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Evaluating the Effects of Vacuum Tumbling with Chitosan Nanoparticles and Water-Soluble Chitosan on the Shelf Life of Cryogenically Frozen Shrimp

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EVALUATING THE EFFECTS OF VACUUM TUMBLING WITH CHITOSAN
NANOPARTICLES AND WATER-SOLUBLE CHITOSAN ON THE SHELF
LIFE OF CRYOGENICALLY FROZEN SHRIMP

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ABSTRACT

Chitosan (CH) is a biopolymer obtained from the deacetylation of chitin, a polysaccharide present in the exoskeleton of crustaceans and cell walls of fungi. Application of CH may be limited by its water insolubility. It can be dissolved in acids and forms relatively high viscosity solutions. CH solutions have antioxidant and antimicrobial properties. CH penetration into shrimp may be facilitated by vacuum tumbling with low viscosity chitosan-sodium tripolyphosphate (CH-TPP) nanoparticle or water-soluble chitosan (WSC) solutions. It is expected that this would reduce lipid oxidation and microbial loads. In this study, CH-TPP and WSC solutions were developed and applied to shrimp, and the quality characteristics during frozen storage were evaluated. This research was conducted in two separate studies. In the first study, four treatment solutions were prepared: (1) a 1% acetic acid (AA) solution, (2) a CH in AA solution, (3) a sodium tripolyphosphate (TPP) in AA solution, and (4) a CH-TPP in AA solution. The solutions were sonicated and then sheared in an ultra-homogenizer to reduce particle size. In the second study, WSC was produced by enzymatic hydrolysis. Two treatment solutions were prepared: (1) a 0.1 M AA solution and (2) a WSC in distilled water (DW) solution. In both studies, fresh shrimp meat was separately vacuum tumbled with the solutions, cryogenically frozen, and evaluated for quality characteristics under frozen storage. Fresh shrimp meat tumbled with DW and fresh shrimp meat without tumbling (NT) were used as controls. Shrimp treated with CH, CH-TPP, and WSC had lower aerobic plate counts (APC) compared to other treatments after 120 days of storage at -20 °C. However, only the WSC treatment decreased yeast and mold counts (YMC) in the shrimp. CH, CH-TPP, and WSC treatments could aid in retention of color, texture, and moisture content of shrimp. Additionally, CH, CH-TPP, and WSC treatments produced the highest reduction in lipid oxidation compared to other treatments. This research indicated that CH, CH-TPP, and WSC solutions, combined with

vacuum tumbling, can be effective at reducing APC and lipid oxidation in shrimp during frozen storage. As WSC can be dissolved in water, it may have greater application potential in seafood than CH or CH-TPP.

CHAPTER 1. INTRODUCTION

Chitosan (CH) is a widely used polysaccharide in edible films and coatings due to its non-toxicity, antimicrobial activity, and antioxidant properties (Elsabee & Abdou, 2013; Jeon, Kamil, & Shahidi, 2002; Kucukgulmez, Kadak, & Gokcin, 2013; No, Meyers, Prinyawiwatkul, & Xu, 2007). CH is derived from chitin, a natural biopolymer found in the shells of crustaceans and cell walls of fungi. Chitin is the second most available biopolymer on earth (Shahidi, Arachchi, & Jeon, 1999). While chitin is mainly composed of poly β-(1-4)-2-acetamido-D-glucose, CH is a copolymer that contains units of β-(1-4)-2-acetamido-D-glucose and β-(1-4)-2-amino-D-glucose (Elsabee & Abdou, 2013). CH has been used to coat the surface of shrimp by glazing (Solval, Espinoza Rodezno, Moncada, Bankston, & Sathivel, 2014), but its penetration into meat tissues and the effect of penetrated chitosan on a seafood have not yet been studied.

Some inherent characteristics of CH, such as its large particle size (Qi, Xu, Jiang, Hu, & Zou, 2004) and high viscosity in solution (Jo, Lee, Lee, & Byun, 2001), may limit its penetration into shrimp muscle tissues. CH nanoparticles and water-soluble low molecular weight CH may penetrate into seafood, especially into shrimp meat, with the assistance of a vacuum tumbling. CH nanoparticles are typically formed by instant ionic gelation in which positively charged CH is combined with an anion, such as sodium tripolyphosphate (Gan, Wang, Cochrane, & McCarron, 2005). CH nanoparticles have been used in drug delivery systems due to their mucoadhesivity and ability to augment large molecule penetration across mucosal surfaces (Xu & Du, 2003). Reduction of particle size to sub-micron levels can allow for deep penetration into tissues through fine capillaries and fenestration in the epithelial lining (Gan et al., 2005). Nanoparticles generally have higher intracellular uptake compared to microparticles. Due to their small size and relative mobility, they are available to a greater number of biological targets as

well (Mohanraj & Chen, 2007). It has been reported that CH nanoparticles may exhibit greater antimicrobial activity than CH particles of larger sizes as a result of their small size and the quantum size effect (Qi et al., 2004).

Although CH has vast potential in many fields, because of its water insolubility at neutral pH values, uses may still be limited in some areas (Ilyina, Tatarinova, & Varlamov, 1999; Xie, Xu, & Liu, 2001), such as in food, health, agriculture (Roncal, Oviedo, de Armentia, Fernandez, & Villaran, 2007), and biomedical applications (Snyman, Hamman, Kotze, Rollings, & Kotze, 2002). CH can be partially hydrolyzed to achieve water solubility by obtaining shorter chain lengths with more free amino groups in D-glucosamine units as a result of a decrease in molecular weight (Qin et al., 2003). Water-soluble low molecular weight CH has been shown to have significant biological activities, including antimicrobial (No, Park, Lee, & Meyers, 2002b; Vishu, Varadaraj, Gowda, & Tharanathan, 2005; Zheng & Zhu, 2003) and antitumor activity (Qin, Du, Xiao, Li, & Gao, 2002; Seo et al., 2000), as well as the ability to slow the progression of diabetes mellitus (Kondo, Nakatani, Hayashi, & Ito, 2000). Water-soluble chitosan (WSC) has a wide array of antioxidant properties, including DPPH radical, superoxide anion radical, and hydrogen peroxide radical scavenging activities, as well as Cu^{2+} ion chelating ability (Lin & Chou, 2004).

Tumbling is used to increase brine uptake and protein extraction in meat through the transfer of kinetic energy with a rotating drum or paddles (Lin, Mittal, & Barbut, 1990; Price & Schweigert, 1987). This process has been shown to enhance tenderness, ensure juiciness, effectively promote cohesion of meat pieces during cooking, and develop a uniform product with increased yield and desirable slicing characteristics (Cassidy et al., 1978; Chow, Ockerman, Cahill, & Parrett, 1986; Gillett, Cassidy, & Simon, 1981; Krause, Ockerman, Krol, Moerman, &

Plimpton, 1978). Vacuum is an important component of the vacuum tumbling process and its function is to prevent air from diffusing into the protein gel structure, ensuring that a tacky exudate is present on the surface of meat proteins after tumbling (Price & Schweigert, 1987). Vacuum tumbling can improve tenderness, decrease cooking losses, and enhance the water holding capacity of meat (Rejt, Kubicka, & Pisula, 1978). Vacuum brining facilitates mass transfer in meat by increasing solution uptake through the pores (Deumier, Trystram, Collignan, Guedider, & Bohuon, 2003). CH penetration into shrimp may be facilitated by vacuum tumbling with low viscosity CH nanoparticle or WSC solutions. The penetrated CH into shrimp muscle is expected to reduce lipid oxidation and microbial loads and maintain the quality of the shrimp in frozen storage.

The objective of this research was to evaluate the effects of vacuum tumbling with CH nanoparticle and WSC solutions on the quality characteristics of cryogenically frozen shrimp.

CHAPTER 2. LITERATURE REVIEW

2.1. Factors affecting frozen food quality

2.1.1. Freezing rate

Freezing is a method of long term food preservation in which heat is drawn out from a food due to the utilization of temperatures below its freezing point. At freezing temperatures, water inside of food undergoes a phase change to ice, resulting in a lower water activity (Fellows, 2009). There is a 9% volume expansion of the water contained in food products as a result of freezing and ice formation (Fellows, 2009). Ice formation is the phase change that occurs when water freezes and it results in lower food quality due to physicochemical changes. Freezing is generally a two step process, including ice crystal formation (nucleation) and acretion. Nucleation is the ordering of molecules into a stable conglomerate that acts as a source of crystal growth. Ice nuclei usually arise in food at extracellular spaces, however intracellular ice formation can occur at high freezing rates. To initiate nucleation a certain activation energy must be reached by supercooling water below 0 ºC (Zaritzky, 2000). Supercooling in food occurs when the product's temperature decreases below its freezing point without ice formation (Hui, Legarretta, Lim, Murrell, & Nip, 2004). Heterogenous nucleation is predominant on active surfaces in food, stemming from the crystallization of water molecules. Homogenous nucleation does not occur at food surfaces, instead it is the result of a random orientation of water molecules in pure water (Fellows, 2009). The freezing point of a system is derived from the molar concentration of solute molecules and the freezing pont of food with high solute concentrations is lower than in foods with greater moisture contents. Extracellular ice formation in a food induces a higher solute concentration and reduces the water activity. To preserve chemical

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potential equilibrium in a food system, intracellular water can move outward and begin to freeze over the extracellular ice crystals, causing some dehydration (Zaritzky, 2000).

The rate of nucleation in a system depends on the freezing temperature and rate. The freezing rate of a food is the rate of temperature change, mainly encompassing rates of ice formation and heat removal. Lower supercooling temperatures generally increase nucleation rate until a point of high viscosity is reached, which can stop nucleation. The occurance of nucleation also depends on the rate of heat transfer and sample volume. To assure the highest quality of frozen products, a fast freezing rate is preferred over slow freezing. Rapid freezing causes smaller ice crystal formation in plant and animal tissues compared to slow freezing, resulting in less structural damage to cell walls (Rahman, 2007). According to Giddings and Hill (1978), large ice crystal formation can lead to the emptying of cellular contents due to the rupture of muscle cell membranes during frozen storage. Another benefit of rapid freezing is a lower rate of cell shrinkage and dehydration (Rahman, 2007). The amount of ice crystal formation is greater at high freezing rates by increasing the activity of nucleation sites. The rate of ice crystal acretion after nuclei formation is contingent on the rate of heat removal and mass transfer of water to ice crystal surfaces during freezing. Ice crystal size is inversely proportionate to the quantity of nuclei formed in a system which is why quick freezing creates small ice crystals, while slow freezing yields larger ice crystals. By using quick freezing techniques, rather than slow freezing, some moisture loss can be avoided (Hui et al., 2004). At temperatures less than -40 ºC, the amount of new ice formation is negligible, leaving some non-freezeable water in the food matrix (Zaritzky, 2000).

Generally, the most commerical freezing methods are air blast freezing, plate contact freezing, immersion freezing, fluidized-bed freezing, and rapid cryogenic freezing with a

liquified gas, such as liquid nitrogen or liquid carbon dioxide (Rahman, 2007). In the seafood industry, the most popular and economical freezing methods are cryogenic freezing and air blast freezing (Goncalves & Ribeiro, 2008). Cryogenic freezing involves liquid nitrogen or liquid carbon dioxide and offers various advantages compared to slow freezing methods, such as rapid processing times, and compatibility with a wide range of food products. Also, cryogenic gases can be used to improve handling and processing of soft products by forming a hard frozen crust layer. Food products can by cryogenically frozen by three methods: (1) blowing vaporized cryogenic liquid over food in a batch freezer or spiral freezer, (2) directly spraying cryogenic liquid over food in a tunnel freezer, and (3) using an immersion freezer to submerge the food in a cryogenic liquid. Liquid nitrogen may be preferred over liquid carbon dioxide because it is odorless, colorless, chemically inert, and has a boiling point of -195.8 °C (Rahman, 2007). The vaporization temperatures at 1 atm for liquid nitrogen and liquid carbon dioxide are -195.56 °C and -77.78 °C, respectively (Kolbe & Kramer, 2007).

Air blast freezers are a type of mechanical freezer used to freeze and store food (Fellows, 2009). This process uses fans to circulate cold air in order to freeze a product (Smith, 2011). When using batch freezing equipment, food is placed on trays and held in cabinets or rooms, while equipment used for continuous freezing consists of food trays on trolleys that pass through insulated tunnels or conveyor belts. Air blast freezing recirculates air at a temperature of -30 to -50 °C, using a velocity of 1.5-6.0 ms^{-1}, which raises the surface heat transfer coefficient by diminishing boundary air film thickness around food. Inside of air blast freezers, the air flow is ducted, either from a parallel or perpendicular direction, to distrubute even freezing throughout the food (Fellows, 2009). A drawback of this process is product dehydration. Products typically experience a moisture loss of 1-2%, and sometimes up to 5% (Kolbe & Kramer, 2007). This type

of equipment is efficient, compact, can be used for a multitude of different foods, and has a low capital cost (Fellows, 2009).

2.1.2. Moisture migration and recrystallization of ice crystals

Moisture migration can occur when the cell contents of a food are supercooled during freezing as a result of osmosis. Moisture migration and recrystallization processes can alter product appearance, texture, juiciness, and incur nutrient losses. Frozen foods experience moisture migration due to the temperature and vapor pressure differential in the storage enclosure (Zaritzky, 2000). Moisture migration in frozen food leads to freezer burn due to a lack of a moisture barrier for frozen tissues. An opaque dessicated layer is formed on the food as a result of ice sublimation on tissue surfaces (Rahman, 2007). As a result of ice crystal withdrawal, microscopic cavities are formed that can change the wavelength of reflected light to appear as a light color (Fellows, 2009). Moisture migration can cause drip loss in meats. Drip is the fluid that is not reabsorbed back into the meat when thawing. (Zaritzky, 2000). Drip loss can provide an avenue for enzymatic activity and microbial growth, especially psychotrophic spoilage and pathogenic microorganisms (Fellows, 2009). During meat processing, the product weight loss is about 1% during freezing, 1-2% during chilling, and 0.5-1% for each month of storage and transport, depending on packaging. Various foods can disperse moisture to other food components because of differences in water activities. To prevent moisture migration, temperature fluctuation during storage should be avoided. Temperature fluctuations result in moisture migration from the inner layer of a food outwards toward the surface. Internal barriers should be incorporated in the food product and packaging (Zaritzky, 2000).

