships of antibacterial activity by chitosan have been reported by various investigators (Jeon et al., 2001; No et al., 2002; Zheng and Zhu, 2003). However, there are lots of reports with conflicting results. One study showed that the antimicrobial effect on *E.coli* decreased as the molecular weight of chitosan increased. In addition, it indicated that the optimum molecular weight of chitosan for antimicrobial activity was 1.5 KDa (Zheng and Zhu, 2003). In contrast, recent studies on antimicrobial activity of chitosan and its oligomers have revealed that chitosan is more effective in inhibiting growth of bacteria than chitosan

oligomers (Jeon et al., 2001; No et al., 2002). In addition, Jeon and Kim (2000) showed that the molecular weight of chitooligosaccharides is critical for microorganism inhibition and is required to be higher than 10 KDa. Another study showed that chitosan with 40 KDa could inhibit 90% of *Staphylococcus aureus* and *E.coli* at a concentration of 0.5% and chitosan with 180 KDa could inhibit completely the growth of *Staphylococcus aureus* and *E.coli* at concentration of 0.05% (Shin et al., 1997). In contrast, according to No et al. (2002), 0.1% chitosan of 746 KDa was most effective against *E.coli*.

In this study, chitosan with molecular weight ranging from 102 to 244 KDa showed greater antimicrobial activity against *Listeria monocytogenes*, *Staphylococcus aureus*, and *Pseudomonas fluorescens*, whereas for *E.coli*, high molecular weight chitosan was more effective in growth inhibition than low molecular weight chitosan. Results indicated that the antimicrobial activities of chitosan differed depending on the different molecular weight, concentration of chitosan, and types of microorganisms. However, the antimicrobial effects strengthened as the concentration of chitosan increased, regardless of molecular size. Uchida et al. (1989) reported the chitosan MIC (minimum inhibition concentration) for *E.coli* and *S. aureus* to be 0.025% and 0.05%, respectively. In another study, Yun et al. (1999) found differences in MIC values of chitosan, ranging from 0.05% to > 0.2% for *E.coli* and 0.04% to 0.1% for *S. aureus*. However, our results were quite different with previous studies. In this study, chitosan did not exhibit any antimicrobial activity against *S. aureus* at the 0.1% of chitosan concentrations. This difference is probably due to differences in experimental methods, chitosan characteristics, or medium pH applied.

Table 5. Antimicrobial activity of 0.1% chitosan against *Listeria monocytogenes* and *Staphylococcus aureus*

Ozone Treatment Time	MW (KDa)	Bacteria			
		<i>Listeria monocytogenes</i>		<i>Staphylococcus aureus</i>	
		Inhibition zone (mm)	Degree of inhibition	Inhibition zone (mm)	Degree of inhibition
0min	1333	0.0 (0.00)	–	0.0 (0.00)	–
5min-1	549	0.0 (0.00)	–	0.0 (0.00)	–
5min-2	406	0.0 (0.00)	–	0.0 (0.00)	–
5min-3	342	0.0 (0.00)	–	0.0 (0.00)	–
10min-1	170	0.0 (0.00)	–	0.0 (0.00)	–
10min-2	190	0.0 (0.00)	–	0.0 (0.00)	–
10min-3	244	0.0 (0.00)	–	0.0 (0.00)	–
15min-1	112	0.0 (0.00)	–	0.0 (0.00)	–
15min-2	159	0.0 (0.00)	–	0.0 (0.00)	–
15min-3	122	0.0 (0.00)	–	0.0 (0.00)	–
20min-1	106	0.0 (0.00)	–	0.0 (0.00)	–
20min-2	106	0.0 (0.00)	–	0.0 (0.00)	–
20min-3	102	0.0 (0.00)	–	0.0 (0.00)	–
Blank	n/a	0.0 (0.00)	–	0.0 (0.00)	–

Strong inhibition with clear zones = +++, Inhibition with several colonies in the zones = ++

Slight inhibition with fuzzy zones = +, No inhibition = –

M* = mixed results, Blank = acetic acid solution without chitosan (pH=5.5)

Numbers (-1, -2, -3) with ozone treatment time indicate different batches of ozone treatment.

Numbers in parentheses are standard deviation of duplicate measurements.

Table 6. Antimicrobial activity of 0.5% chitosan against *Listeria monocytogenes* and *Staphylococcus aureus*

Ozone Treatment Time	MW (KDa)	Bacteria			
		<i>Listeria monocytogenes</i>		<i>Staphylococcus aureus</i>	
		Inhibition zone (mm)	Degree of inhibition	Inhibition zone (mm)	Degree of inhibition
0min	1333	0.0 (0.00)	–	7.0 (0.00)	+
5min-1	549	0.0 (0.00)	–	9.0 (0.00)	+
5min-2	406	0.0 (0.00)	–	9.5 (0.71)	+
5min-3	342	0.0 (0.00)	–	8.5 (0.71)	++
10min-1	170	0.0 (0.00)	–	8.5 (0.71)	++
10min-2	190	0.0 (0.00)	–	9.0 (0.00)	++
10min-3	244	0.0 (0.00)	–	9.0 (0.00)	++
15min-1	112	0.0/8.0 ^{M*} (5.66)	–/+ ^{M*}	9.5 (0.71)	++
15min-2	159	0.0/9.0 ^{M*} (6.36)	–/+ ^{M*}	9.0 (0.00)	++
15min-3	122	0.0/9.0 ^{M*} (6.36)	–/+ ^{M*}	9.0 (0.00)	++
20min-1	106	8.0 (1.41)	+	8.5 (0.71)	++
20min-2	106	8.0 (0.00)	+	9.0 (1.41)	++
20min-3	102	9.0 (0.00)	+	8.0 (0.00)	++
Blank	n/a	0.0 (0.00)	–	0.0 (0.00)	–

Strong inhibition with clear zones = +++, Inhibition with several colonies in the zones = ++

Slight inhibition with fuzzy zones = +, No inhibition = –

M* = mixed results, Blank = acetic acid solution without chitosan (pH=5.5)

Numbers (-1, -2, -3) with ozone treatment time indicate different batches of ozone treatment.

Numbers in parentheses are standard deviation of duplicate measurements.

Table 7. Antimicrobial activity of 1.0% chitosan against *Listeria monocytogenes* and *Staphylococcus aureus*

Ozone Treatment Time	MW (KDa)	Bacteria			
		<i>Listeria monocytogenes</i>		<i>Staphylococcus aureus</i>	
		Inhibition zone (mm)	Degree of inhibition	Inhibition zone (mm)	Degree of inhibition
0min	1333	7.0 (0.00)	++	7.0 (0.00)	++
5min-1	549	7.0 (0.00)	++	7.5 (0.71)	++
5min-2	406	8.5 (0.71)	++	9.5 (0.71)	+++
5min-3	342	7.5 (0.71)	++	9.5 (0.71)	+++
10min-1	170	9.5 (0.71)	+++	10.5 (2.12)	+++
10min-2	190	9.0 (0.00)	+++	10.0 (1.41)	+++
10min-3	244	10.0 (0.00)	+++	10.5 (0.71)	+++
15min-1	112	10.5 (0.71)	+++	10.0 (0.00)	+++
15min-2	159	9.0 (0.00)	+++	10.5 (0.71)	+++
15min-3	122	10.0 (1.41)	+++	9.5 (0.71)	+++
20min-1	106	9.5 (2.12)	+++	8.0 (1.41)	+++
20min-2	106	9.0 (1.41)	+++	10.0 (0.00)	+++
20min-3	102	9.0 (1.41)	+++	11.5 (2.12)	+++
Blank	n/a	0.0 (0.00)	–	0.0 (0.00)	–

Strong inhibition with clear zones = +++, Inhibition with several colonies in the zones = ++

Slight inhibition with fuzzy zones = +, No inhibition = –

M* = mixed results, Blank = acetic acid solution without chitosan (pH=5.5)

Numbers (-1, -2, -3) with ozone treatment time indicate different batches of ozone treatment.

Numbers in parentheses are standard deviation of duplicate measurements.

Table 8. Antimicrobial activity of 0.1% chitosan against *Escherichia coli* and *Pseudomonas fluorescens*

Ozone Treatment Time	MW (KDa)	Bacteria			
		<i>Escherichia coli</i>		<i>Pseudomonas fluorescens</i>	
		Inhibition zone (mm)	Degree of inhibition	Inhibition zone (mm)	Degree of inhibition
0min	1333	0.0 (0.00)	–	0.0 (0.00)	–
5min-1	549	10.0 (1.41)	+	0.0 (0.00)	–
5min-2	406	9.5 (0.71)	+	0.0 (0.00)	–
5min-3	342	9.5 (0.71)	+	0.0 (0.00)	–
10min-1	170	8.5 (0.71)	+	9.0/0.0 ^{M*} (6.36)	-/++ ^{M*}
10min-2	190	8.5 (0.71)	+	8.0/0.0 ^{M*} (5.66)	-/++ ^{M*}
10min-3	244	9.0 (0.00)	+	8.0/0.0 ^{M*} (5.66)	-/++ ^{M*}
15min-1	112	8.5 (0.71)	+	10.5 (0.71)	+++
15min-2	159	0.0/9.0 ^{M*} (6.36)	-/+ ^{M*}	10.5 (0.71)	+++
15min-3	122	0.0/9.0 ^{M*} (6.36)	-/+ ^{M*}	10.5 (0.71)	+++
20min-1	106	0.0 (0.00)	–	10.0 (1.41)	+++
20min-2	106	0.0 (0.00)	–	10.5 (0.71)	+++
20min-3	102	0.0 (0.00)	–	9.5 (0.71)	+++
Blank	n/a	0.0 (0.00)	–	0.0 (0.00)	–

Strong inhibition with clear zones = +++, Inhibition with several colonies in the zones = ++

Slight inhibition with fuzzy zones = +, No inhibition = –

M* = mixed results, Blank = acetic acid solution without chitosan (pH=5.5)

Numbers (-1, -2, -3) with ozone treatment time indicate different batches of ozone treatment.