The recrystallization of ice crystals in food is a physical transition that results in a loss of quality (Fellows, 2009). Generally, it is known as an increase in average ice crystal size.

Recrystallization can dissipate the edge that fast freezing provides over slow freezing by reducing product quality through physicochemical changes. Also, recrystallization can increase the rate of drip loss (Zaritzky, 2000). The liklihood of recrystallization is lowest belowa food's glass transition temperature and the rate of recrystallization is greatest when the temperature is between melting and glass transition (Rahman, 2007). The glass transition temperature of a food is the temperature at which an amorphous liquid undergoes a phase change to a glassy or solid state. Foods are the most stable when stored below their glass transition temperature. The three different forms of food recrystallization are accretive recrystallization, isomass recrystallization, and migratory recrystallization. Accretive recrystallization occurs when the quantity of crystals in food is decreased by the formation of larger crystals through the fusion of adjacent ice crystals. Isomass recrystallization results in a smaller surface area to volume ratio due to changes in internal structure or surface shape. Migratory recrystallization is a reduction in the quantity of crystals as well as a growth in the size of crystals and it is triggered by storage temperature fluctutations. As warm air enters a cold storage unit, the ice crystals in some of the foods in proximity to the heat slightly melt and increase water vapor pressure, causing moisture migration to areas of low vapor pressure in addition to dehydration. After the heat in the enclosure dissipates, Ostwald ripening occurs due to the attachment of water vapor onto present ice crystals. To prevent ice recrystallization in food, a low and stable storage temperature should be used as well as the use of compact packaging that limits moisture migration (Fellows, 2009).

2.1.3. Protein denaturation

Protein denaturation caused by freezing is typically the result of a number of factors, such as dehydration, oxidation, salt concentration, ice formation, recrystallization, and cellular metabolite activity. The functionality of proteins can be measured by water holding capacity,

foaming, gelation, viscosity, emulsification, as well as whipping properties. Protein denaturation can cause changes in muscle texture, primarily due to the presence of free fatty acids and formaldehyde (Zaritzky, 2000). Formaldehyde is present due to the decomposition of trimethylamine oxide and it can promote aggregation and cross-linking of proteins (Ang & Hultin, 1989). In frozen fish, lipase activity can lead to an increase in free fatty acid content (Erickson, 1993). An increased concentration of peptides, amino acids, and nucleic acids show evidence of protein denaturation and structural cell damage (Rahman, 2007). During freezing lysosome breakage can occur, which may result in the release of enzymes, especially when slow freezing rates are used. Temperature fluctuations can also cause harm to lysosome structure (Geromel & Montgomery, 1980). Freezing can lower the water holding capacity of muscle by exchanging water-protein interactions with protein-protein interactions. This occurs because as water moves out from the interior of cells during freezing, proteins interact with compounds of a lower polarity, thus exposing hydrophobic chains that can alter protein conformation and lead to protein denaturation and aggregate formation.

The structure of proteins can be changed by the rise of ionic strength due to competition with electrostatic bonds by virtue of a higher salt concentration during freezing. Studies done on beef muscle meausuring differential scanning calorimetry and ATPase activity indicate that a slower freezing rate results in greater myofibrillar protein denaturation due to myosin unfolding and hydrophobic interactions. While myofibrillar proteins undergo denaturation during freezing, sarcoplasmic proteins do not (Zaritzky, 2000).

2.1.4. Lipid oxidation

Lipid oxidation causes rancidity, color deterioration, and off-flavors in foods (Rahman, 2007). The majority of flavor changes in frozen foods caused by lipid oxidation results from the degradation of hydroperoxides into ketones and aldehydes (Erickson & Hung, 1997). Lipid

oxidation also lowers the nutritive value of food while degrading flavor and taste, and it may cause heart diseases, aging, stroke, emphysema, mutagenesis, and carcinogenesis (Bera, Lahiri, & Nag, 2006). Oxidation in saturated lipids can take place anywhere along the hydrocarbon chain, but in unsaturated lipids, oxygen permeates close to the unsaturated center of the molecule (Schultz, 1962). The autooxidation process begins with an initiation stage in which a fatty acid (Rhoades & Roller, 2000) discards a hydrogen atom, resulting in a fatty acid alkyl radical (R•). During propogation, in the presence of oxygen, the fatty acid alkyl radical transforms into a fatty acid peroxyl radical (ROO•). Then a hydroperoxide is produced along with another fatty acid alkyl radical from the deduction of a hydrogen atom from an adjacent RH (ROO• + RH → R• + ROOH) by a peroxyl radical. Decomposition of the hydroperoxide occurs to extend the free radical process. Hydroperoxide decomposition leads to formation of aldehydes and ketones, a source of rancidity (Zaritzky, 2000). For termination to transpire, a free radical or free radical inhibitor must transform the peroxyl radical into a hydroperoxide to achieve resonance stabilization (Ingold, 1962). Lipid oxidation may occur through enzymatic and non-enzymatic processes (Zaritzky, 2000).

In frozen foods, the major type of oxidation present is typically autooxidation, in addition to some enzymatically derived oxidation (Schultz, 1962). An enzyme that can catalyze oxidation is lipoxygenase and it is found in several plants and animals. These catalyst enzymes should be inactivated by blanching, otherwise off-flavors may form, as well as loss of pigments. In addition to enzymes, iron, and other transition metals are effective catalysts for lipid oxidation. Lipid oxidation in frozen foods leads to deterioration of several quality attributes, such as appearance, nutritional value, protein functionality, and flavor. Frozen muscle is more prone to oxidative flavor deterioration compared to vegetables, which are usually blanched before freezing, thus

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inactivating catalyst enzymes. Chlorophyll can be used as an oxidative subrstrate and heme pigments in red meat can also be degraded (Zaritzky, 2000). The concentration of solutes formed as a result of freezing can catalyze the initiation of lipid oxidation as well as damage and dehydrate cell membranes, subjecting uncovered phospholipids to lipid oxidation (Rahman, 2007).

2.1.5. Microbial and enzyme activity

Freezing reduces the rate of microbiological and biochemical changes in food to extend its shelf life. The rate of microbial death is highest during the initial freezing process and it gradually declines over time in frozen storage (Adams & Moss, 1995). Yeasts, molds, and bacteria are largely inhibited at temperatues lower than -12, -18, and -10 °C, respectively.

Freezing can inhibit microbial growth due to factors such as temperature shock, dehydration, ice formation, concentration of extracellular solutes, and the toxicity of intracellular solutes. Freezing degrades the lipid containing membranes of microorganisms by dehydration (Zaritzky, 2000). Osmotic dehydration is promoted by extracellular ice formation and it results in a greater extracellular solute concentration. As intracellular macromolecules converge near the membranes, emerging repulsive forces cause phase seperation, deformation, and nonlamellar phase formation. When quick freezing is done at very low temperatures, lipid crystallization occurs readily in microbial membranes, limiting the rearrangement of intramembrane particles. During quick freezing, only the region outside of the cytoplasmic membrane of microorganisms is damaged. In products that are slowly frozen there is breakdown of the outer and cytoplasmic membranes, resulting in the detachment of the outer membrane due to extracellular ice crystal formation. This membrane damage reduces cell viability by the leakage of β-galactosidase, potassium ions, amino acids, low molecular solutes, RNA, and DNA from the cells of

microorganisms (Rahman, 2007). Slow freezing and thawing is more effective at inhibiting microorganisms compared to quick freezing and thawing. A higher lethal effect on microorganisms is observed at storage temperatures of -2 to -10 °C than at -15 to -30 °C due to protein denaturation that occurrs at the higher temperatures. When storage temperatures below -60 °C are used, damage to microorganisms is even lower.

Microorganisms in the log phase of bacterial growth are more easily damaged by freezing than those in the stationary phase. Also, microorganisms in the lag phase can withstand sharp temperature decreases (Zaritzky, 2000). Factors that can influence microbiological proliferation in frozen foods include pre-freezing microbiological quality, product handling during and after freezing, as well as the physicochemical characteristics of the food. The temperature resistance of different microorganisms is a key element in determining the quantity and activity of microbes present in a food system. Yeast and mold vegetative cells, in addition to gram-negative bacteria, are the least resistant to low temperatures. Mold spores and gram-positive bacteria possess a greater resistance, while bacterial spores are almost immune to low temperatures (Fellows, 2009).

Although freezing slows microbial growth, it does not inactivate enzymes (Fellows, 2009). Cold storage can slow enzymatic activity in food tissues. In meats, hydrolytic and lipolytic enzymes cause quality deterioration during frozen storage. Hydrolytic enzyme activity can lead to textural changes, color loss, hydrolytic rancidity, and an increased rate of lipid oxidation. Lipid hydrolysis, catalyzed by lipolytic enzymes, can result in off-flavors and textural changes in frozen foods. Also, lipase activity in frozen fish results in free fatty acid accumulation which can alter texture and cause hydrolytic rancidity. During freezing, the rate of lipolysis can increase, particularly with fluctuating temperatures and low freezing rates (Zaritzky, 2000).

2.2. Freezing and frozen storage of seafood

Freezing reduces the rate at which foods deteriorate by slowing physicochemical and biochemical reactions, as well as enzymatic and microbial activity. During frozen storage, foods can be deteriorated by enzymatic activity and lipid oxidation, causing vitamin loss, pigment degradation. The various factors that affect the quality of frozen foods include storage temperature, storage time, and thawing process (Fellows, 2009). Freezing generally preserves the nutritional quality of food, although vitamin losses can be observed, especially for water soluble vitamins, albeit at a much lower rate than at room temperature or chilled storage (Zaritzky, 2000). During frozen storage, color change in products can occur due to pigment oxidation, lipolysis, and freezer burn (Hui, 2006).

Freezing and subsequent frozen storage of shrimp is necessary because it is easily spoiled at ambient temperatures. Shrimp contains greater amounts of free amino acids and volatile nitrogenous bases than chicken or red meat, which amplifies the need for proper storage conditions (Elsabee & Abdou, 2013). White shrimp (*Litopenaeus setiferus*) have a proximate composition (g/100 g) of 77.18±1.16 moisture, 18.57±1.07 protein, 1.78±0.23 ash, and 1.12±0.13 total lipids (Solval et al., 2014). The fatty acids found in white shrimp are mainly polyunsaturated and contain 12.8±1.4% Eicosapentaenoic acid C20-5 ω-3 (EPA) and 9.8±2.6% Docosahexaenoic acid C22-6 ω-3 (DHA) (Bottino, Gennity, Lilly, Simmons, & Finne, 1980). This may make shrimp susceptible to oxidation during frozen storage. During chilled and frozen storage of shrimp, discoloration (melanosis) can appear. This is caused by the post-mortem activity of polyphenol oxidase, which is naturally found in shrimp. This enzyme oxidizes phenolic substrates to form o-benzquinones which then produce melanins that constitute a dark color in shrimp (Nirmal & Benjakul, 2009). Typically, the indigenous microbiota of marine fish

consists mainly of gram-negative facultative anaerobic bacteria, such as *Vibrio* and *Pseudomonas* (Balcazar et al., 2006). Gram-negative microorganisms of the genus *Pseudomonas, Achromobacter, and Flavobacterium* predominantely inhabit the microbial flora of fresh iced shrimp (Chander & Lewis, 1980). The practical shelf life of frozen shrimp is 6 months at -18 °C, 12 months at -25 °C, and 12 months at -30 °C (Johnston, 1994).

With regard to the freezing process, Johnston (1994) reported that three phases are observed in the freezing of fish muscle (Figure 2.1). In the first phase, cooling of the muscle occurred during which the temperature was lowered to slightly below 0 ºC. In the second phase, most of the water in the system was converted to ice as the temperature of the muscle decreased slightly. If the second phase was extended, there could be larger ice crystal formation and acretion. In the third phase, the majority of remaining water in the fish muscle froze. The third phase ensued after about 55% of the water in the fish muscle was frozen at which point the temperature decreased quickly (Johnston, 1994).
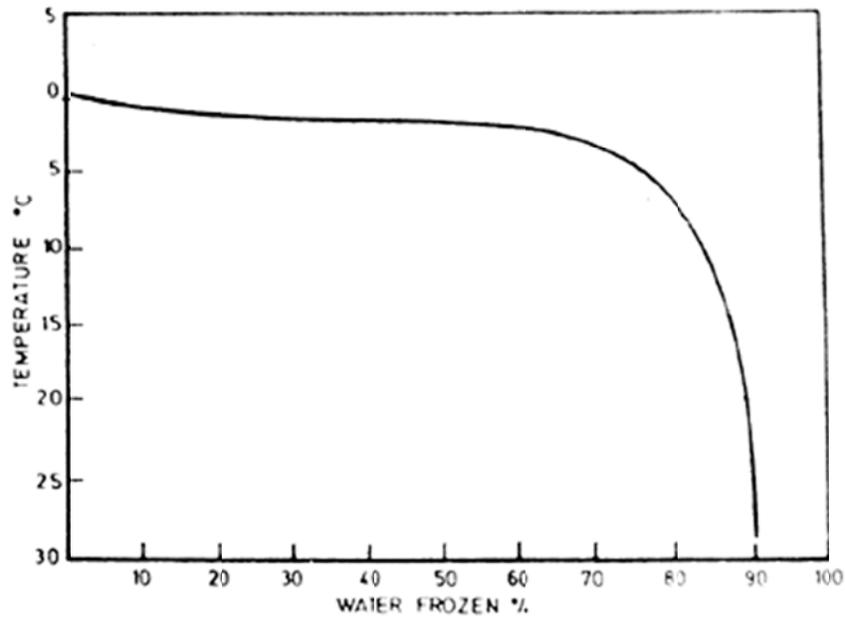
Figure 2.1. Quantity of water frozen in fish muscle at various temperatures.
Source: Johnston (1994)

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Seafood can degrade during frozen storage due to ice formation, moisture migration, recrystallization of ice crystals, protein denaturation, as well as microbial and enzyme activity (Boonsumrej, Chaiwanichsiri, Tantratian, Suzuki, & Takai, 2007). Because of seafood's high polyunsaturated phospholipid content, lipid oxidation during storage is also a concern. Over time, seafood in frozen storage undergoes surface dehydration, which results in protein denaturation. This causes a decreased water holding capacity in tissues, possibly leading to changes in meat texture (Shenouda, 1980). The lower water holding capacity of frozen seafood may be due to the shortening of sarcomeres in muscles (Jarenback & Liljemark, 1975). After fish die, they begin to spoil and undergo quality changes, such as meat softening, off-flavors, and loss of fluid containing protein and fat.