Numbers in parentheses are standard deviation of duplicate measurements.

Table 9. Antimicrobial activity of 0.5% chitosan against *Escherichia coli* and *Pseudomonas fluorescens*

Ozone Treatment Time	MW (KDa)	Bacteria			
		<i>Escherichia coli</i>		<i>Pseudomonas fluorescens</i>	
		Inhibition zone (mm)	Degree of inhibition	Inhibition zone (mm)	Degree of inhibition
0min	1333	8.5 (0.71)	+	0.0 (0.00)	-
5min-1	549	9.0 (0.00)	+	0.0 (0.00)	-
5min-2	406	9.5 (0.71)	+	6.0/0.0 ^{M*} (4.24)	-/++ ^{M*}
5min-3	342	9.0 (1.41)	+	7.0/0.0 ^{M*} (4.95)	-/++ ^{M*}
10min-1	170	9.5 (2.12)	+	10.5 (0.71)	+++
10min-2	190	9.5 (0.71)	+	11.0 (0.00)	+++
10min-3	244	9.0 (1.41)	+	11.0 (1.41)	+++
15min-1	112	9.0 (0.00)	+	12.0 (0.00)	+++
15min-2	159	8.5 (0.71)	+	12.0 (0.00)	+++
15min-3	122	9.0 (1.41)	+	11.0 (0.00)	+++
20min-1	106	9.5 (0.71)	+	11.5 (0.71)	+++
20min-2	106	9.0 (1.41)	+	12.0 (0.00)	+++
20min-3	102	9.0 (0.00)	+	12.0 (0.00)	+++
Blank	n/a	0.0 (0.00)	-	0.0 (0.00)	-

Strong inhibition with clear zones = +++, Inhibition with several colonies in the zones = ++

Slight inhibition with fuzzy zones = +, No inhibition = -

M* = mixed results, Blank = acetic acid solution without chitosan (pH=5.5)

Numbers (-1, -2, -3) with ozone treatment time indicate different batches of ozone treatment.

Numbers in parentheses are standard deviation of duplicate measurements.

Table 10. Antimicrobial activity of 1.0% chitosan against *Escherichia coli* and *Pseudomonas fluorescens*

Ozone Treatment Time	MW (KDa)	Bacteria			
		<i>Escherichia coli</i>		<i>Pseudomonas fluorescens</i>	
		Inhibition zone (mm)	Degree of inhibition	Inhibition zone (mm)	Degree of inhibition
0min	1333	9.0 (0.00)	++	7.5 (0.71)	++
5min-1	549	8.0 (0.00)	++	8.0 (1.41)	++
5min-2	406	9.0 (0.00)	++	9.0 (0.00)	+++
5min-3	342	8.5 (2.12)	++	10.5 (0.71)	+++
10min-1	170	9.5 (0.71)	+	11.5 (0.71)	+++
10min-2	190	10.0 (1.41)	+	11.5 (0.71)	+++
10min-3	244	9.5 (0.71)	+	13.0 (1.41)	+++
15min-1	112	9.0 (0.00)	+	13.0 (0.00)	+++
15min-2	159	9.5 (0.71)	+	13.0 (1.41)	+++
15min-3	122	9.0 (1.41)	+	12.0(1.41)	+++
20min-1	106	9.5 (0.71)	+	12.0 (0.00)	+++
20min-2	106	8.5 (0.71)	+	12.0 (0.00)	+++
20min-3	102	9.0 (0.00)	+	12.0 (0.00)	+++
Blank	n/a	0.0 (0.00)	–	0.0 (0.00)	–

Strong inhibition with clear zones = +++, Inhibition with several colonies in the zones = ++

Slight inhibition with fuzzy zones = +, No inhibition = –

M* = mixed results, Blank = acetic acid solution without chitosan (pH=5.5)

Numbers (-1, -2, -3) with ozone treatment time indicate different batches of ozone treatment.

Numbers in parentheses are standard deviation of duplicate measurements.

CHAPTER 5

SUMMARY AND CONCLUSION

In this research, the effects of ozone treatment on depolymerization and decolorization of chitosan were investigated. Physicochemical changes of chitosan were also examined. Obviously, this study has demonstrated that ozone treatment of chitosan in water was not effective in degradation of chitosan molecules and removal of chitosan pigments. Ozonation of chitosan in water over 15 min resulted in deamination by undesirable side reactions. On the other hand, ozone treatment of chitosan in acetic acid solution resulted in reduction of molecular size with destruction of chitosan pigments. Ozone treatment for 20 min reduced molecular weight of chitosan by 92% compared to untreated chitosan with a decrease in viscosity of chitosan solution from 331 to 10 cP. In addition, ozone treatment of chitosan for 5 min markedly increased the whiteness of chitosan. However, further ozonation gradually reduced whiteness of chitosan and the whiteness of ozone-treated chitosan for 20 min to levels similar to those of untreated chitosan. It was also demonstrated that ozone treatment of chitosan in both water and acetic acid solution was not effective in removing acetyl groups (Deacetylation) in chitosan molecules.

In the antimicrobial test, ozone-treated chitosan (102~1333 KDa) showed different antimicrobial activities depend on its molecular weight, concentration, and different bacterial species. Chitosan with molecular weight ranging from 102 to 244 KDa showed greater antimicrobial activity against *Listeria monocytogenes*, *Staphylococcus aureus*, and *Pseudomonas fluorescens*, whereas for *E.coli*, high molecular weight chitosan was more effective in inhibition of growth than low molecular weight chitosan.

Currently depolymerization is accomplished by chemical or enzymatic methods. Therefore, ozone treatment showed the potential to replace time consuming and expensive

chemical and enzymatic methods that are currently used to depolymerize and decolorize chitosan. Moreover, ozone treatment may reduce chemical wastes resulted from depolymerization and decolorization of chitosan by strong acids and bases. However, ozone treatment also caused some undesirable side effects such as development of yellowness as ozone treatment time increased in a similar manner to other chemical methods. To elucidate undesirable reactions during ozonation, structural conformation and molecular weight distribution of the depolymerized chitosan have to be analyzed.

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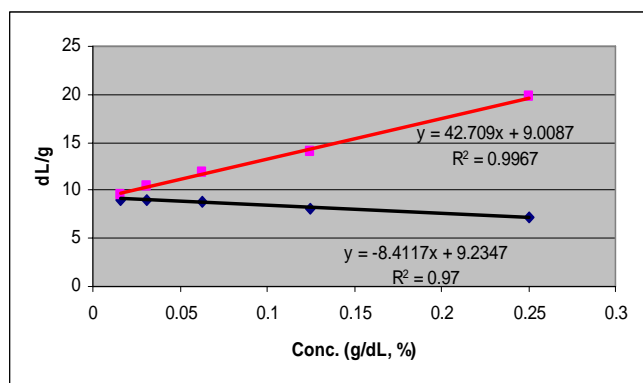
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APPENDIX A. DATA OF MOLECULAR WEIGHT CALCULATION

1. Ozone-treated chitosan in water

Sample: 0 min-1

Conc. %	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.015625	227	228	228	227.67	1.149832	0.149832	8.935395	9.589226
0.03125	264	261	263	262.67	1.326599	0.326599	9.043801	10.451178
0.0625	343	347	346	345.33	1.744108	0.744108	8.899890	11.905724
0.125	546	543	543	544.00	2.747475	1.747475	8.085458	13.979798
0.25	1180	1176	1179	1178.33	5.951178	4.951178	7.134357	19.804714



Mark Houwink equation : $[\eta] = KMw^a$

$$K = 1.81 \cdot 10^{-5} \text{ dl/g}$$

$$a = 0.93$$

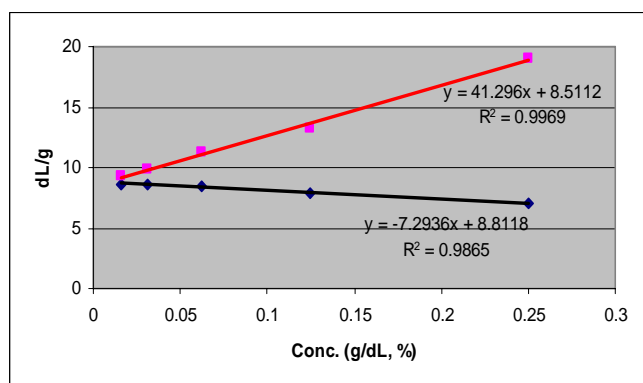
$$[\eta] = (9.0087 + 9.2347) / 2 = 9.1217 \text{ (dL/g)}$$

$$9.1217 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$$

$$Mw = 1353973 \text{ g/mol}$$

Sample: 5 min-1

Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.015625	227	226	227	226.67	1.144781	0.144781	8.653663	9.265993
0.03125	260	258	258	258.67	1.306397	0.306397	8.552742	9.804714
0.0625	336	339	335	336.67	1.700337	0.700337	8.493221	11.205387
0.125	526	528	527	527.00	2.661616	1.661616	7.831468	13.292929
0.25	1135	1141	1138	1138.00	5.747475	4.747475	6.995042	18.989899