The unfrozen water in a food system is reactive and can reduce the shelf life of a food by allowing physicochemical and enzymatic reactions. When freezing a food, the thermal center of that product should reach a temperature of at least -18 ºC to ensure termination of microbial growth and to delay physicochemical changes (Rahman, 2007). Frozen fish can experience varying degrees of lipid oxidation, depending on oxygen availability, tissue composition, free fatty acid content, and lipid hydrolysis (Rahman, 2007). Losses of ω-3 polyunsaturated fatty acids, as well as the formation of off odors and flavors can be a problem in fatty fish, moreso than in white fish (Fellows, 2009). Although, oxygen can permeate to the unsaturated phospholipids in the membranes of lean fish, which would promote greater oxidation than in adipose triglycerols.

The muscle proteins of fish are prone to protein denaturation (Reid, Doong, Snider, & Foin, 1987). During frozen storage, fish degradation occurs due to a loss of protein extractibility, solubility, and nutritional value (Rahman, 2007). Shrimp myosin may undergo deterioration due

to cathepsins that can damage sarcomeres and z-lines (Smolinska & Abdul-Halim, 1992). According to the findings of Jiang, Hwang, Moody, and Chen (1991), the solubility of actomyosin in grass prawn reduced with increasing frozen storage time. Slow freezing of seafood may result in moisture loss due to the puncturing of muscle by large ice crystals, leading to undesirable texture changes (Shenouda, 1980). Seafood that is rapidly frozen experiences a lower protein denaturation rate than slow frozen products due to less time spent in the zone where a majority of ice crystal formation occurs (-1 to -2 °C) (Johnston, 1994). It has been reported by Goncalves and Ribeiro (2008) that shrimp which are quickly frozen and stored at low temperatures experience little drip loss compared to slowly frozen shrimp. Various characteristics than can influence the amount of drip loss in frozen meats are the magnitude of water reabsorption, condition of tissues before freezing, tissue water holding capacity, rate of thawing, as well as the location and size of ice crystals (Zaritzky, 2000). Lampila, Mohr, and Reid (1985) investigated patterns of ice crystal formation by subjecting rockfish fillets to varying freezer storage temperatures. At higher temperatures ice crystal size increased, indicating that lower frozen storage temperatures may better prevent muscle damage and drip loss (Lampila et al., 1985).

2.3. Edible films and coatings

Edible films and coatings are non-pollutant products made of natural and biodegradable substances. The application of these materials results in decreased moisture transfer in frozen foods, as well as lower oxygen permeability. They should have high barrier and mechanical efficiencies, good sensory qualities, freedom from toxic compounds, safety, simplicity, low cost, as well as adequate physicochemical, biochemical, and microbial stability. Coatings can be spread onto foods or be made as part of a food, while films are used to wrap food after it is

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produced. They are typically used as oxygen and moisture barriers to preserve the quality of foods. Also, edible films and coatings can encapsulate antioxidants, pigments, aroma compounds, antimicrobial agents, and vitamins. Edible films and coatings should be considered as additives rather than ingredients because they generally provide insignificant nutritional value (Debeaufort, Quezada-Gallo, & Voilley, 1998). A desired quality of edible films and coatings is tastelessness, to limit interference with the sensory qualities of the product (Labuza & Contreras-Medellin, 1981). If a certain taste or flavor is present, it must complement the food product. Edible packagings may be able to improve the mechanical properties of food as well as sensorial characteristics, for instance, shininess, color, transparency, sticking, or roughness. The selective barrier and permeability capacity of edible films and coatings for a variety of substances is shown in Figure 2.2.
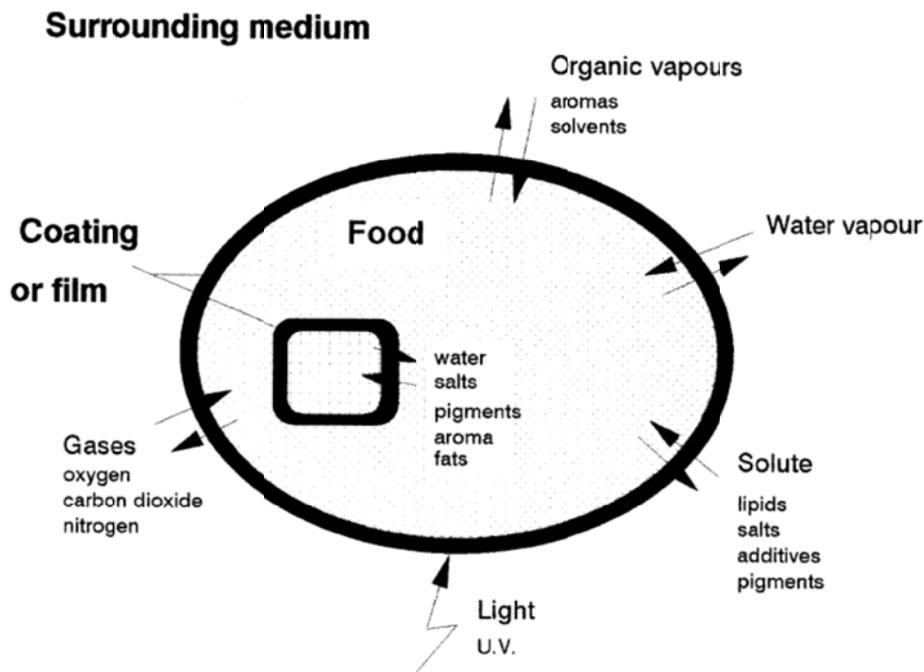
Figure 2.2. Selective barrier and permeability capacity of edible films and coatings.
Source: Debeaufort et al. (1998)

Edible films and coatings can provide an effective barrier to limit surface dehydration in fresh or frozen meats, fruits, and vegetables. For example, water absorption could be limited in food powders and dry cakes to prevent caking and loss of crispiness, respectively. Also, fruit ripening can be more effectively modulated by controlling oxygen permeability. Foods with high amounts of polyunsaturated fats can be better protected from rancidity with edible films and coatings. In composite food products that contain multiple layers or components, edible films and coatings can reduce moisture migration. With the inclusion of pigments or light absorbers, edible packagings may reduce UV light interaction with the food product, resulting in less radicalair reactions. Transfer of solutes within foods can also be controlled using edible films and coatings (Debeaufort et al., 1998). Hydrophobic materials, such as lipids, resins, and waxes are very effective for delaying moisture transfer in foods (Gontard & Guilbert, 1994). Water soluble hydrocolloids, typically proteins and polysaccharides, are less effective moisture barriers. However, they provide better mechanical properties to edible films and coatings than hydrophobic materials. Typically, multilayered films and coatings exhibit superior mechanical and barrier properties than emulsion methods. Although, due to the multiple production steps required for multilayered films and coatings, emulsion methods have more industrial applications (Debeaufort et al., 1998). Some commercial applications of edible films and coatings include chocolate coatings for confections, water-soluble cellulose ether-based coatings used for food ingredients, gelatin-based pharmaceutical coatings, and collagen casings used to enclose sausages. Some of the advantages of using edible films and coatings for meat, poultry, and fish include less moisture and oxygen transfer, antimicrobial and antioxidant activity, less volatile flavor loss and foreign odor pick-up, as well as additional nutritional value, depending on the type and concentration of film or coating that is applied (Gennadios, Hanna, & Kurth, 1997).

Chitosan-based edible films and coatings have been widely used to preserve food quality and extend shelf life due to their antimicrobial activity. Because chitosan-based films exhibit high water vapor permeability, they are typically combined with other hydrocolloids to lower water vapor transmission rates. In a study conducted by Sathivel, Liu, Huang, and Prinyawiwatkul (2007), it was found that glazing skinless pink salmon filets with a 1% w/w chitosan solution delayed lipid oxidation and elicited a higher thaw yield than in the non-glazed control after frozen storage for eight months. It was determined by Fan et al. (2009) that coating silver carp with 2% chitosan dip treatment can preserve its quality characteristics and extend the shelf life during frozen storage. Compared to silver carp treated using the 1% acetic acid control, the 2% chitosan coated carp had lower total viable counts and thiobarbituric-acid-reactive-substances (TBARS) values, as well as higher sensory scores during the 30 day frozen storage time. Edible films and coatings consisting of chitosan blends that may have potential to preserve food quality include: chitosan-starch, chitosan-gelatin, chitosan-alginate, chitosan-essential oil, and chitosan-clay (Elsabee & Abdou, 2013).

2.4. Chitosan

2.4.1 Structure and production

Chitosan is a non-toxic biopolymer composed of β-(1-4)-linked 2-amino-2-deoxy-glucopyranose and 2-acetamido-2-deoxy-β-D-glucopyranose (Rampino, Borgogna, Blasi, Bellich, & Cesaro, 2013). Chitosan is derived from chitin, a polysaccharide found in the exoskeleton of insects and crustaceans, as well as the cell walls of fungi (Dash, Chiellini, Ottenbrite, & Chiellini, 2011). Chitin is the second most available biopolymer on earth after cellulose (Shahidi et al., 1999). Chitin and chitosan have been used in their native and modified structures in food, material science, biotechnology, drugs, and pharmaceuticals, as well as gene

therapy (Prashanth & Tharanathan, 2007). The structure of chitin is similar to that of cellulose, except that it contains acetamide groups instead of C-2 hydroxyl residues (Kurita, 1998). Chitin consists of poly β-(1-4)-N-acetyl-D-glucosamine and is insoluble. Once its degree of deacetylation (DD) reaches around 50%, it may be solubilized in acidic media due to the -NH_2 function at the C-2 position of D-glucosamine becoming protonated. After solubility is obtained, the polymer can be classified as chitosan (Rinaudo, 2006). Lowering the molecular weight of chitosan can increase water solubility by altering the content of N-acetylglucosamine units, resulting in different chitosan conformations (Kubota, Tatsumoto, Sano, & Toya, 2000). The concentration of glucosamine units in chitosan determines properties such as solubility, biodegradability, antimicrobial activity, and wound healing attributes (Cho, Jang, Park, & Ko, 2000). In addition to its amino/acetamido reactive functional group, chitosan has primary and secondary hydroxyl groups at C-3 and C-6 positions, respectively (Xia, Liu, Zhang, & Chen, 2011). Due to the removal of acetyl groups, chitin can become chitosan with free amino (NH_2) groups (Figure 2.3). The pKa value of the D-glucosamine residues in chitosan is around 6.2-7.0, rendering it insoluble at neutral and alkaline pH (Rojanarata, Opanasopit, Techaarpornkul, Ngawhirunpat, & Ruktanonchai, 2008). In acidic solutions, the free amino groups of chitosan become protonated on the C-2 position of the pyranose ring (Hsu, Don, & Chiu, 2002). Also, the acidic conditions promote greater interactions between positively charged chitosan and negatively charged molecules, such as proteins, fats, cholesterols, and metal ions (Li, Dunn, Grandmaison, & Goosen, 1992).
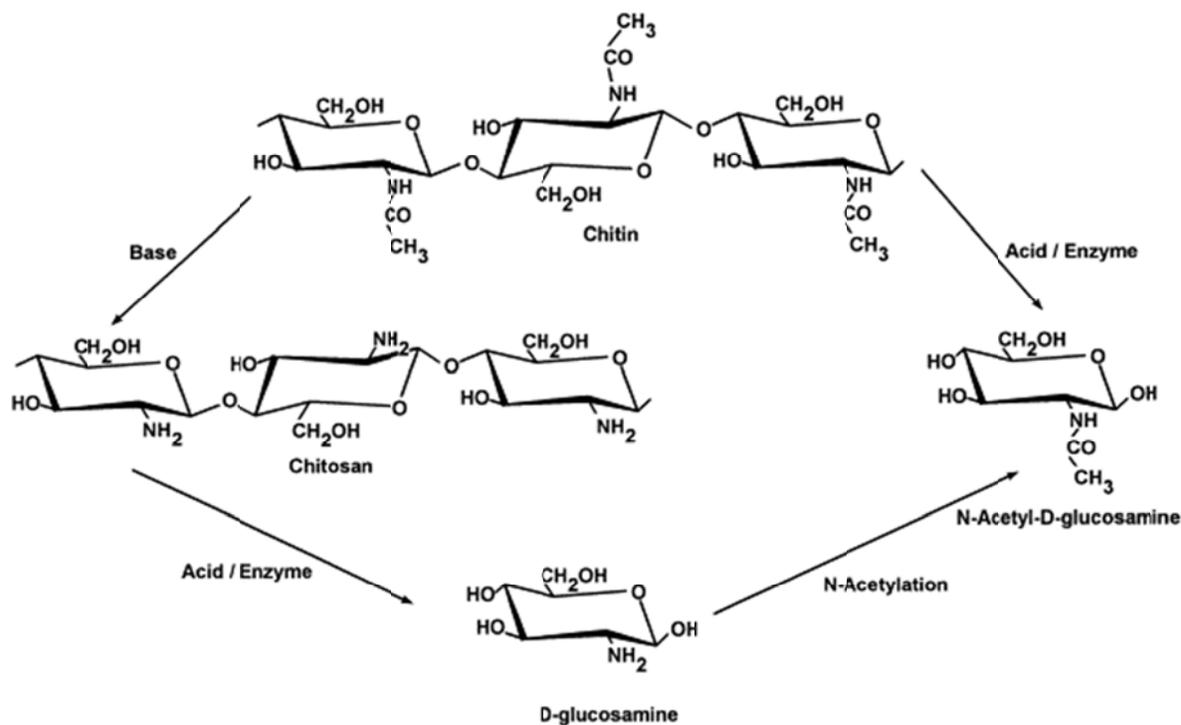
Figure 2.3. Preparation and chemical structure of chitin derivatives
Source: Shahidi et al. (1999)

Shrimp and crab shells are the main sources of commercial chitosan production. There are about 10^9-10^{10} tons of shrimp and crab shell waste produced every year worldwide (Peter, 1995). The composition of crustacean shells is 30-50% calcium carbonate, 30-40% protein, and 20-30% chitin, depending on the species and season. The physicochemical properties of chitin and chitosan vary with source, species, and preparation technique. Some of the attributes likely to be affected by these factors are the DD, acetyl group distribution, as well as chitosan chain length and conformational structure (Brine & Austin, 1981). Chitosan can be found in a wide range of molecular weights, from a few hundred to over a thousand kDa, and it can be grouped into three categories: low molecular weight chitosan, medium molecular weight chitosan, and high molecular weight chitosan (Kumirska et al., 2011). Commercial chitosan generally has a molecular weight of 10-1000 kDa and DD of 70 to 95% (Moura, Moura, Soares, & de Almeida Pinto, 2011).