Mark Houwink equation : $[\eta] = KMw^a$

$$K = 1.81 \cdot 10^{-5} \text{ dl/g}$$

$$a = 0.93$$

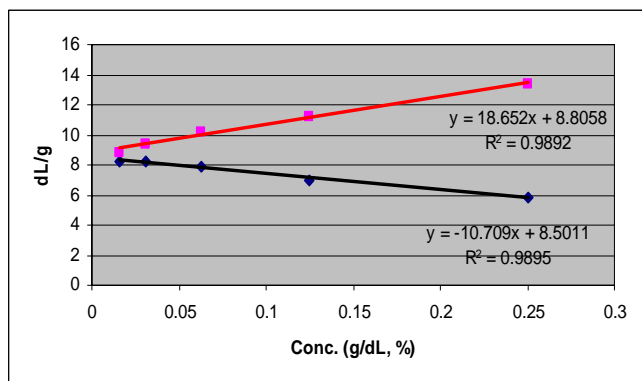
$$[\eta] = (8.5112 + 8.8118) / 2 = 8.6615 \text{ (dL/g)}$$

$$8.6615 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$$

$$Mw = 1280663 \text{ g/mol}$$

Sample: 10 min-1

Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.015625	226	226	224	225.33	1.138047	0.138047	8.276080	8.835017
0.03125	257	256	256	256.33	1.294613	0.294613	8.262773	9.427609
0.0625	324	325	324	324.33	1.638047	0.638047	7.896076	10.208754
0.125	472	478	476	475.33	2.400673	1.400673	7.005994	11.205387
0.25	857	861	864	860.67	4.346801	3.346801	5.877761	13.387205



Mark Houwink equation : $[\eta] = KMw^a$

$$K = 1.81 \cdot 10^{-5} \text{ dl/g}$$

$$a = 0.93$$

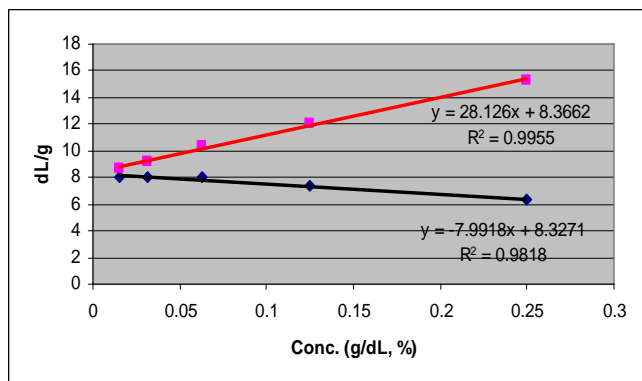
$$[\eta] = (8.8058 + 8.5011) / 2 = 8.65345 \text{ (dL/g)}$$

$$8.65345 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$$

$$Mw = 1279384 \text{ g/mol}$$

Sample: 15 min-1

Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.015625	225	225	224	224.67	1.134680	0.134680	8.086451	8.619529
0.03125	254	255	255	254.67	1.286195	0.286195	8.054031	9.158249
0.0625	327	325	327	326.33	1.648148	0.648148	7.994437	10.370370
0.125	495	497	494	495.33	2.501684	1.501684	7.335711	12.013468
0.25	966	948	951	955.00	4.823232	3.823232	6.293777	15.292929



Mark Houwink equation : $[\eta] = KMw^a$

$$K = 1.81 \cdot 10^{-5} \text{ dl/g}$$

$$a = 0.93$$

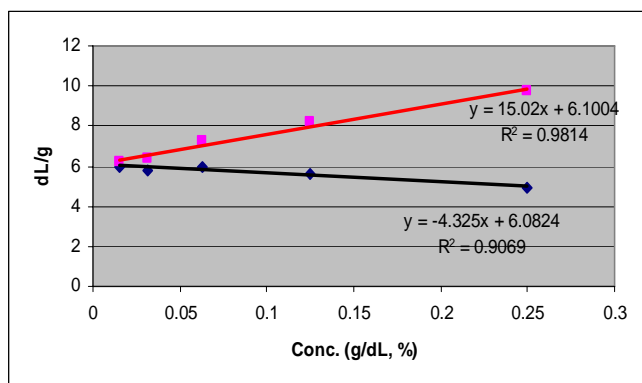
$$[\eta] = (8.3662 + 8.3271) / 2 = 8.34665 \text{ (dL/g)}$$

$$8.34665 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$$

$$Mw = 1230676 \text{ g/mol}$$

Sample: 20 min-1

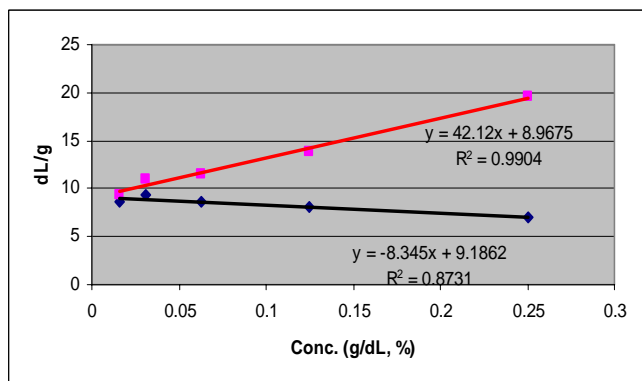
Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.015625	217	218	217	217.33	1.097643	0.097643	5.962576	6.249158
0.03125	239	236	237	237.33	1.198653	0.198653	5.798355	6.356902
0.0625	289	287	288	288.00	1.454545	0.454545	5.995095	7.272727
0.125	399	400	401	400.00	2.020202	1.020202	5.625580	8.161616
0.25	678	682	680	680.00	3.434343	2.434343	4.935303	9.737374



Mark Houwink equation : $[\eta] = KMw^a$
 $K = 1.81 \cdot 10^{-5} \text{ dl/g}$
 $a = 0.93$
 $[\eta] = (6.1004 + 6.0824) / 2 = 6.0914 \text{ (dL/g)}$
 $6.0914 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$
 $Mw = 877106 \text{ g/mol}$

Sample: 0 min-2

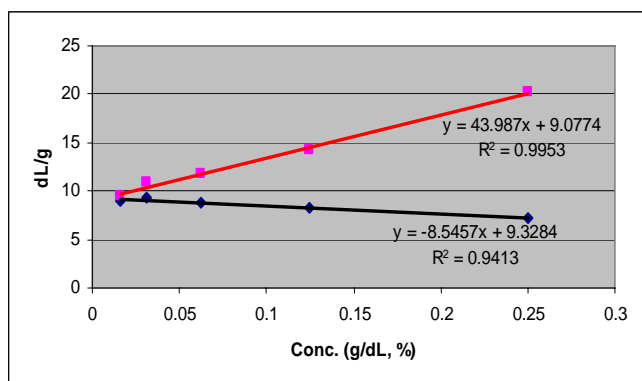
Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.015625	227	227	226	226.67	1.144781	0.144781	8.653663	9.265993
0.03125	265	266	265	265.33	1.340067	0.340067	9.367036	10.882155
0.0625	341	342	341	341.33	1.723906	0.723906	8.713480	11.582492
0.125	539	542	544	541.67	2.735690	1.735690	8.051070	13.885522
0.25	1164	1171	1173	1169.33	5.905724	4.905724	7.103688	19.622896



Mark Houwink equation : $[\eta] = KMw^a$
 $K = 1.81 \cdot 10^{-5} \text{ dl/g}$
 $a = 0.93$
 $[\eta] = (8.9675 + 9.1862) / 2 = 9.0769 \text{ (dL/g)}$
 $9.0769 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$
 $Mw = 1346824 \text{ g/mol}$

Sample: 5 min-2

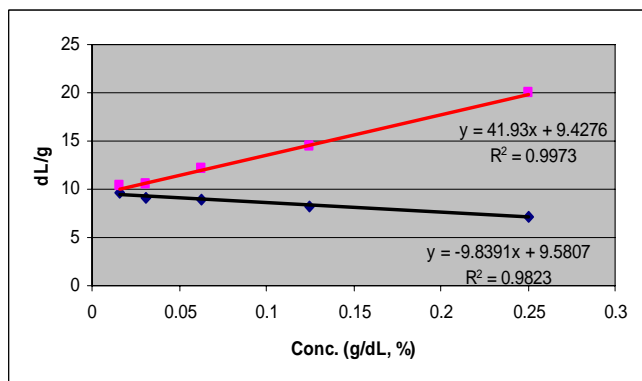
Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.015625	228	228	227	227.67	1.149832	0.149832	8.935395	9.589226
0.03125	264	266	266	265.33	1.340067	0.340067	9.367036	10.882155
0.0625	344	343	343	343.33	1.734007	0.734007	8.806956	11.744108
0.125	551	551	552	551.33	2.784512	1.784512	8.192580	14.276094
0.25	1196	1199	1199	1198.00	6.050505	5.050505	7.200567	20.202020



Mark Houwink equation : $[\eta] = KMw^a$
 $K = 1.81 \cdot 10^{-5} \text{ dl/g}$
 $a = 0.93$
 $[\eta] = (9.0774 + 9.3284)/2 = 9.2029 \text{ (dL/g)}$
 $9.2029 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$
 $Mw = 1366937 \text{ g/mol}$