Chitosan is typically manufactured through chemical or microbiological processes (Tsai, Su, Chen, & Pan, 2002). As shown in Figure 2.4, production of chitosan from shellfish waste using the chemical process involves four steps: (1) protein separation by deproteinization, (2) calcium carbonate separation by demineralization, (3) pigment separation by decoloration, and (4) removal of acetyl groups by deacetylation. After the first three steps, chitin is isolated and with further deacetylation chitosan is produced (Shahidi et al., 1999). NaOH, HCl, and a de-coloring agent are used to remove protein, calcium carbonate, and pigment, respectively. Then NaOH is used again for the final deacetylation step (Tsai et al., 2002).
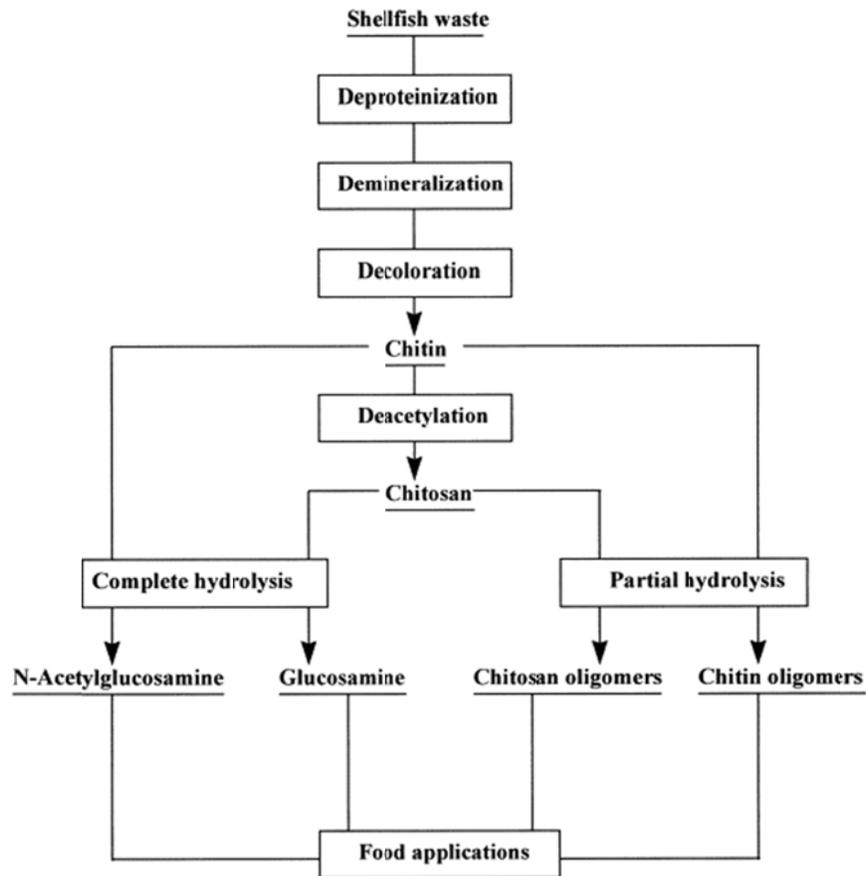
Figure 2.4. Flowchart of chitin, chitosan, and chitooligosaccharide production from shellfish waste.
Source: Shahidi et al. (1999)

2.4.2 Physicochemical properties and applications

Chitosan is distinguished by its structural characteristics, such as the DD and molecular weight. These characteristics can influence the various functional properties of chitosan, as shown in Table 2.1. The DD measures the concentration of free amino groups in polysaccharides. This characteristic can be used to differentiate chitin from chitosan and it has a significant effect on chitosan's physicochemical properties and applications (Muzzarelli, 1977).

Table 2.1. Effect of structural characteristics on chitosan functional properties

Property	Structural characteristics[a]
Solubility	↑ DD
Crystallinity	↓ DD
Biodegradability	↓ DD, ↓ Molecular weight
Viscosity	↑ DD
Biocompatibility	↑ DD
Biological	
Mucoadhesion	↑ DD, ↑ Molecular weight
Analgestic	↑ DD
Antimicrobial	↑ DD, Molecular weight
Permeation enhancing effect	↑ DD
Antioxidant	↑ DD, ↓ Molecular weight
Hemostatic	↑ DD

[a] ↑–Directly propotional to property; ↓–inversely propotional to property
Source: Dash et al. (2011)

Chitosan with low DD degrades more rapidly than high DD chitosan and the rate of degradation can be affected by the arrangement of acetyl groups and length of the polymer chains (molecular weight). Generally, crystallinity is inversely related to DD, but it is highest for 0% deacetylated chitin and 100% deacetylated chitosan (Dash et al., 2011). This is because fully deacetylated chitosan and fully acetylated chitin have greater chemical regularity than partially deacetylated chitosan or chitin. The chemical regularity of the polymer chain decreases from 0 to 50% DD and increases from 50 to 100% DD. Also the polymer chains of chitosan with higher

DD are more flexibile, promoting the formation of hydrogen bonds and ensuing crystallization (Wenling et al., 2005).

Chitosan's antimicrobial activity is mainly contingent on molecular weight, concentration, DD, and type of bacteria (Jeon, Park, & Kim, 2001; No et al., 2002b; Tsai et al., 2002; Zheng & Zhu, 2003). Also, it has been shown that chitosan exhibits superior antibacterial properties compared to chitosan oligomers (Jeon et al., 2001; Uchida, 1989). The interaction between protonated amine (NH_3^+) groups of chitosan and the negatively charged surface components of various bacteria and fungi may play an extensive role in the antimicrobial mechanisms of chitosan (Raafat & Sahl, 2009). The lethal action of chitosan on microorganisms can be described in six steps: (1) adsorption onto the bacterial cell surface, (2) diffusion through the cell wall, (3) adsorption onto the cytoplasmic membrane, (4) disruption of the cytoplasmic membrane, (5) leakage of the cytoplasmic constituents, and (6) death of the cell (Kong, Chen, Xing, & Park, 2010).

In another suggested mode of action, chitosan may be able to form a polymer membrane on the cell surface, preventing nutrients from reaching the cell (Zheng & Zhu, 2003). Diffused chitosan hydrolysis products may be able to interact with microbial DNA, resulting in inhibition of mRNA and protein synthesis (Sudarshan, Hoover, & Knorr, 1992). This polymer possesses antifungal properties that are executed through the suppression of sporulation and spore germination (Hernandez-Lauzardo et al., 2008). Chitosan has higher antimicrobial activity at lower pH values, as well as with increasing temperatures (Raafat & Sahl, 2009). According to a study conducted by Jarry et al. (2001) on the antimicrobial activity of chitosan against different microorganisms, it was shown that bacteria that were separated from the chitosan solution by membrane filtration could grow rapidly. The bacteriostatic activity of chitosan may be linked to

its ability to bind to microbial cells, negating any further activity against unbound microorganisms (Rhoades & Roller, 2000).

Chitosan has been shown to have a wide range of antimicrobial activities depending on bacterial type. Chung et al. (2004) reported that gram-negative bacteria could be inhibited more effectively than gram-positive bacteria, citing greater hydrophilicity and adsorptive characteristics between chitosan and the gram-negative bacterial cell as the mechanism of action. While it was observed by No et al. (2002b) that chitosan had more potent bactericidal effects against gram-positive bacteria than gram-negative bacteria, also noting that chitosan had higher antimicrobial activity at lower pH. Tsai et al. (2002) showed that chitosan was more effective at inhibiting bacteria than fungi. Also, the findings of Wang, Du, and Liu (2004) showed no difference in the antimicrobial activities of chitosan against gram-positive bacteria or gram-negative bacteria, although it was suggested that chitosan with higher DD confers a higher positive charge, resulting in greater antimicrobial activity. In a study conducted by Tsai et al. (2002), the minimal lethal concentrations of chitosans ranging from 53 to 98% DD were obtained using several gram-positive and gram-negative microorganisms, as well as fungi. Chitosan with 98% DD was the most effective at inhibiting microorganisms, thus it was then used as a coating to conduct a shelf life study. The shelf life of Sockeye salmon was extended from 5 to 9 days at 4 °C by soaking for 3 h in a solution prepared by dissolving 1% chitosan (98%DD) in 0.1 N HCl. Chitosan treated salmon exhibited lower mesophillic, psychotrophic, coliforms, *Aeromonas*, and *Vibrio* species counts, as well as lower volatile basic nitrogen values compared to control samples that were soaked in 0.1 N HCl for 3 h. According to the findings of Kong et al. (2008), chitosan microspheres with high DD (97.5%) had greater antibacterial activity against *Staphylococcus aureus* than chitosan with moderate DD (83.7%) at pH 5.5. The

higher positive charge density of chitosan with high DD was associated with strong electrostatic interactions. Sagoo, Board, and Roller (2002) showed that adding 0.3 or 0.6% chitosan glutamate to an unseasoned minced pork mixture decreased total viable counts, yeasts and molds, as well as lactic acid bacteria up to 3 log CFU/g in the first 3 days of storage at 4 °C. Chitosan treated pork had a lower number of all microbial counts during the 18 day storage period compared to the untreated control. Antimicrobial activity was concentration dependent, with higher chitosan concentrations having a greater effect.

The viscosity of chitosan solutions is another important functional property that depends on a number of factors, such as molecular weight, DD, concentration, pH, ionic strength, and temperature (Li et al., 1992). The viscosity of chitosan can help identify its commercial applications and range of antimicrobial activity. In a study conducted on the antimicrobial activity of chitosan against *Escherichia.coli* and *Bacillus* sp., greater antimicrobial effect was noted when the viscosity of chitosan decreased from 1000 to 10 cP (Cho, No, & Meyers, 1998). The viscosity of chitosan is associated with its molecular weight, exhibiting higher molecular weights with increasing viscosities and lower molecular weights with decreasing viscosities. Increasing the time that chitosan is treated with techniques such as heating, grinding, autoclaving, ultrasonication, and ozonation, results in decreased viscosity (No, Kim, & Meyers, 1999).

While chitosan may be able to inhibit a large spectrum of bacteria, its antimicrobial effects vary with different molecular weight and type of bacteria. It was reported by No et al. (2002b) that chitosans of molecular weights between 28 and 1671 kDa inhibited growth of most of the bacteria tested (four gram-negative and seven gram-positive) with generally a stronger effect against gram-positive bacteria compared to gram-negative bacteria. According to Zheng

and Zhu (2003), gram-negative and gram-positive bacteria are inhibited by chitosan through different mechanisms. The antimicrobial activity, with regard to gram-positive *S. aureus*, increased as the molecular weight of chitosan increased. However, for gram-negative *E. ecoli*, higher antimicrobial activity was observed as molecular weight decreased. The antimicrobial mechanism for *S. aureus* may have resulted from the formation of a polymer membrane on the surface of the cell by the higher molecular weight chitosan, which could prevent nutrients from enetering the cell. In the case of *E.coli*, the lower molecular weight chitosan may have been able to enter the cell through pervasion (Zheng & Zhu, 2003). The polymer chains of low molecular weight chitosan have the ability to bind to more than one cell due to greater flexibility. This allows for rapid bridge formation between the polymer chains of chitosan and bacterial cells, leading to immediate inactivation of bacteria (Wu, Zeng, Mo, & Ruan, 2006).

Tsai et al. (2002) showed that the shelf life of salmon fillets that were dipped in 49.1 kDa molecular weight chitosan solution (1% in 0.1 N HCl) for 3 h could be extended from 5 to 9 days through reduction of psychotroph, mesophile, coliform, *Aeromonas* spp., and *Vibrio* sp. counts, as well as by slowing the rate at which volatile basic nitrogen content was produced. Jeon et al. (2002) found that chitosan coatings with molecular weights of 660, 960, and 1800 kDa could significantly reduce chemical spoilage (total volatile basic nitrogen, trimethylamine, and hypoxanthine), lipid oxidation, and microbial growth in Atlantic cod (*Gadus morhua*) and herring (*Clupea harengus*) compared to uncoated samples. Chitosans with molecular weights of 960 and 1800 kDa preserved the fish more effectively than 660 kDa chitosan. In a study conducted by (Raafat, Von Bargen, Haas, & Sahl, 2008) in which microbial inhibition was tested using several chitosan preparations, antimicrobial activity was not signficantly different for any chitosan with molecular weight of more than 10 kDa.