Sample: 10 min-2

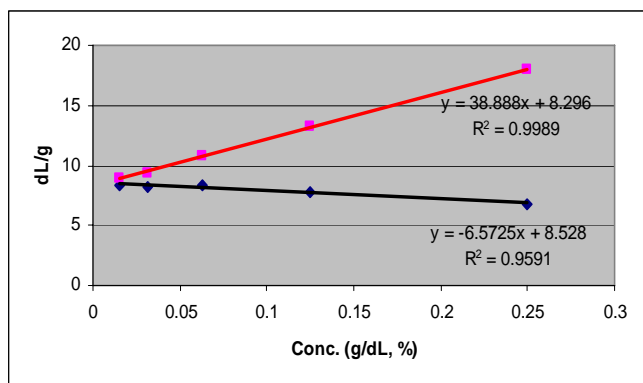
Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.015625	230	230	230	230.00	1.161616	0.161616	9.587986	10.343434
0.03125	264	263	263	263.33	1.329966	0.329966	9.124916	10.558923
0.0625	348	347	348	347.67	1.755892	0.755892	9.007634	12.094276
0.125	553	557	555	555.00	2.803030	1.803030	8.245609	14.424242
0.25	1189	1188	1191	1189.33	6.006734	5.006734	7.171525	20.026936



Mark Houwink equation : $[\eta] = KMw^a$
 $K = 1.81 \cdot 10^{-5} \text{ dl/g}$
 $a = 0.93$
 $[\eta] = (9.4276 + 9.5807)/2 = 9.50415 \text{ (dL/g)}$
 $9.50415 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$
 $Mw = 1415109 \text{ g/mol}$

Sample: 15 min-2

Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.015625	226	226	225	225.67	1.139731	0.139731	8.370685	8.942761
0.03125	257	254	256	255.67	1.291246	0.291246	8.179439	9.319865
0.0625	332	333	332	332.33	1.678451	0.678451	8.285943	10.855219
0.125	524	526	526	525.33	2.653199	1.653199	7.806128	13.225589
0.25	1086	1089	1088	1087.67	5.493266	4.493266	6.814092	17.973064



Mark Houwink equation : $[\eta] = KMw^a$

$$K = 1.81 \cdot 10^{-5} \text{ dl/g}$$

$$a = 0.93$$

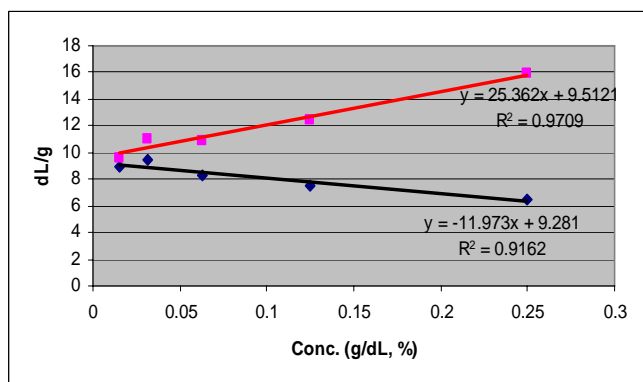
$$[\eta] = (8.296 + 8.528)/2 = 8.412 \text{ (dL/g)}$$

$$8.4125 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$$

$$Mw = 1241040 \text{ g/mol}$$

Sample: 20 min-2

Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.015625	228	228	227	227.67	1.149832	0.149832	8.935395	9.589226
0.03125	267	265	266	266.00	1.343434	0.343434	9.447337	10.989899
0.0625	332	333	332	332.33	1.678451	0.678451	8.285943	10.855219
0.125	504	507	507	506.00	2.555556	1.555556	7.506157	12.444444
0.25	987	991	987	988.33	4.991582	3.991582	6.431012	15.966330



Mark Houwink equation : $[\eta] = KMw^a$

$$K = 1.81 \cdot 10^{-5} \text{ dl/g}$$

$$a = 0.93$$

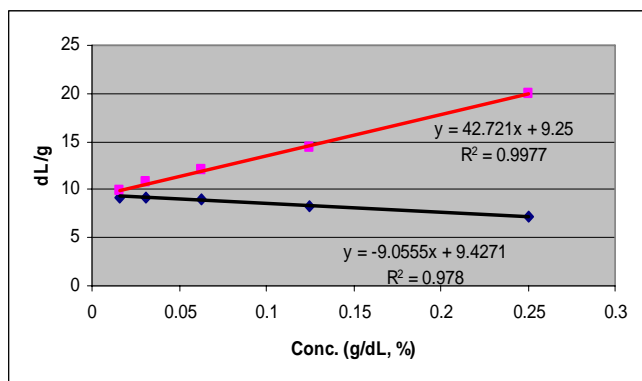
$$[\eta] = (9.5121 + 9.281)/2 = 9.39655 \text{ (dL/g)}$$

$$9.39655 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$$

$$Mw = 1397890 \text{ g/mol}$$

Sample: 0 min-3

Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.015625	228	228	229	228.33	1.153199	0.153199	9.122529	9.804714
0.03125	264	263	266	264.33	1.335017	0.335017	9.246205	10.720539
0.0625	347	348	348	347.67	1.755892	0.755892	9.007634	12.094276
0.125	554	551	551	552.00	2.787879	1.787879	8.202248	14.303030
0.25	1192	1188	1187	1189.00	6.005051	5.005051	7.170403	20.020202



Mark Houwink equation : $[\eta] = KMw^a$

$$K = 1.81 \cdot 10^{-5} \text{ dl/g}$$

$$a = 0.93$$

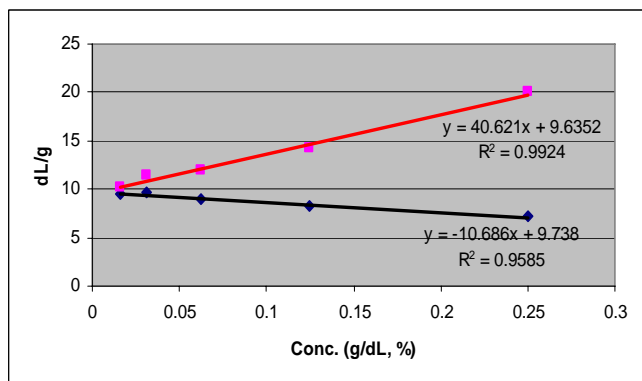
$$[\eta] = (9.25 + 9.4271) / 2 = 9.3386 \text{ (dL/g)}$$

$$9.3386 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$$

$$Mw = 1388622 \text{ g/mol}$$

Sample: 5 min-3

Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.015625	230	230	229	229.67	1.159933	0.159933	9.495165	10.235690
0.03125	269	268	268	268.33	1.355219	0.355219	9.726815	11.367003
0.0625	345	345	347	345.67	1.745791	0.745791	8.915326	11.932660
0.125	553	552	553	552.67	2.791246	1.791246	8.211904	14.329966
0.25	1186	1188	1188	1187.33	5.996633	4.996633	7.164793	19.986532



Mark Houwink equation : $[\eta] = KMw^a$

$$K = 1.81 \cdot 10^{-5} \text{ dl/g}$$

$$a = 0.93$$

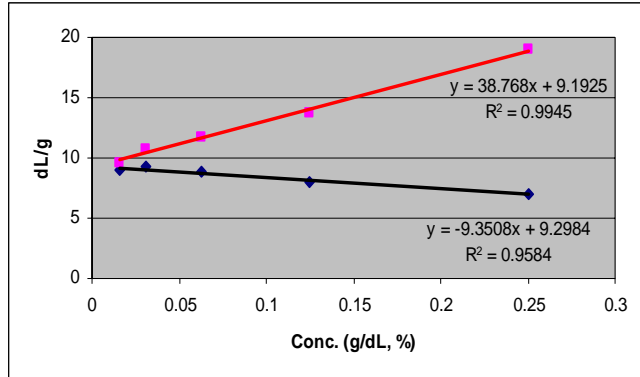
$$[\eta] = (9.6352 + 9.738) / 2 = 9.6866 \text{ (dL/g)}$$

$$9.6866 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$$

$$Mw = 1444341 \text{ g/mol}$$

Sample: 10 min-3

Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.015625	228	228	227	227.67	1.149832	0.149832	8.935395	9.589226
0.03125	264	264	265	264.33	1.335017	0.335017	9.246205	10.720539
0.0625	342	344	344	343.33	1.734007	0.734007	8.806956	11.744108
0.125	538	535	537	536.67	2.710438	1.710438	7.976881	13.683502
0.25	1137	1140	1139	1138.67	5.750842	4.750842	6.997385	19.003367



Mark Houwink equation : $[\eta] = KMw^a$

$$K = 1.81 \cdot 10^{-5} \text{ dl/g}$$

$$a = 0.93$$

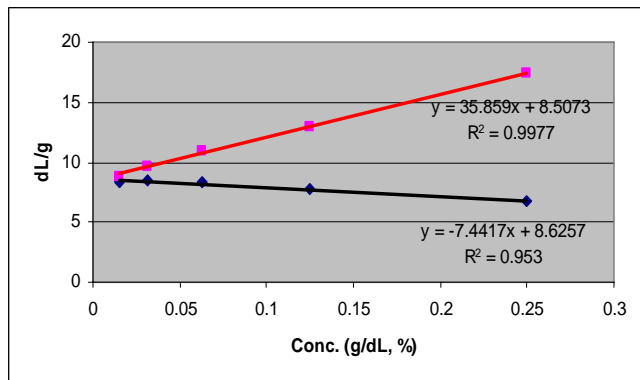
$$[\eta] = (9.1925 + 9.2984) / 2 = 9.24545 \text{ (dL/g)}$$

$$9.24545 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$$

$$Mw = 1373734 \text{ g/mol}$$

Sample: 15 min-3

Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.015625	225	226	225	225.33	1.138047	0.138047	8.276080	8.835017
0.03125	258	258	258	258.00	1.303030	0.303030	8.470162	9.696970
0.0625	333	335	333	333.67	1.685185	0.685185	8.350007	10.962963
0.125	518	520	520	519.33	2.622896	1.622896	7.714231	12.983165
0.25	1061	1058	1063	1060.67	5.356902	4.356902	6.713544	17.427609



Mark Houwink equation : $[\eta] = KMw^a$

$$K = 1.81 \cdot 10^{-5} \text{ dl/g}$$

$$a = 0.93$$

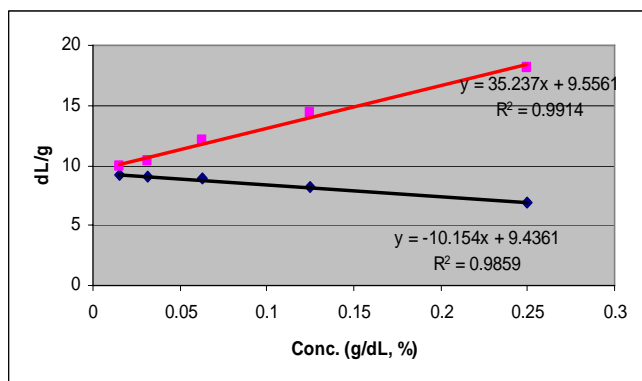
$$[\eta] = (8.5073 + 8.6257) / 2 = 8.5665 \text{ (dL/g)}$$

$$8.5665 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$$

$$Mw = 1265566 \text{ g/mol}$$

Sample: 20 min-3

Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.015625	229	229	228	228.67	1.154882	0.154882	9.215892	9.912458
0.03125	263	262	262	262.33	1.324916	0.324916	9.003166	10.397306
0.0625	346	347	348	347.00	1.752525	0.752525	8.976924	12.040404
0.125	552	554	554	553.33	2.794613	1.794613	8.221548	14.356902
0.25	1099	1098	1091	1096.00	5.535354	4.535354	6.844622	18.141414



Mark Houwink equation : $[\eta] = KMw^a$

$K = 1.81 \cdot 10^{-5} \text{ dl/g}$

$a = 0.93$

$[\eta] = (9.5561 + 9.4361) / 2 = 9.4961 \text{ (dL/g)}$

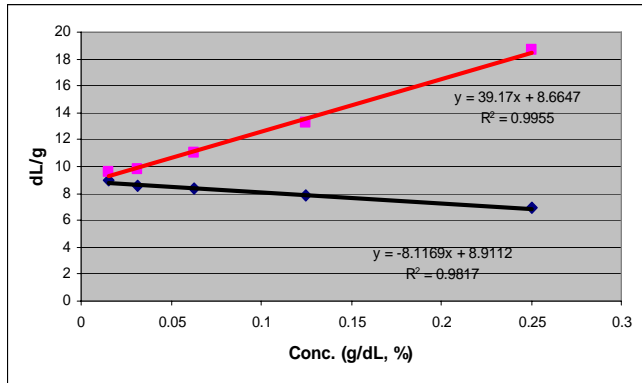
$9.4961 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$

$Mw = 1413821 \text{ g/mol}$

2. Ozone-treated chitosan in acetic acid

Sample: 0 min-1

Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.015625	227	228	228	227.67	1.149832	0.149832	8.935395	9.589226
0.03125	260	257	259	258.67	1.306397	0.306397	8.552742	9.804714
0.0625	335	333	336	334.67	1.690236	0.690236	8.397888	11.043771
0.125	526	523	527	525.33	2.653199	1.653199	7.806128	13.225589
0.25	1117	1121	1123	1120.33	5.658249	4.658249	6.932458	18.632997



Mark Houwink equation : $[\eta] = KMw^a$

$$K = 1.81 \cdot 10^{-5} \text{ dl/g}$$

$$a = 0.93$$

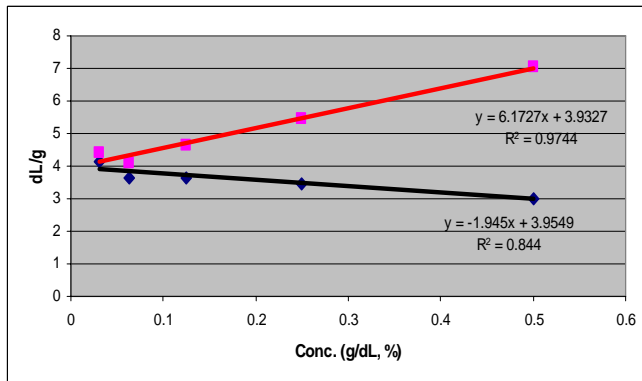
$$[\eta] = (8.6647 + 8.9112) / 2 = 8.78795 \text{ (dL/g)}$$

$$8.78795 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$$

$$Mw = 1300778 \text{ g/mol}$$

Sample: 5 min-1

Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.03125	226	225	225	225.33	1.138047	0.138047	4.138040	4.417508
0.0625	249	248	249	248.67	1.255892	0.255892	3.645540	4.094276
0.125	311	313	313	312.33	1.577441	0.577441	3.646432	4.619529
0.25	467	468	468	467.67	2.361953	1.361953	3.437955	5.447811
0.5	895	899	898	897.33	4.531987	3.531987	3.022321	7.063973



Mark Houwink equation : $[\eta] = KMw^a$

$$K = 1.81 \cdot 10^{-5} \text{ dl/g}$$

$$a = 0.93$$

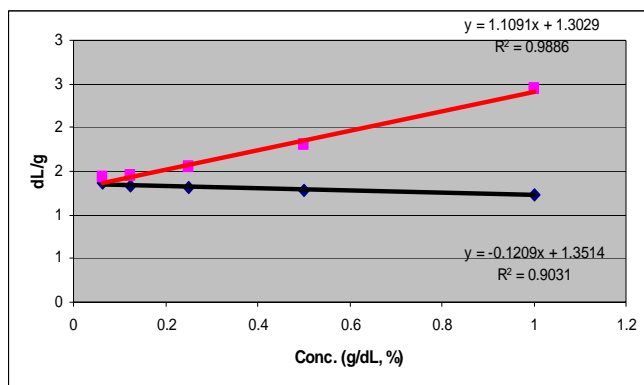
$$[\eta] = (3.9327 + 3.9549) / 2 = 3.9438 \text{ (dL/g)}$$

$$3.9438 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$$

$$Mw = 549590 \text{ g/mol}$$

Sample: 10 min-1

Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.03125	206	206	205	205.67	1.038721	0.038721	1.215671	1.239057
0.0625	216	216	215	215.67	1.089226	0.089226	1.367472	1.427609
0.125	234	234	233	233.67	1.180135	0.180135	1.325029	1.441077
0.25	275	275	274	274.67	1.387205	0.387205	1.309165	1.548822
0.5	376	377	376	376.33	1.900673	0.900673	1.284416	1.801347
1	680	683	683	682.00	3.444444	2.444444	1.236763	2.444444



Mark Houwink equation : $[\eta] = KMw^a$

$$K = 1.81 \cdot 10^{-5} \text{ dl/g}$$

$$a = 0.93$$

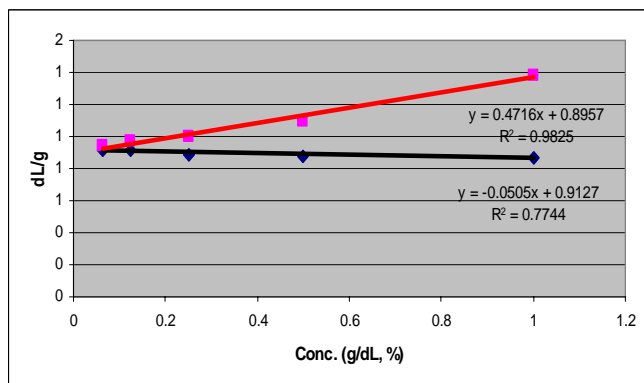
$$[\eta] = (1.3029 + 1.3514)/2 = 1.32715 \text{ (dL/g)}$$

$$1.32715 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$$

$$Mw = 170389 \text{ g/mol}$$

Sample: 15 min-1

Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.03125	205	204	204	204.33	1.031987	0.031987	1.007540	1.023569
0.0625	210	210	209	209.67	1.058923	0.058923	0.916031	0.942761
0.125	222	223	221	222.00	1.121212	0.121212	0.915283	0.969697
0.25	247	247	248	247.33	1.249158	0.249158	0.889880	0.996633
0.5	307	306	307	306.67	1.548822	0.548822	0.874989	1.097643
1	471	473	473	472.33	2.385522	1.385522	0.869418	1.385522