Chitosan possesses antioxidant properties which can be used to preserve food during storage, especially seafood because it typically contains highly unsaturated fatty acids which are sensitive to oxidation (Prashanth & Tharanathan, 2007). The antioxidant activity of chitosan is related to its DD and molecular weight, exhibiting greater activity at higher DD and lower molecular weight (Dash et al., 2011). Kamil, Jeon, and Shahidi (2002) evaluated the antioxidative activity of chitosans with different viscosity (14 cP, 57 cP, and 360 cP) in cooked comminuted herring. All chitosan treated herring samples showed lower peroxide values and total volatile aldehyes compared to untreated samples. The low viscosity chitosan (14 cP) was found to have the greatest antioxidative effect. The antioxidant effect of chitosans with different viscosity in cooked comminuted fish model systems could be affected by the molecular weight of the polymer, which may be able to regulate chelation of metal ions. The protonated amino groups of chitosan are able to increase hydrodynamic volume by extended chain conformation through intramolecular electric repulsive forces. This may explain why high viscosity (high molecular weight) chitosans display inferior chelation properties compared to lower viscosity chitosans.

Because the degree of polymerization is related to chitosan viscosity, the DD may have an effect on chelation ability. It was reported by Kim and Thomas (2007) that DPPH radical scavenging activity depended on chitosan molecular weight. When comparing scavenging activites of chitosans with different molecular weights (30, 90, and 120 kDa), 30 kDa chitosan had the highest scavenging activity. This is because lower molecular weight chitosan could have higher mobility than 120 kDa chitosan, decreasing the possibility of inter and intramolecular bonding between high molecular weight chitosan molecules and perhaps enhancing the exposure of amino groups. Additionally, higher scavenging activities at greater chitosan concentrations

may be due to the availability of a higher quantity of amine groups. Also, the antioxidant activities *in vitro* of different chitosans with different molecular weights were observed by Xing et al. (2005). It was found that low molecular weight (9 kDa) chitosan had stronger scavenging effects than high molecular weight (760 kDa) chitosan on hydroxyl and superoxide radicals. Scavenging activity was greater with increasing concentration of chitosan. In a study conducted by Yen, Yang, and Mau (2008), crab chitosans that had been exposed to varying alkaline N-deacetylation times (60, 90, and 120 min) were tested for antioxidant activity. All chitosans had similar chelating ability on ferrous ions, but hydroxyl radical scavenging ability was related to their N-deacetylation times. Crab chitosan with higher DD had superior antioxidant properties because more amino groups were present on the C-2 atom. Chitosan has extraordinary potential for applications in food and nutrition, material science, biotechnology, drugs and pharmaceuticals, agriculture and environmental protection, as well as gene therapy (Prashanth & Tharanathan, 2007).

The application potential of chitin/chitosan is shown in Figure 2.5. The FDA has not officially designated chitosan as GRAS (Generally Recognized As Safe), but it has received approval for utilization in medicine, such as for bandages and drug encapsulation. In 2001, a Norwegian company (Primex Ingredients ASA) announced that their product, ChitoClear®, a shrimp-derived purified chitosan, has attained self-affirmed GRAS status in the U.S. market (Raafat & Sahl, 2009).

2.4.3. Water-soluble chitosan

Although chitosan has vast potential in many fields, because of its water insolubility at neutral pH values, uses may still be limited in some areas (Ilyina et al., 1999; Xie et al., 2001), such as in food, health, agriculture (Roncal et al., 2007), and biomedical applications (Snyman et

al., 2002). It is water-insoluble because phase separation occurs in chitosan solutions with pH values of greater than 6.5, which is the approximate pKa value of chitosan's amino group.
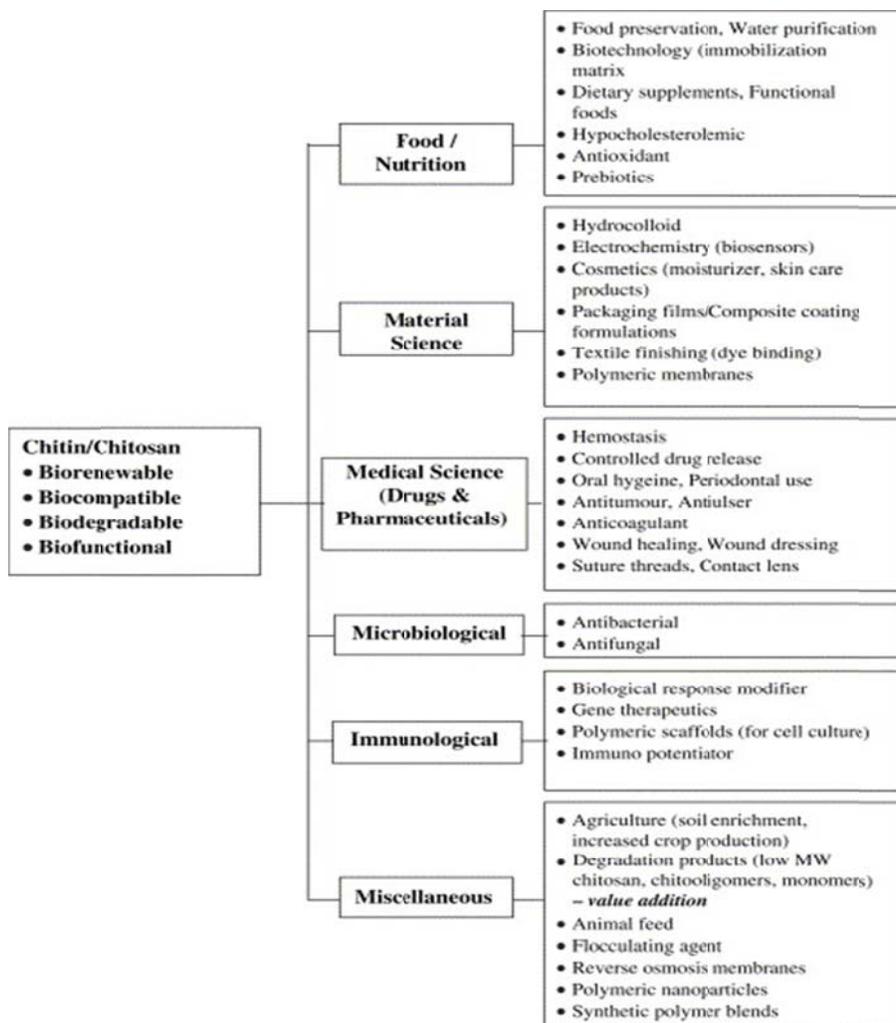
Figure 2.5. Possible applications of chitin/chitosan
Source: Prashanth and Tharanathan (2007)

Chitosan retains a positive charge in solutions that have pH values lower than around 6.5. The majority of the amino groups of chitosan should be protonated at pH values lower than 4. This contributes to enhanced swelling of the polymer network due to greater electrostatic repulsion between charged groups of the same sign at low pH values (Nystrom, Kjoniksen, & Iversen, 1999). Chitosan can be partially hydrolyzed to achieve water solubility by obtaining shorter chain lengths with more free amino groups in D-glucosamine units as a result of a

decrease in molecular weight (Qin et al., 2003). Although chitin/chitosan derivatives possess strong biological activities, they may have limited uses because of their high viscosities and molecular weights (Jo et al., 2001). Due to its highly viscous properties, high molecular weight chitosan with visocity greater than 500 cP at 1% concentration in 1% acetic acid was reported to be difficult to prepare, which restricted its viability in some antimicrobial experiments (No, Park, Lee, Hwang, & Meyers, 2002a). Also, primary amino and hydroxyl groups are present in the linear polyglucosamine chain structure of chitosan. These groups can be chemically modified to allow for side group attachment using various mild reaction conditions. The addition of a side chain disrupts the material's crystal structure and increases the amorphous fraction, resulting in a material with lower stiffness and altered solubility (Francis Suh & Matthew, 2000).

There are numerous studies documenting the production of water-soluble chitosan derivatives using chemical modification techniques, such as PEG-grafting (Gorochovceva & Makuska, 2004), chitosan carrying phosphonic and alkyl groups (Ramos, Heras, & Agullo, 2003), chitosan-saccharide derivatives (Chung, Kuo, & Chen, 2005), O-succinyl-chitosan (Zhang, Ping, Zhang, & Shen, 2003), hydroxypropyl chitosan (Xie, Xu, Liu, & Xue, 2002), branching with oligosaccharides (Tommeraas et al., 2002), carboxymethylated chitosan (Chen & Park, 2003), partial N-acetylation (Kubota et al., 2000), N-acetylation, and quaternisation (Francis Suh & Matthew, 2000). Water-soluble low molecular weight chitosan has been shown to have significant biological activities, including antimicrobial (No et al., 2002b; Vishu et al., 2005; Zheng & Zhu, 2003) and antitumor activity (Caiqin Qin et al., 2002; Seo et al., 2000), as well as the ability to slow the progression of diabetes mellitus (Kondo et al., 2000). It also has potential as a DNA delivery system (Richardson, Kolbe, & Duncan, 1999). Chemical or enzymatic methods may be used to prepare low molecular weight chitosan (Akiyama, Kawazu,

& Kobayashi, 1995). Unlike enzymatic methods, the chemical procedure involves harsh hydrolysis conditions, low product yields, and chemical modifications, such as formation of carboxyl groups and deamination (Qin, Du, & Xiao, 2002). Enzymatic hydrolysis may be suitable for large scale production of low molecular weight chitosan which retains its native biological properties due to the higher specificity of enzymes and use of milder conditions (Yalpani & Pantaleone, 1994).

Water-soluble chitosan has a wide array of antioxidant properties, including DPPH radical, superoxide anion radical, and hydrogen peroxide radical scavenging activities, as well as Cu^{2+} ion chelating ability (Lin & Chou, 2004). Moreover, it was shown by Xie et al. (2001) that water-soluble chitosan can scavenge hydroxyl radicals (•OH). There are three modes of action for chitosan's •OH scavenging activity: (1) the hydroxyl groups in the polysaccharide unit can react with •OH by the H-abstraction reaction, (2) •OH can react with the residual free amino groups, specifically the active hydrogen atoms, to form stable macromolecule radicals, and (3) the amino groups can form NH_3^+ groups by absorbing hydrion from the solution, then reacting with •OH through addition reaction. According to a study conducted by Matsugo et al. (1998), water-soluble chitosan derivatives were able to inhibit the formation of TBARS in *t*-butyl hydroperoxide and benzoyl peroxide induced lipid peroxidations as a result of their radical chain-breaking activity.

Xie, Xu, Wang, and Liu (2002) reported that water-soluble chitosan derivatives that were produced through etherification reaction and graft copolymerization showed greater antimicrobial activity against *S.aureus* and E.*coli* than either chitosan or hydroxypropyl chitosan. The mechanism of action may have been related to the amphiphilic structure and strong coordination capability of the water-soluble chitosan derivatives. Gerasimenko, Avdienko,

Bannikova, Zueva, and Varlamov (2004) investigated the antibacterial activity of water-soluble low molecular weight (5-27 kDa) chitosans against different microorganisms. Water-soluble chitosans with 85% DD and viscosity-average molecular weights of 5, 6, 10, 12, 15.7, and 27 kDa had high antibacterial activities against *Pseudomonas aureofaciens*, *Enterobacter agglomerans*, *Bacillus subtilis*, and *Bifidobacterium bifidum* 791, inducing 80-100% cell death. *E.coli* cell death rate was also high for every chitosan with the exception of the 5 kDa treatment. Chitosans with viscosity-average molecular weights of 5, 12, and 27 kDa were effective against *S.aureus*. *Bifidobacterium bifidum* ATCC 14893 was unaffected by 5, 15.7, and 27 kDa chitosans. Also, it was observed that the antibacterial activity of 4 kDa chitosan increased with increasing DD (55-85%) when tested against *E. coli* and *B. bifidum* 791. The addition of water-soluble chitosan (0.03%) to processed milk for reduction of microbial (bacterial and yeast) spoilage was reported by Ha and Lee (2001). Banana-flavored milk containing water-soluble chitosan experienced total inhibition of microbial growth during 15 days of storage at 4 and 10 °C, in contrast to the results obtained for the control milk that did not incorporate water-soluble chitosan. Park and Chong (2002) reported that the shelf life of rice cake could be extended with the addition of water-soluble chitosan. Water-soluble chitosan that had been previously dissolved in water was applied to rice cake in concentrations of 0.05, 0.1, 0.3, or 0.5%. The total microbial counts decreased with increasing concentrations of water-soluble chitosan during four weeks of storage at 5 °C. After the four week storage period, it was shown that 0.3 and 0.5% water-soluble chitosan treated rice cakes had total microbial counts two log cycles lower than that (8.2×10^4 CFU/g) of the control.