Mark Houwink equation : $[\eta] = KMw^a$

$$K = 1.81 \cdot 10^{-5} \text{ dl/g}$$

$$a = 0.93$$

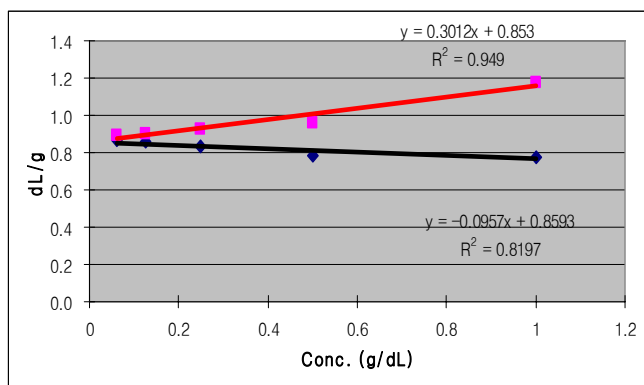
$$[\eta] = (0.8957 + 0.9127)/2 = 0.9042 \text{ (dL/g)}$$

$$0.9042 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$$

$$Mw = 112783 \text{ g/mol}$$

Sample: 20 min-1

Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.03125	204	203	204	203.67	1.028620	0.028620	0.902964	0.915825
0.0625	209	209	209	209.00	1.055556	0.055556	0.865076	0.888889
0.125	220	221	220	220.33	1.112795	0.112795	0.854996	0.902357
0.25	243	244	244	243.67	1.230640	0.230640	0.830137	0.922559
0.5	293	293	293	293.00	1.479798	0.479798	0.783811	0.959596
1	430	430	432	430.67	2.175084	1.175084	0.777067	1.175084



Mark Houwink equation : $[\eta] = KMw^a$

$$K = 1.81 \cdot 10^{-5} \text{ dl/g}$$

$$a = 0.93$$

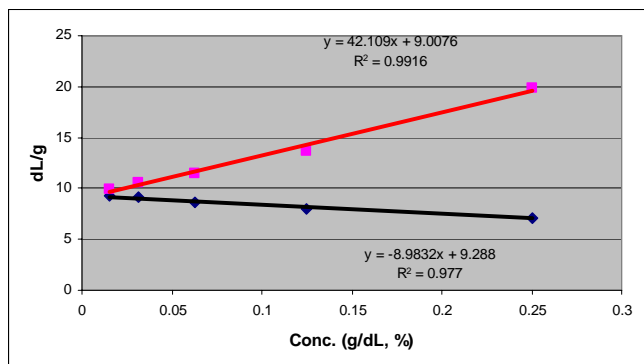
$$[\eta] = (0.853 + 0.8593)/2 = 0.85615 \text{ (dL/g)}$$

$$0.85615 = 1.81 \cdot 10^{-5} * Mw^{0.93}$$

$$Mw = 106351 \text{ g/mol}$$

Sample: 0 min-2

Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.015625	228	229	229	228.67	1.154882	0.154882	9.215892	9.912458
0.03125	262	264	264	263.33	1.329966	0.329966	9.124916	10.558923
0.0625	341	339	338	339.33	1.713805	0.713805	8.619454	11.420875
0.125	539	536	538	537.67	2.715488	1.715488	7.991774	13.723906
0.25	1183	1175	1179	1179.00	5.954545	4.954545	7.136619	19.818182



Mark Houwink equation : $[\eta] = KMw^a$

$$K = 1.81 \cdot 10^{-5} \text{ dl/g}$$

$$a = 0.93$$

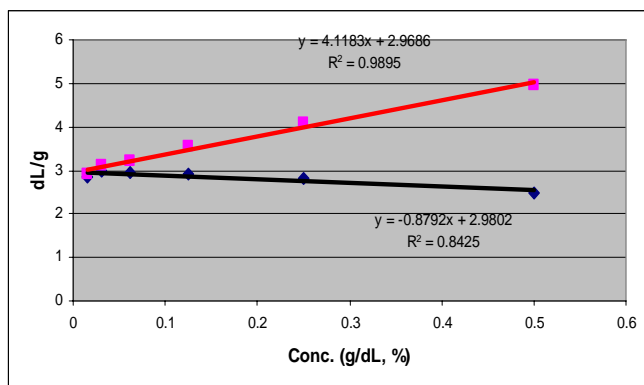
$$[\eta] = (9.0076 + 9.288)/2 = 9.1478 \text{ (dL/g)}$$

$$9.1478 = 1.81 \cdot 10^{-5} * Mw^{0.93}$$

$$Mw = 1358139 \text{ g/mol}$$

Sample: 5 min-2

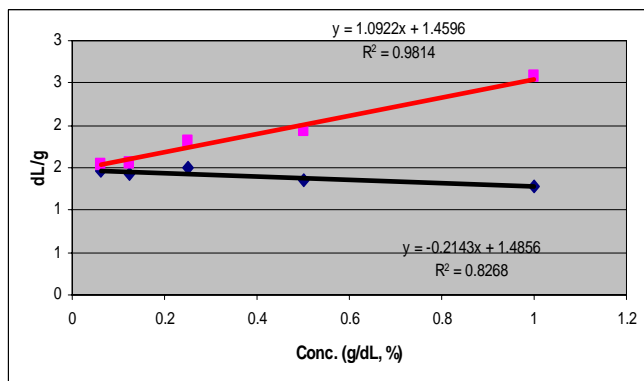
Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.015625	207	207	207	207.00	1.045455	0.045455	2.844913	2.909091
0.03125	217	217	218	217.33	1.097643	0.097643	2.981288	3.124579
0.0625	238	239	237	238.00	1.202020	0.202020	2.944058	3.232323
0.125	286	286	285	285.67	1.442761	0.442761	2.932469	3.542088
0.25	399	401	401	400.33	2.021886	1.021886	2.816122	4.087542
0.5	688	692	690	690.00	3.484848	2.484848	2.496849	4.969697



Mark Houwink equation : $[\eta] = KMw^a$
 $K = 1.81 \cdot 10^{-5} \text{ dl/g}$
 $a = 0.93$
 $[\eta] = (2.9789 + 2.98329) / 2 = 2.98105 \text{ (dl/g)}$
 $2.98105 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$
 $Mw = 406766 \text{ g/mol}$

Sample: 10 min-2

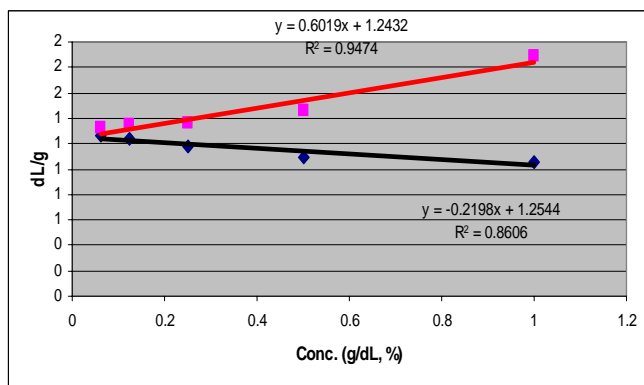
Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.0625	217	217	217	217.00	1.095960	0.095960	1.466085	1.535354
0.125	236	237	237	236.67	1.195286	0.195286	1.427085	1.562290
0.25	289	286	288	287.67	1.452862	0.452862	1.494141	1.811448
0.5	389	390	389	389.33	1.966330	0.966330	1.352338	1.932660
1	706	707	709	707.33	3.572391	2.572391	1.273235	2.572391



Mark Houwink equation : $[\eta] = KMw^a$
 $K = 1.81 \cdot 10^{-5} \text{ dl/g}$
 $a = 0.93$
 $[\eta] = (1.4596 + 1.4856) / 2 = 1.4726 \text{ (dl/g)}$
 $1.4726 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$
 $Mw = 190549 \text{ g/mol}$

Sample: 15 min-2

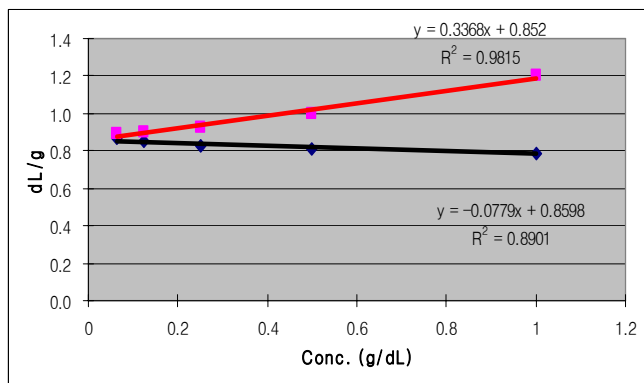
Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.0625	215	215	213	214.33	1.082492	0.082492	1.268246	1.319865
0.125	231	231	232	231.33	1.168350	0.168350	1.244741	1.346801
0.25	264	265	268	265.67	1.341751	0.341751	1.175901	1.367003
0.5	342	343	343	342.67	1.730640	0.730640	1.096982	1.461279
1	571	571	573	571.67	2.887205	1.887205	1.060289	1.887205



Mark Houwink equation : $[\eta] = KMw^a$
 $K = 1.81 \cdot 10^{-5} \text{ dl/g}$
 $a = 0.93$
 $[\eta] = (1.2432 + 1.2544) / 2 = 1.2488 \text{ (dL/g)}$
 $1.2488 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$
 $Mw = 159597 \text{ g/mol}$

Sample: 20 min-2

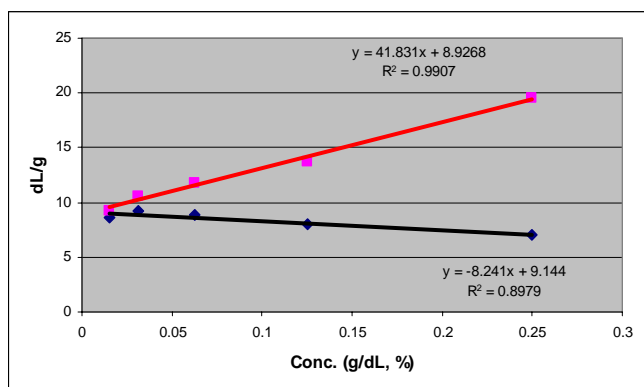
Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.0625	209	209	209	209.00	1.055556	0.055556	0.865076	0.888889
0.125	220	220	221	220.33	1.112795	0.112795	0.854996	0.902357
0.25	244	243	244	243.67	1.230640	0.230640	0.830137	0.922559
0.5	297	296	297	296.67	1.498316	0.498316	0.808684	0.996633
1	436	437	435	436.00	2.202020	1.202020	0.789375	1.202020