2.4.4 Chitosan-sodium tripolyphosphate nanoparticles

Nanoparticles are particulate dispersions or solid particles within the size range of 10-1000 nm. Particle size and size distribution are vital components of nanoparticle systems. These characteristics determine *in vivo* distribution, toxicity, targeting ability, and the biological fate of nanoparticle systems. Nanoparticles generally have higher intracellular uptake compared to microparticles. Due to their small size and relative mobility, they are available to a greater number of biological targets (Mohanraj & Chen, 2007). Desai, Labhasetwar, Walter, Levy, and Amidon (1997) reported that the uptake for nanoparticles (100 nm) was 2.5 fold higher than microparticles (1 μm) and 6 fold higher than microparticles (10 μm) in a Caco-2 cell line. Chitosan is well suited for nanoparticle production due to its low toxicity and biodegradability, in addition to immunostimulating and high mucoadhesive properties. Currently, chitosan nanoparticles are mainly used in drug delivery systems (Rampino et al., 2013). Chitosan nanoparticles are typically produced by electrostatic interactions between the positively charged amine groups of chitosan and negatively charged groups of a polyanion. Sodium tripolyphosphate is a commonly used cross-linker with chitosan because it is multivalent, non-toxic, and has the ability to form gels through ionic interactions (Azeredo, 2013). The interactions between chitosan and sodium tripolyphosphate are related to charge density, which can be regulated by pH (Zhao et al., 2011). Chitosan's amine groups are positively charged in acidic medium, creating a large charge density. Chitosan-sodium tripolyphosphate nanoparticles are spontaneously formed when sodium tripolyphosphate is mixed with chitosan due to the molecular linkages between phosphate and amino groups.

Chitosan nanoparticles possess an overall positive surface charge, which can be used to facilitate a number of biological interactions. Particle size is a characteristic that is strongly

related to the uptake and intracellular trafficking of nanoparticles. The sub-cellular and sub-micron size of chitosan-sodium tripolyphosphate nanoparticles allows for deep penetration into tissues through fine capillaries and fenestration in the epithelial lining (Gan et al., 2005). In an *ex vivo* canine carotid artery model, smaller nanoparticles (~100 nm) showed more than three times greater arterial uptake compared to larger nanoparticles (~275 nm). This was because the smaller nanoparticles could penetrate through the sub-mucosal layers, unlike the larger nanoparticles which were generally localized in the epithelial lining (Desai, Labhasetwar, Amidon, & Levy, 1996; Song, Labhasetwar, Cui, Underwood, & Levy, 1998). According to findings of Gan et al. (2005), the chitosan to sodium tripolyphosphate mass ratio is linearly correlated with particle size. Increasing the chitosan to sodium tripolyphosphate mass ratio from 3:1 to 7:1 using chitosans with three different molecular weights (low, medium, and high) dissolved in 1% (w/v) acetic acid yielded nanoparticles around 90 nm larger in size within their respective molecular weights. With this linear relationship it may be possible to control chitosan-sodium tripolyphosphate nanoparticle adhesion and transport properties. Also, treatment of chitosan nanoparticles with low mass ratio (4:1) using ultra-sonication was examined by Antoniou et al. (2015). Ultra-sonication at 500 W for 10 min was able to decrease particle size of chitosan nanoparticles by almost 50%. In a study conducted by Qi et al. (2004), chitosan nanoparticles showed higher antimicrobial activity compared to chitosan particles of larger size. Smaller particle size and the quantum size effect could explain the greater activity of the nanoparticles. The polycationic chitosan nanoparticles may have been able to interact with bacteria more effectively than chitosan because of their high surface charge density and greater affinity for bacterial cells. It was likely that the larger surface area of chitosan nanoparticles aided in bacterial cell inhibition as a result of a change in membrane penetrability caused by particle

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adsorption, membrane disruption, and leakage of cellular protein. Huang, Khor, and Lim (2004) found that chitosan did not possess significantly different *in vitro* cytotoxicity on A549 cells compared to chitosan nanoparticles. By decreasing DD from 88 to 61%, the cytotoxicity of chitosan and chitosan nanoparticles was significantly lowered.

The efficacy of chitosan nanoparticles as a glazing material for cryogenically frozen shrimp was assessed by Solval et al. (2014). After 30 days of frozen storage at -20 °C, shrimp that were glazed with chitosan nanoparticles showed lower lipid oxidation (TBARS), aerobic plate counts, as well as yeast and mold counts than shrimp glazed with chitosan, sodium tripolyphosphate, acetic acid, or the controls, consisting of shrimp glazed with distilled water and untreated shrimp. Chitosan nanoparticles were able to preserve the quality of the shrimp without affecting texture and color. The antioxidant activity of chitosan nanoparticles was likely related to the hydroxyl radial scavenging activity of chitosan, which may have been amplified from the high surface area per unit volume of chitosan nanoparticles. Similarly, the superior antimicrobial activity of chitosan nanoparticles, compared to chitosan, was likely the result of their greater surface area per unit volume and higher charge density, both factors contributing to the interaction with anionic bacterial cell membranes. The bioactive free amine groups of chitosan are also an important determinant of chitosan nanoparticle antimicrobial activity (Azeredo, 2013). Chitosan nanoparticles reportedly had strong antibacterial activity against gram-positive (*S.auerus*) and gram-negative (*E.coli*) bacteria (Tsai et al., 2002).

2.4.5. Chitooligosaccharides

Chitooligosaccharides can be obtained by partial hydrolysis of chitosan and they are comprised of 2-10 glucosamine units which are bound with β-(1-4)-glycoside linkages. Possible applications can be found in fields such as food, agriculture, biomedicine, cosmetics,

environmental protection, and wastewater management (Kim & Rajapakse, 2005). Chitooligosacharides, especially pentamers and hexamers, may have a multitude of uses in medicines and functional foods due to their antimicrobial and antitumor activities, as well as immunoenhancing effects (Kuroiwa et al., 2008). Although chitosan possesses a wide spectrum of functional properties, its high molecular weight and viscosity, as well as water insolubility can limit the uses *in vivo* (Je, Park, & Kim, 2004). Chitooligosaccharides have a greater amount of free amino groups in D-glucosamine units than chitosan and they possess a low viscosity, as well as high water solubility at neutral pH. Chitooligosaccharides obtain a positive charge after acetyl units are removed from D-glucosamine residues. This mediates the strong binding of chitooligosaccharides to negatively charged surfaces which can aid in a variety of biological activities (Kim & Rajapakse, 2005). The anti-inflammatory and antioxidant capabilities of chitooligosaccharides may allow for applications in biological systems (Benhabiles et al., 2012).

Chitooligosaccharides are produced by either chemical hydrolysis using acids or enzymatic hydrolysis. Product mixtures of chemical hydrolysis contain a large number of monomeric D-glucosamine units because the process is nonselective. However, chitosanolytic enzymes that have appropriate selectivity produce small amounts of D-glucosamine. Thus, enzymatic hydrolysis of chitosan in a bioreactor can be an effective option for chitooligosaccharide production, specifically the multifunctional pentamers and hexamers (Kuroiwa et al., 2009). Chemical hydrolysis is more common for industrial scale production, but some of the drawbacks, such as development of toxic compounds, risk of environmental pollution, and lower production yields tend to make enzymatic processes preferable. The production cost of enzymatic processes is higher than for chemical hydrolysis, but this factor can be minimized by reusing hydrolytic enzymes (Kim & Rajapakse, 2005). Additionally, Izume and

Ohtakara (1987) reported that the yield of high degree of polymerization chitooligosaccharides was greater following enzymatic hydrolysis than after acid hydrolysis. Chitooligosaccharides of different molecular weights can be fractioned using an ultrafiltration membrane, making it possible to separate chitooligosaccharides into low, medium, and high molecular weight fractions (Jeon et al., 2001).

Kuroiwa et al. (2009) obtained chitosan oligosaccharides (namely pentamers and hexamers) using a continuous enzymatic membrane bioreactor with immobilized chitosanase from *Bacillus pumilus* BN-262. Class I enzymes, such as the chitosanase *Streptomyces* sp. N174, can split both GlcN-GlcN and GlcNAc-GlcN linkages, preferably utilizing chitosan with low DD. Also, class II enzymes can only split GlcN-GlcN linkages, while class III enzymes split GlcN-GlcN and GlcN-GlcNAc linkages (Tremblay, Yamaguchi, Fukamizo, & Brzezinski, 2001). Generally, the types of randomly distributed glycosidic bonds present in differentially deacetylated chitosans are linkages between two N-acetylated units (A-A), deacetylated and acetylated units (D-A), acetylated and deacetylated units (A-D), and two deacetylated units (D-D). Chitosanase catalytic action and specificity regarding cleavage of glycosidic linkages is dependent upon the identity of reducing and non-reducing ends, as well as the DD of chitosan (Kim & Rajapakse, 2005).

The molecular weight of chitooligosaccharides plays a significant role in determining their functional properties (Jeon & Kim, 2000). Typically, chitooligosaccharides have a molecular weight of up to 10 kDa (Kim & Rajapakse, 2005; Xia et al., 2011). A study conducted by Jeon et al. (2001) tested the antimicrobial activity of chitosan and chitooligosaccharides with different molecular weights: high molecular weight (7-24 kDa), medium molecular weight (1.5-6 kDa), and low molecular weight (<1.5 kDa) on different bacteria. Although their effects were

lower than those shown by chitosan, high molecular weight chitooligosaccharides were found to have superior antimicrobial activity compared to medium and low molecular weight chitooligosaccharides. While the inhibitory effects of chitosan and chitooligosaccharides varied depending on the microorganism, inhibition of gram-positive bacteria was greater than gram-negative bacteria for both. Chitosan had good antimicrobial activity against all microorganisms tested, with the exception of *Pseudomonas aeruginosa*, however chitooligosaccharides were more effective against pathogens than non-pathogens. For significant inhibition of microorganisms, a chitooligosaccharide molecular weight of around 10 kDa or greater was required, suggesting that a minimum degree of polymerization is required for effective antimicrobial activity. No et al. (2002b) also reported in their findings that the antimicrobial activity of chitosan and chitooligosaccharides varied depending on their molecular weight and microorganism tested. Chitosan generally was more effective at inhibiting gram-positive bacteria than gram-negative bacteria. Chitosan oligomers with lower molecular weights, especially 1 kDa, were more potent against gram-negative bacteria. Compared to other molecular weight (1, 7, 10, and 22 kDa) chitosan oligmers, those with molecular weights of 2 and 4 kDa had higher antimicrobial actvity against gram-positive bacteria. Overall, chitosan showed greater antimicrobial activity compared to chitooligosaccharides. In another study, Uchida (1989) determined that chitosan hydrolysate with 50 mg total reducing sugar per gram of chitosan (chitosan oligomer I) was largely composed of tetramers, pentamers, and hexamers. Chitosan oligomer I exhibited antimicrobial and antifungal effects, but chitosan oligomer II, which was mainly composed of trimers and tetramers had no activity. The inhibitory effects of chitosan oligomers increased with increasing DD. According to Benhabiles et al. (2012), chitosan oligomers with the molecular weight of 12 kDa displayed a bacteriostatic effect on all of the

tested microorganisms, four gram-positive and seven gram-negative bacteria. The antimicrobial activity of the oligomers varied with microorganism. Wei and Xia (2003) found that chitooligosaccharide antimicrobial activity depended on the quantity of protonated amino groups present, as well as the relative molecular weights. Also, Tsai and Su (1999) reported that chitosan oligomers could pass through the cell wall of *E.coli*, causing leakage of glucose and lactate dehydrogenase. This suggests that the antibacterial mechanism of chitosan occurs through cross-linkage between chitosan polycations and the anions on the bacterial surface, which can alter membrane permeability.

In a study conducted by Je et al. (2004), chitooligosaccharides with different DD (50, 75, and 90%) and molecular weights, high (5-10 kDa), medium (1-5 kDa), and low (below 1 kDa) were assessed for antioxidant activity by scavenging. The medium molecular weight chitooligosaccharides with 90% DD exhibited the highest free radical scavenging activities for all radicals examined (DPPH, hydroxyl, superoxide anion, and carbon-centered). While the function of molecular weight was not clear, the higher DD resulted in a greater number of free amino groups present at the C-2 position of chitooligosaccharides. The nitrogen on the C-2 position can quench different radicals. The residual free amino groups may be able to react with free radicals to form stable macromolecule radicals and the amino groups can form ammonium groups through the absorption of hydrogen ion from solution. Free radical scavenging activity increased as the concentration of chitooligosaccharides that were used increased. There was no free radical activity for any tested radical species with the application of N-acetylglucosamine, a monomer of chitooligosaccharides. Sun, Zhou, Xie, and Mao (2007) tested the free radical scavenging activity of chitosan oligomers with four different molecular weights (2.30, 3.27, 6.12, and 15.25 kDa) against superoxide anion and hydroxyl radicals. Free radical scavenging activity

against both radicals increased as the molecular weight of the chitosan oligomers decreased, with the 2.30 kDa oligomer showing the highest scavenging activity. Lower molecular weights were more effective because the relatively short chains of chitosan oligomers decrease the potential of intramolecular hydroxyl bond formation, thus making hydroxyl and amino groups are more active and likely to contribute to free radical scavenging.

2.5. Vacuum tumbling

Tumbling is used to increase brine uptake and protein extraction in meat through the transfer of kinetic energy in a rotating drum or paddles (Lin et al., 1990; Price & Schweigert, 1987). This process has been shown to enhance tenderness, ensure juiciness, effectively promote cohesion of meat pieces during cooking, and develop a uniform product with increased yield and desirable slicing characteristics (Cassidy et al., 1978; Chow et al., 1986; Gillett et al., 1981; Krause et al., 1978). Higher brine uptake during tumbling may be related to structural component damage, which can result in higher swelling potential (Cheng & Sun, 2008; Theno, Siegel, & Schmidt, 1978). Siegel, Theno, Schmidt, and Norton (1978) reported that tumbling could improve binding quality and reduce cooking losses in excised muscles from cured hams. In a study conducted by Krause et al. (1978), it was shown that tumbling could improve the external appearance, color, sliceability, taste, aroma, and yield of cured hams. With the addition of 3.3% sodium tripolyphosphate to the curing pickle solution, (80.1% water, 16.5% sodium chloride, 3.9% glucose, and 0.09% sodium nitrite) the hams experienced significant improvement in sliceability, external color, taste, aroma, and yield that was independent of the tumbling effect. Hams that were tumbled continuously for 3 h had less improvement in product quality and yield compared to those that were tumbled for 18 h intermittently. Lin et al. (1990) reported that restructured hams processed at 9000 tumbler revolutions had lower hardness, gumminess, and chewiness, compared to hams processed using 3000 and 6000 revolutions. Increasing tumbling

speed from 15 to 25 rpm increased hardness, elasticity, gumminess, and chewiness due to higher cell disruption and myofibrillar protein extraction. The effect of tumbling with sodium tripolyphosphate (0, 0.25, 0.4, and 0.5%) on lipid oxidation in precooked roast beef during 0, 2, 4, and 7 days of storage was investigated by Cheng and Ockerman (2003). They found that after 7 days the roast beef that had been tumbled with 0.5% sodium tripolyphosphate, an iron chelator, had significantly lower TBARS values than the other treatments.