Mark Houwink equation : $[\eta] = KMw^a$
 $K = 1.81 \cdot 10^{-5} \text{ dl/g}$
 $a = 0.93$
 $[\eta] = (0.852 + 0.8598) / 2 = 0.8559 \text{ (dL/g)}$
 $0.8559 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$
 $Mw = 106318 \text{ g/mol}$

Sample: 0 min-3

Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.015625	227	226	227	226.67	1.144781	0.144781	8.653663	9.265993
0.03125	263	263	265	263.67	1.331650	0.331650	9.165397	10.612795
0.0625	343	345	345	344.33	1.739057	0.739057	8.853490	11.824916
0.125	534	536	538	536.00	2.707071	1.707071	7.966937	13.656566
0.25	1169	1160	1166	1165.00	5.883838	4.883838	7.088837	19.535354



Mark Houwink equation : $[\eta] = KMw^a$

$$K = 1.81 \cdot 10^{-5} \text{ dl/g}$$

$$a = 0.93$$

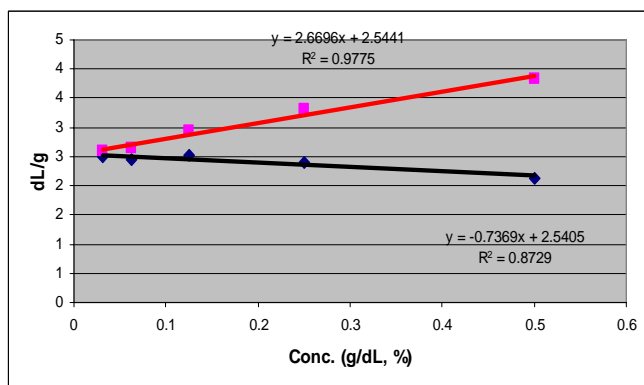
$$[\eta] = (8.9268 + 9.144) / 2 = 9.0354 \text{ (dL/g)}$$

$$9.0354 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$$

$$Mw = 1340204 \text{ g/mol}$$

Sample: 5 min-3

Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.03125	214	214	214	214.00	1.080808	0.080808	2.486687	2.585859
0.0625	231	230	231	230.67	1.164983	0.164983	2.443306	2.639731
0.125	270	272	271	271.00	1.368687	0.368687	2.510814	2.949495
0.25	360	362	363	361.67	1.826599	0.826599	2.409824	3.306397
0.5	575	578	577	576.67	2.912458	1.912458	2.137995	3.824916



Mark Houwink equation : $[\eta] = KMw^a$

$$K = 1.81 \cdot 10^{-5} \text{ dl/g}$$

$$a = 0.93$$

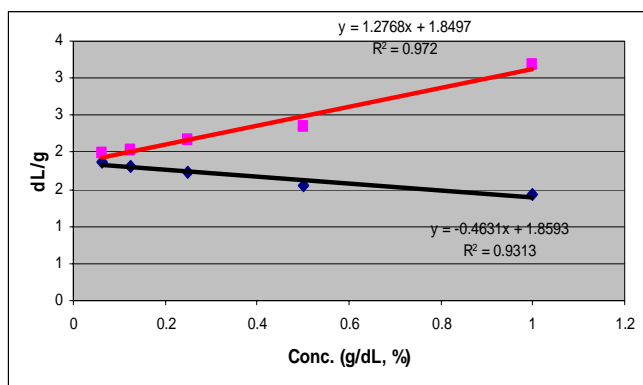
$$[\eta] = (2.5441 + 2.5405) / 2 = 2.5423 \text{ (dL/g)}$$

$$2.5423 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$$

$$Mw = 342766 \text{ g/mol}$$

Sample: 10 min-3

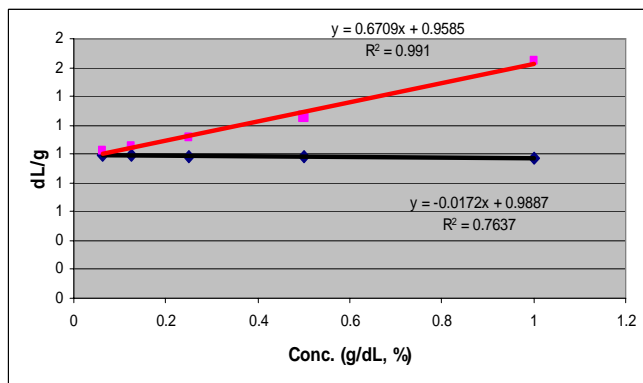
Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.0625	222	223	223	222.67	1.124579	0.124579	1.878542	1.993266
0.125	247	249	248	248.00	1.252525	0.252525	1.801294	2.020202
0.25	304	306	306	305.33	1.542088	0.542088	1.732548	2.168350
0.5	430	431	431	430.67	2.175084	1.175084	1.554135	2.350168
1	831	828	830	829.67	4.190236	3.190236	1.432757	3.190236



Mark Houwink equation : $[\eta] = KMw^a$
 $K = 1.81 \cdot 10^{-5} \text{ dl/g}$
 $a = 0.93$
 $[\eta] = (1.8497 + 1.8593)/2 = 1.8545 \text{ (dL/g)}$
 $1.8545 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$
 $Mw = 244167 \text{ g/mol}$

Sample: 15 min-3

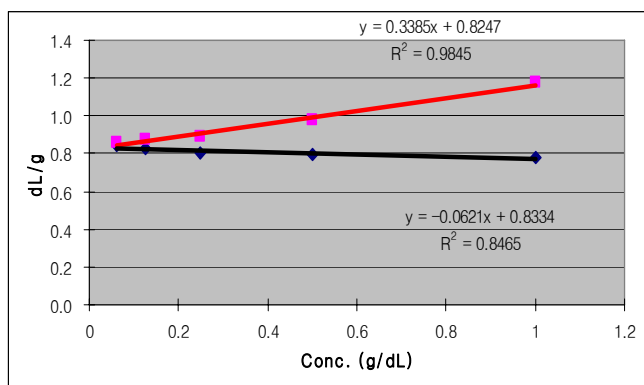
Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.0625	210	211	211	210.67	1.063973	0.063973	0.992161	1.023569
0.125	225	223	224	224.00	1.131313	0.131313	0.987032	1.050505
0.25	252	254	253	253.00	1.277778	0.277778	0.980490	1.111111
0.5	322	323	323	322.67	1.629630	0.629630	0.976706	1.259259
1	524	525	524	524.33	2.648148	1.648148	0.973861	1.648148



Mark Houwink equation : $[\eta] = KMw^a$
 $K = 1.81 \cdot 10^{-5} \text{ dl/g}$
 $a = 0.93$
 $[\eta] = (0.9585 + 0.9887)/2 = 0.9736 \text{ (dL/g)}$
 $0.9736 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$
 $Mw = 122117 \text{ g/mol}$

Sample: 20 min-3

Conc.(%)	Reading (sec)			Average	η_{rel}	η_{sp}	η_{inh}	η_{red}
	1	2	3					
Solvent	198	198	198	198.00				
0.0625	208	209	209	208.67	1.053872	0.053872	0.839537	0.861953
0.125	220	219	220	219.67	1.109428	0.109428	0.830754	0.875421
0.25	242	242	242	242.00	1.222222	0.222222	0.802683	0.888889
0.5	295	295	295	295.00	1.489899	0.489899	0.797417	0.979798
1	430	432	429	430.33	2.173401	1.173401	0.776293	1.173401



Mark Houwink equation : $[\eta] = KMw^a$

$$K = 1.81 \cdot 10^{-5} \text{ dl/g}$$

$$a = 0.93$$

$$[\eta] = (0.8247 + 0.8334) / 2 = 0.82905 \text{ (dL/g)}$$

$$0.82905 = 1.81 \cdot 10^{-5} \cdot Mw^{0.93}$$

$$Mw = 102736 \text{ g/mol}$$

APPENDIX B. DATA OF DEGREE OF DEACETYLATION

1. Ozone-treated chitosan in water

Sample	Chitosan (g)	5% formic acid (g)	PVSK (ml)	X	Y	DD(%)	Average
0min-1	0.5008	99.50	8.70	0.003536767	0.001463233	75.29	76.02
			8.90	0.003618072	0.001381928	76.75	
5min-1	0.5008	99.50	8.80	0.00357742	0.00142258	76.02	76.02
			8.80	0.00357742	0.00142258	76.02	
10min-1	0.5008	99.50	8.80	0.00357742	0.00142258	76.02	76.02
			8.80	0.00357742	0.00142258	76.02	
15min-1	0.5008	99.50	8.50	0.003455462	0.001544538	73.83	73.83
			8.50	0.003455462	0.001544538	73.83	
20min-1	0.5008	99.50	8.60	0.003496115	0.001503885	74.56	74.20
			8.50	0.003455462	0.001544538	73.83	
0min-2	0.5004	99.50	8.90	0.003618072	0.001381928	76.75	76.75
			8.90	0.003618072	0.001381928	76.75	
5min-2	0.5008	99.50	8.90	0.003618072	0.001381928	76.75	76.39
			8.80	0.00357742	0.00142258	76.02	
10min-2	0.5002	99.50	8.80	0.00357742	0.00142258	76.02	76.39
			8.90	0.003618072	0.001381928	76.75	
15min-2	0.5003	99.50	8.50	0.003455462	0.001544538	73.83	73.46
			8.40	0.00341481	0.00158519	73.09	
20min-2	0.5004	99.50	8.60	0.003496115	0.001503885	74.56	74.20
			8.50	0.003455462	0.001544538	73.83	
0min-3	0.5007	99.50	8.80	0.00357742	0.00142258	76.02	76.39
			8.90	0.003618072	0.001381928	76.75	
5min-3	0.5004	99.50	8.80	0.00357742	0.00142258	76.02	75.66
			8.70	0.003536767	0.001463233	75.29	
10min-3	0.5004	99.50	8.70	0.003536767	0.001463233	75.29	75.66
			8.80	0.00357742	0.00142258	76.02	
15min-3	0.5006	99.50	8.40	0.00341481	0.00158519	73.09	73.46
			8.50	0.003455462	0.001544538	73.83	
20min-3	0.5003	99.50	8.60	0.003496115	0.001503885	74.56	74.56
			8.60	0.003496115	0.001503885	74.56	

$$DD(\%) = \left[\frac{X / 161}{X / 161 + Y / 203} \right] \times 100$$

Where, X (Amount of glucosamine in molecule) = $1/400 \times 1/1000 \times f \times 161 \times V$

Y (Amount of N-acetylglucosamine in molecule) = $0.5 \times 1/100 - X$

V: Titrated volume (ml) of n/400 PVSK; f: Factor of PVSK solution

2. Ozone-treated chitosan in acetic acid solution

Sample	Chitosan (g)	5% formic acid (g)	PVSK (ml)	X	Y	DD(%)	Average
0min-1	0.5004	99.50	8.80	0.00357742	0.00142258	76.02	76.02
			8.80	0.00357742	0.00142258	76.02	
5min-1	0.5000	99.50	8.80	0.00357742	0.00142258	76.02	76.02
			8.80	0.00357742	0.00142258	76.02	
10min-1	0.5005	99.50	8.70	0.003536767	0.001463233	75.29	75.66
			8.80	0.00357742	0.00142258	76.02	
15min-1	0.5005	99.50	8.90	0.003618072	0.001381928	76.75	76.39
			8.80	0.00357742	0.00142258	76.02	
20min-1	0.5003	99.50	8.80	0.00357742	0.00142258	76.02	75.66
			8.70	0.003536767	0.001463233	75.29	
0min-2	0.5006	99.50	8.80	0.00357742	0.00142258	76.02	76.02
			8.80	0.00357742	0.00142258	76.02	
5min-2	0.5003	99.50	8.80	0.00357742	0.00142258	76.02	76.02
			8.80	0.00357742	0.00142258	76.02	
10min-2	0.5001	99.50	8.80	0.00357742	0.00142258	76.02	76.02
			8.80	0.00357742	0.00142258	76.02	
15min-2	0.5001	99.50	8.70	0.003536767	0.001463233	75.29	75.66
			8.80	0.00357742	0.00142258	76.02	
20min-2	0.5004	99.50	8.90	0.003618072	0.001381928	76.75	76.39
			8.80	0.00357742	0.00142258	76.02	
0min-3	0.5007	99.50	8.80	0.00357742	0.00142258	76.02	76.39
			8.90	0.003618072	0.001381928	76.75	
5min-3	0.5000	99.50	8.80	0.00357742	0.00142258	76.02	76.02
			8.80	0.00357742	0.00142258	76.02	
10min-3	0.5005	99.50	8.80	0.00357742	0.00142258	76.02	76.02
			8.80	0.00357742	0.00142258	76.02	
15min-3	0.5005	99.50	8.80	0.00357742	0.00142258	76.02	76.39
			8.90	0.003618072	0.001381928	76.75	
20min-3	0.5003	99.50	8.80	0.00357742	0.00142258	76.02	76.02
			8.80	0.00357742	0.00142258	76.02	

$$DD(\%) = \left[\frac{X/161}{X/161 + Y/203} \right] \times 100$$

Where, X (Amount of glucosamine in molecule) = $1/400 \times 1/1000 \times f \times 161 \times V$

Y (Amount of N-acetylglucosamine in molecule) = $0.5 \times 1/100 - X$

V: Titrated volume (ml) of n/400 PVSK; f: Factor of PVSK solution

APPENDIX C. DATA OF COLOR

1. Ozone-treated chitosan in water

Sample	color value					
	L*	a*	b*	c	h	whiteness
0min-1	72.62	2.82	16.43	16.67	80.26	67.94
5min-1	72.54	2.75	16.47	16.70	80.53	67.86
10min-1	72.58	2.76	16.55	16.78	80.54	67.85
15min-1	72.87	2.76	16.69	16.92	80.60	68.03
20min-1	72.72	2.71	16.71	16.94	80.80	67.89
0min-2	72.70	2.80	16.45	16.69	80.34	68.00
5min-2	72.66	2.79	16.47	16.70	80.40	67.96
10min-2	72.71	2.73	16.51	16.74	80.62	67.99
15min-2	72.72	2.73	16.59	16.82	80.67	67.96
20min-2	72.76	2.75	16.66	16.89	80.64	67.95
0min-3	72.74	2.83	16.47	16.72	80.26	68.03
5min-3	72.70	2.80	16.47	16.71	80.35	67.99
10min-3	72.68	2.78	16.53	16.77	80.44	67.95
15min-3	72.73	2.75	16.57	16.80	80.58	67.97
20min-3	72.76	2.73	16.64	16.87	80.67	67.96

2. Ozone-treated chitosan in acetic acid solution

Sample	color value					
	L*	a*	b*	c	h	whiteness
0min-1	72.87	2.80	16.34	16.57	80.29	68.21
5min-1	81.58	-0.95	13.08	13.11	94.17	77.39
10min-1	78.33	0.88	19.79	19.81	87.46	70.64
15min-1	81.41	-2.28	23.73	23.84	95.48	69.94
20min-1	82.28	-2.36	23.43	23.55	95.75	70.72
0min-2	73.07	2.80	16.46	16.70	80.35	68.31
5min-2	82.60	-1.10	13.17	13.21	94.78	78.21
10min-2	82.36	-1.84	18.36	18.45	95.72	74.61
15min-2	81.49	-2.00	23.19	23.28	94.92	68.79
20min-2	81.07	-1.95	25.21	25.28	94.41	68.53
0min-3	72.93	2.80	16.46	16.69	80.34	68.20
5min-3	82.27	-1.16	13.36	13.41	94.96	77.83
10min-3	82.42	-1.65	17.63	17.70	95.34	75.16
15min-3	81.86	-2.02	22.37	22.46	95.15	71.27
20min-3	79.39	-1.14	27.94	27.97	92.34	65.30

APPENDIX D. DATA OF NITROGEN CONTENT

Sample	Ozone-treated chitosan in water		Ozone-treated chitosan in acetic acid solution	
	Weight (mg)	Nitrogen %	Weight (mg)	Nitrogen %
0min-1	48.5	7.23	48.7	7.59
	48.9	7.69	49.0	7.75
5min-1	48.8	7.14	49.0	8.04
	48.6	8.01	49.5	7.29
10min-1	49.3	8.08	48.9	7.68
	48.5	7.10	49.1	8.15
15min-1	48.4	7.11	48.8	7.55
	48.7	7.05	48.4	7.38
20min-1	48.7	6.90	48.3	7.44
	48.6	7.10	48.9	7.40
0min-2	48.3	7.58	48.6	7.53
	48.4	7.54	48.4	7.45
5min-2	48.3	7.33	48.8	7.34
	46.7	7.68	48.5	7.43
10min-2	48.6	7.81	49.7	7.49
	46.8	6.93	48.4	7.34
15min-2	48.5	7.01	48.6	7.57
	49.3	7.21	48.9	7.66
20min-2	48.3	7.18	49.0	7.78
	49.3	7.01	49.0	7.57
0min-3	49.0	7.61	49.1	7.45
	49.1	7.77	48.5	7.51
5min-3	44.2	7.68	48.7	7.53
	47.8	7.13	48.7	7.59
10min-3	47.1	7.16	48.9	7.31
	48.3	7.38	49.2	7.56
15min-3	49.0	6.88	48.3	7.59
	49.4	6.73	48.7	7.30
20min-3	48.8	6.79	48.4	7.31
	48.3	7.06	48.8	7.66

APPENDIX E. DATA OF VISCOSITY

Sample	Viscosity (cPs)	
	Ozone-treated chitosan in water	Ozone-treated chitosan in acetic acid solution
0min-1	347	331
0min-2	340	330
0min-3	346	333
5min-1	311	30
5min-2	313	28
5min-3	301	28
10min-1	302	14
10min-2	312	14
10min-3	322	14
15min-1	319	11
15min-2	317	12
15min-3	324	12
20min-1	318	10
20min-2	317	10
20min-3	311	10

VITA

Seung-wook Seo was born in Seoul, Republic of Korea, on March 25, 1977. In 1996, he entered Chung-Ang University where he obtained a Bachelor of Science degree in food science and technology in 2003. During school he joined the Republic of Korea Air Force and finished the military service in 2000. After graduation, he worked as a research assistant at Daesang R&D in Korea for one year. He began pursuing a master's degree in the Department of Food Science at Louisiana State University in Baton Rouge, Louisiana, in August 2004. He is a candidate for the degree of Master of Science in food science in August 2006.