Vacuum is an important component of the vacuum tumbling process and its function is to prevent air from diffusing into the protein gel structure, ensuring that a tacky exudate is present on the surface of meat proteins after tumbling (Price & Schweigert, 1987). The tacky exudate consists mainly of the salt soluble proteins, actin and myosin. Its function is to effectively promote cohesion during thermal processing and to aid in the development of desirable slicing characteristics (Krause et al., 1978). Incorporation of air into the meat during tumbling may cause exudate foaming, which could result in greater protein denaturation and possibly lead to decreased binding capacity (Solomon, Norton, & Schmidt, 1980). The use of vacuum can promote greater exchange between occluded internal gases in food and the external soaking medium (Collignan, Bohuon, Deumier, & Poligne, 2001; Fito, 1994; Fito, Chiralt, Barat, Spiess, & Behsnilian, 2001; Lenart & Flink, 1984; Raoult-Wack, 1994; Rastogi & Raghavarao, 1996; Roa, Tapia, & Millan, 2001). Changes in pressure can also have an effect on capillary uptake (Chiralt et al., 2001). The hydrodynamic mechanism (HDM) is a mass transfer mechanism that occurs during solid-liquid interactions. According to the HDM, vacuum allows for the internal gas in a food product to expand and flow out, while promoting the transfer of liquid into the new void spaces (capillary penetration). After atmospheric pressure is regained,

the residual gas in the pores compresses and results in greater infiltration of liquid into the pores (Fito, 1994; Fito & Pastor, 1994).

Vacuum tumbling was found to be effective in decreasing cooking losses, as well as improving tenderness and water hold capacity of hams compared to controls that were not tumbled (Rejt et al., 1978). It has been shown that gases can dissolve when coming into contact with meat (Bruce, Wolfe, Jones, & Price, 1996; Gros, Dussap, & Brient, 1996). Kilic and Cassens (1998) observed the presence of oxymyoglobin at a depth of 1 cm from the muscle surface following exposure of meat to oxygen for 12 h. This indicates that oxygen can penetrate into meat by binding with myoglobin. According to Deumier (2004), pulsed vacuum brining of chicken skin in an acid solution at 7 °C resulted in 3.7, 2.5, 2.4, and 2.0 decimal reductions in total viable counts, Enterobacteriaceae, total coliforms, and *E.coli*, respectively. Pulsed vacuum brining could increase water gain and acidification, leading to a higher decontamination effect. This process, shown in Figure 2.6, likely promotes greater penetration of solution into pores of the skin. Immersing the product in solution under vacuum causes occluded gases to expand and be expelled from the matrix through the pores. After atmospheric pressure is regained, the occluded residual gases in the pores are compressed and their volume is lowered, allowing for greater infiltration of solution into the pores. It was shown by Deumier et al. (2003) that turkey meat, which was immersed in a 35% salt solution, had greater solution uptake during pulsed vacuum brining compared to atmospheric pressure brining. They also measured mass transfer in non-porous gel cubes that were pulsed vacuum brined or atmospheric pressure brined. There were no significant differences found in mass transfer from brining solution to the gel cubes when comparing the two methods, indicating that a porous structure is required for increasing mass transfer through pulsed vacuum brining. Porosity may possibly be enhanced during vacuum

processing due to the likely breakdown of meat tissues near areas of weaker cohesion, such as along muscle fibers around the perimysium.

Occluded gases are expelled from the meat.

A return to atmospheric pressure induces solution infiltration via the meat pores.

Continued processing under atmospheric pressure leads to dilution of the infiltrated solution.
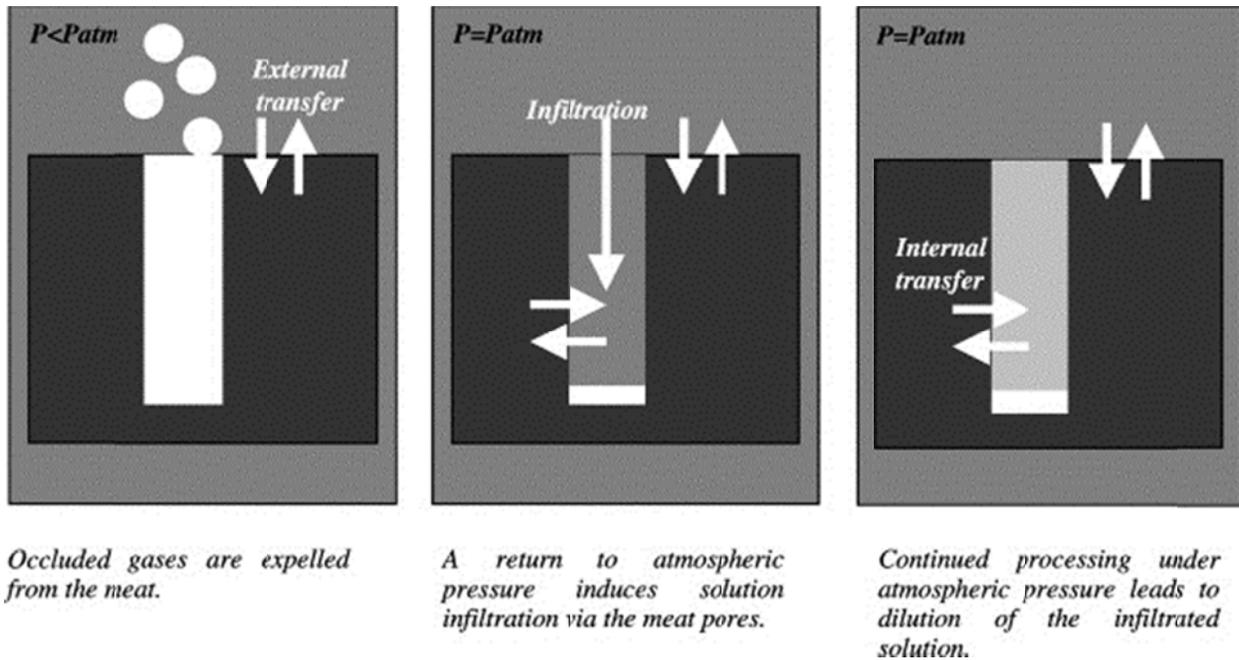
Figure 2.6. Mass transfers that occur during pulsed vacuum brining. Concentrated solution (■); meat (■); occluded gas (□); diluted solution (■).
Source: Deumier et al. (2003)

In addition, both occluded and dissolved gases may have an effect on meat porosity. Solomon et al. (1980) reported that tumbling ham muscles with applied vacuum could provide greater cure (11.16% sodium chloride, 1.67% sodium tripolyphosphate, 2.22% sucrose, 0.087% sodium nitrite, and 84.89% deionized water) absorption and binding functionality than tumbling alone. Along with vacuum, the utilization of prerigor muscles could independently increase cure absorption more than postrigor treatments. Although vacuum could facilitate higher cure absorption, there was an unequal distribution of marinade within whole muscles as measured by the sodium chloride content at three different depths. Sodium chloride content was found to be higher in exterior muscle layers, showing a decrease in concentration from outer to inner depths of the muscle.

Also, the application of vacuum to the tumbling process could independently increase the breaking strengths of cooked ham slices. It was observed by Solomon and Schmidt (1980) that vacuum tumbled beef muscles had a higher amount of extracted crude myosin compared to beef muscles tumbled without vacuum. An increase in mixing time, from 0.5 to 2.5 hr, caused a linear decrease in functional properties, such as binding ability and least protein gel concentration. Xiong and Kupski (1999) evaluated the penetration of different marinade solutions (tetrasodium pyrophosphate, sodium tripolyphosphate, and sodium hexametaphosphate) into tumbled chicken filets using a dye-tracing method. Solution concentrations of 1.6 and 3.2% were used and the filets were tumbled for 0, 5, 10, 15, or 30 min. They found that the marinades penetrated into the filets most rapidly during the first 5 min. It was also shown that tumbling with low concentrations (1.6%) of phosphates could promote deep water penetration into the filets, however tumbling with high concentrations (3.2%) of phosphates, with the addition of salt (8%), enhanced surface layer water penetration. Lower deep water penetration into tumbled filets using high conentrations of phosphates, with the addition of salt (8%), may have been the result of decreased solubility of phosphates due to competition between the salt and phosphates for available water. Alvarado and Sams (2004) observed that tumbling chicken breast under vacuum with a solution containing 0.54% sodium chloride and 0.42% sodium tripolyphosphate could facilitate migration of sodium ions from the solution into the center of the muscle. After tumbling, it was found that the sodium ion distribution was largely centralized in the outer muscle layers.

The effect of vacuum tumbling on the extent of sucrose penetration and functionality in trout muscle was evaluated in a study done by Jittinandana, Kenney, and Slider (2005). Trout chunks were tumbled with 4% (w/w) powdered sucrose, under vacuum pressures of 0, 50, or 70

kPa, at 7 or 14 rpm, for 30 or 60 min. The trout chunks were then frozen and stored at -20 °C for 6, 12, 18, and 24 weeks. The application of vacuum during tumbling, as well as utilization of a longer tumbling time, increased sucrose penetration into the center portion of trout muscle chunks. During 24 weeks of frozen storage, vacuum tumbled trout chunks had higher gel hardness and cohesiveness, as well as lower thaw loss than trout chunks that were not tumbled. The processing conditions for optimal sucrose penetration and trout muscle chunk functionality involved tumbling under 70 kPa vacuum at 7 rpm for 30 min.

Deumier (2006) reported that vacuum tumbling deboned chicken legs in a 1-5% lactic acid solution for 1-10 min could improve microbiological quality. Processing with higher tumbling speeds resulted in greater decontamination of chicken legs, reducing total viable counts and Enterobacteriaceae. The relative contributions of acid-soaking, continuous vacuum, pulsed vacuum, and tumbling on mass variation in whole deboned chicken legs treated for 5 min with a 1% lactic acid solution were 5, 10, 16, and 68%, respectively. At 2% lactic acid concentration, the relative contributions of acid-soaking, continuous vacuum, pulsed vacuum, and tumbling were 19, 13, 30, and 38%, respectively. Also, a significant decontaminating effect on *Salmonella spp.* was shown in fresh chicken sausages after vacuum tumbling for 1 min using a 1% lactic acid solution. The decontaminating effect of lactic acid was likely related to acidification, instead of lactate ions. Moreover, it has been shown that organic acids can bolster the denaturation of muscle proteins, myofibrils, (Berge et al., 2001) and connective tissue (Aktas & Kaya, 2001).

Tumbling can increase water and acid transport between meat and solution likely due to a greater transfer surface area resulting from meat surface destructuring. This can lead to accelerated marinade penetration and diffusion (Ghavimi, Rogers, Althen, & Ammerman, 1986; Katsaras & Budras, 1993). The water holding capacity of meat is dependent on muscle pH. The

isoelectric point of myofibrillar proteins (actin and myosin) is around 5.1. As the pH of muscle

approaches this value, it experiences lower water holding capacity. At the isoelectric point, there

would be minimum net charges on the protein, which would decrease the space between

filaments for water binding (Alvarado & McKee, 2007).

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CHAPTER 3. EFFECTS OF VACUUM TUMBLING WITH CHITOSAN NANOPARTICLES ON THE QUALITY CHARACTERISTICS OF CRYOGENICALLY FROZEN SHRIMP

3.1. Abstract

Chitosan (CH) has been utilized in edible films and coatings for many food systems, mainly due to its powerful antimicrobial and antioxidant abilities. It can be dissolved in acids and forms relatively high viscosity solutions. CH penetration into shrimp may be facilitated by vacuum tumbling with a low viscosity chitosan-sodium tripolyphosphate (CH-TPP) nanoparticle solution. It is expected that this would reduce lipid oxidation and microbial loads. In this study, CH-TPP solution was developed and applied to shrimp, and the quality characteristics during frozen storage were evaluated. Four solutions were prepared for this study: (1) a 1% acetic acid (AA) solution in distilled water (DW), (2) a 0.5% CH solution in the 1% AA solution, (3) a 0.167% sodium tripolyphosphate (TPP) solution in the 1% AA solution, and (4) a CH-TPP solution, prepared by adding 0.167% TPP in the CH solution. With the addition of TPP to the CH solution, CH-TPP nanoparticles were instantaneously formed by ionotropic gelation. Fresh shrimp meat was separately vacuum tumbled with the solutions, cryogenically frozen, and evaluated for quality characteristics under frozen storage. Fresh shrimp meat tumbled with DW and fresh shrimp meat without tumbling (NT) were used as controls. CH and CH-TPP treated shrimp had lower aerobic plate counts (APC) compared to other treatments during the entire storage time. CH and CH-TPP treated shrimp retained their color, texture, and moisture contents. In addition, CH and CH-TPP treatments produced the highest reduction in lipid oxidation compared to other treatments at 120 days of storage at -20 °C. This study showed that a CH or CH-TPP solution, combined with vacuum tumbling, can be effective at reducing APC and lipid oxidation in shrimp during frozen storage, while maintaining desired physicochemical properties.

Keywords: chitosan, nanoparticles, shrimp, freezing, vacuum tumbling

3.2. Introduction

Chitosan (CH) is a widely used polysaccharide in edible films and coatings due to its non-toxicity, antimicrobial activity, and antioxidant properties (Elsabee & Abdou, 2013; Jeon, Kamil, & Shahidi, 2002; Kucukgulmez, Kadak, & Gokcin, 2013; No, Meyers, Prinyawiwatkul, & Xu, 2007). CH is derived from chitin, a natural biopolymer found in the shells of crustaceans and cell walls of fungi. Chitin is the second most available biopolymer on earth (Shahidi, Arachchi, & Jeon, 1999). While chitin is mainly composed of poly β-(1-4)-2-acetamido-D-glucose, CH is a copolymer that contains units of β-(1-4)-2-acetamido-D-glucose and β-(1-4)-2-amino-D-glucose (Elsabee & Abdou, 2013). CH is typically produced from the partial deacetylation of chitin with sodium hydroxide. At a degree of deacetylation (DD) of over 50%, chitin becomes soluble in acidic solutions and can then be classified as CH. The DD of CH, in addition to molecular weight, strongly influences its antimicrobial activity (Tsai, Su, Chen, & Pan, 2002). Some inherent characteristics of CH, such as its large particle size (Qi, Xu, Jiang, Hu, & Zou, 2004) and high viscosity in solution (Jo, Lee, Lee, & Byun, 2001), may limit its penetration into shrimp muscle tissues.

Reduction of particle size to sub-micron levels can allow for deep penetration into tissues through fine capillaries and fenestration in the epithelial lining (Gan, Wang, Cochrane, & McCarron, 2005). CH nanoparticles are formed by instant ionic gelation in which positively charged CH is combined with an anion, such as sodium tripolyphosphate (TPP) (Gan et al., 2005). CH nanoparticles have been used in drug delivery systems due to their mucoadhesivity and ability to augment large molecule penetration across mucosal surfaces (Xu & Du, 2003). It has been reported that CH nanoparticles may exhibit greater antimicrobial activity than CH particles of larger sizes as a result of their small size and the quantum size effect (Qi et al., 2004).

Many studies have been conducted using CH as a coating for seafood preservation (Fan et al., 2009; Sathivel, Liu, Huang, & Prinyawiwatkul, 2007; Soares, Oliveira, & Vicente, 2015; Solval, Espinoza Rodezno, Moncada, Bankston, & Sathivel, 2014). Although, limited research has been done on the efficacy of vacuum tumbling seafood with CH. CH penetration into shrimp may be facilitated by vacuum tumbling with a low viscosity CH nanoparticle solution.

Tumbling is used to increase brine uptake and protein extraction in meat through the transfer of kinetic energy with a rotating drum or paddles. (Lin, Mittal, & Barbut, 1990; Price & Schweigert, 1987). This process has been shown to enhance tenderness, ensure juiciness, effectively promote cohesion of meat pieces during cooking, and develop a uniform product with increased yield and desirable slicing characteristics (Cassidy et al., 1978; Chow, Ockerman, Cahill, & Parrett, 1986; Gillett, Cassidy, & Simon, 1981; Krause, Ockerman, Krol, Moerman, & Plimpton, 1978). Vacuum is an important component of the vacuum tumbling process and its function is to prevent air from diffusing into the protein gel structure, ensuring that a tacky exudate is present on the surface of meat proteins after tumbling, rather than frothy foam (Price & Schweigert, 1987). In addition to improving tenderness and decreasing cooking losses, vacuum tumbling may be able to improve the water holding capacity of meat (Rejt, Kubicka, & Pisula, 1978). Vacuum brining facilitates mass transfer in meat by increasing solution uptake through the pores (Deumier, Trystram, Collignan, Guedider, & Bohuon, 2003). The objective of this study was to evaluate the effects of vacuum tumbling with a CH nanoparticle solution on the quality characteristics of cryogenically frozen shrimp.

3.3. Materials and methods

3.3.1. Preparation of treatment solutions

Medium molecular weight chitosan (CH) and technical grade sodium tripolyphosphate (TPP) was purchased from Sigma Aldrich (St. Louis, MO). Reagent-grade acetic acid (AA) was obtained from EMD Chemicals Inc. (Gibbstown, NJ). Solutions used for vacuum tumbling with fresh shrimp were formulated according to the process described by Solval et al. (2014). As shown in Figure 3.1, four solutions were prepared for this study: (1) a 1% AA solution in distilled water (DW), (2) a 0.5% CH solution in the 1% AA solution, (3) a 0.167% TPP solution in the 1% AA solution, and (4) a chitosan-sodium tripolyphosphate (CH-TPP) solution, prepared by adding 0.167% TPP in the CH solution. With the addition of TPP to the CH solution, CH-TPP nanoparticles were instantaneously formed by ionotropic gelation. This occurs due to an interaction between TPP anions and the positively charged amino groups of CH (Fernandez-Urrusuno, Calvo, Remunan-Lopez, Vila-Jato, & Alonso, 1999). The solutions were stirred for 1 h at room temperature. The mixtures were then sonicated for 30 min in an ice bath at 4 °C with amplitude of 80% and pulser set to 2 sec using an ultrasonic processor (Model WU-04711-70, Cole-Parmer Inc., Vernon, IL) fitted with a 22 mm tip diameter. To further reduce particle size, the solutions were processed using an ultra-homogenizer (Omni, Ultra shear M, Omni International, Kennesaw, GA) at 25000 rpm for 30 min. DW was compared with other treatment solutions for free radical scavenging activity.

3.3.2. Vacuum tumbling with treatment solutions and freezing of shrimp

Peeled fresh white shrimp meat was separately tumbled with treatment solutions (AA, CH, TPP, CH-TPP, DW) at a 1:1 ratio by weight in a 2270 g capacity Reveo MariVac vacuum tumbler (Eastman Outdoors, Flushing, MI) in a cold processing room (4 °C) for 10 min. The shrimp processing and storage methods are shown in Figure 3.2.
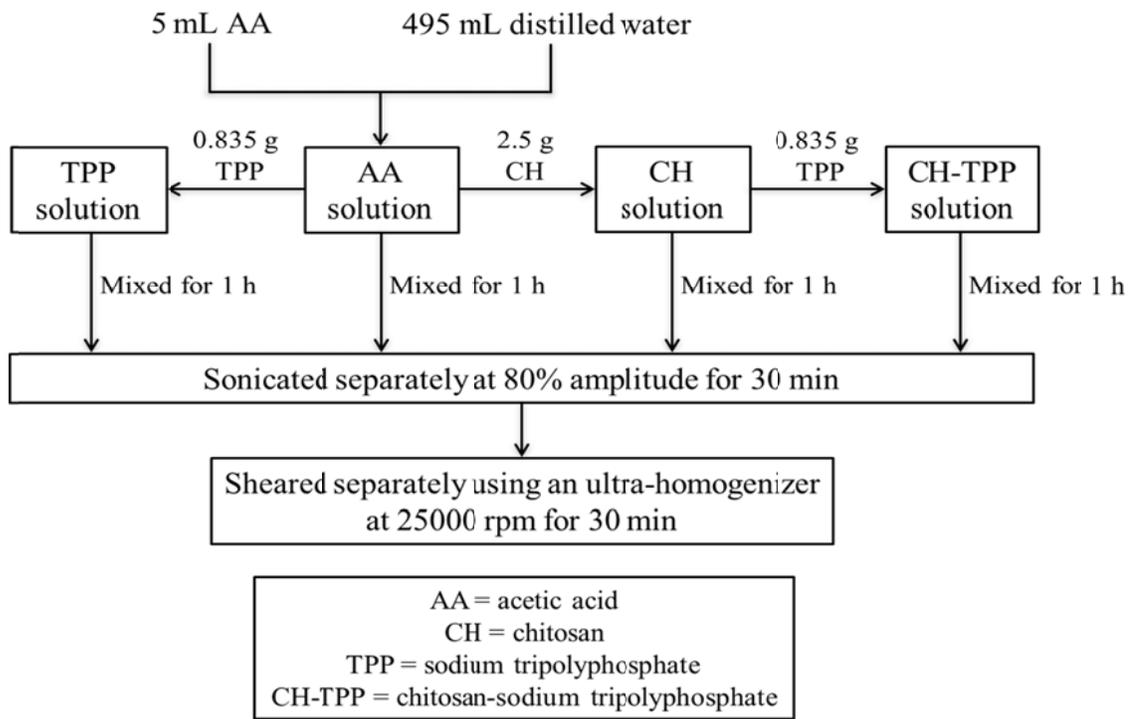
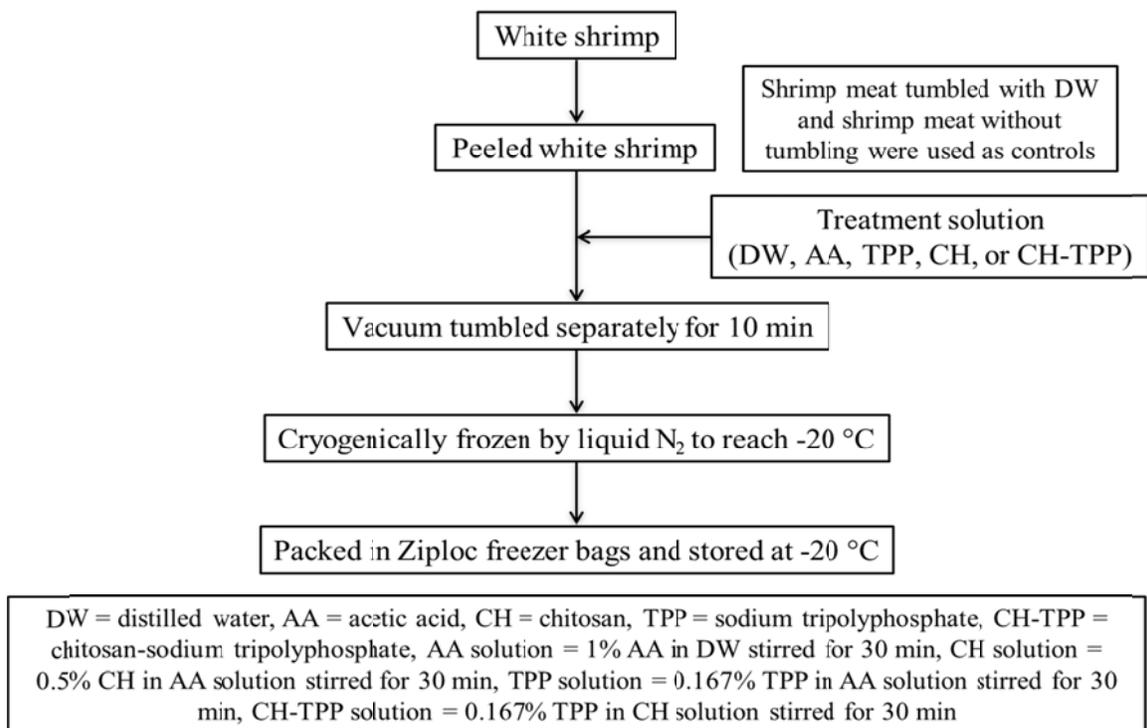Figure 3.1. Flowchart of treatment solution preparation.

Figure 3.2. Flow diagram of shrimp processing and storage.

Fresh shrimp meat tumbled with DW, as well as fresh shrimp meat without tumbling were used as controls. After tumbling, excess solution was allowed to drain for 30 sec. The shrimp were then frozen with liquid nitrogen using a cabinet type cryogenic freezer (Air Liquide, Houston, TX). During cryogenic freezing, thermocouples attached to a data logger (Comark, Comark Ltd. Stevenage, Herts, UK) were embedded into the middle of the second abdominal segment of the shrimp to record temperature changes until a core temperature of -20 °C was reached. Frozen shrimp samples were packed into one quart Ziploc freezer bags (SC Johnson, Racine, WI) and stored at -20 °C for 120 days, undergoing physicochemical analysis every 30 days and microbiological analysis in 60 day intervals. Frozen shrimp was thawed at 4 °C for 22 h prior to analysis.

3.3.3. Molecular weight and degree of deacetylation of chitosan

CH (2.5 g) was dissolved in 497.5 mL of 0.5 M AA/0.5 M sodium acetate (NaAc) for 1 h to produce CH in AA/NaAc solution before processing with sonication and ultra-shearing. The solution was sonicated for 30 min at 80% amplitude and sheared using an ultra-homogenizer for 30 min to obtain CH in AA/NaAc solution after processing with sonication and ultra-shearing. The CH in AA/NaAc solutions before and after processing with sonication and ultra-shearing were analyzed for intrinsic viscosity [η]. To remove insoluble materials the solutions were passed through Whatman #4 filter paper. Then 7 mL of known concentrations (0.1-0.5%) of the solution were individually placed in a Cannon-Fenske routine viscometer (Cannon-Fenske, No. 100). The flow time of the solutions through the viscometer capillary in a 25 °C constant-temperature water bath were recorded as seconds in order to calculate [η].

$$\eta_{rel} \text{(Relative viscosity)} = \frac{t_{solution(efflux\ time\ of\ solution)}}{t_{solvent\ (efflux\ time\ of\ solvent)}} \qquad (3.1)$$

$$\eta_{sp} \text{(Specific viscosity)} = \eta_{rel} - 1 \qquad (3.2)$$

$$\eta_{inh} \text{(Inherent viscosity)} = \frac{(Ln\,\eta_{rel})}{C} \qquad (3.3)$$

$$\eta_{red} \text{(Reduced viscosity)} = \frac{\eta_{sp}}{C} \qquad (3.4)$$

$$[\eta] = \lim_{c \to 0} \frac{\eta_{sp}}{c} \equiv \lim_{c \to 0} c^{-1} \ln \eta_{rel} \qquad (3.5)$$

Where C = Concentration of CH in AA/NaAc solution (g/dL, %)

η_{inh} and η_{red} were plotted on a graph and $[\eta]$ (dL/g) was obtained from the common intercept of both plots on the ordinate at C = 0. η_{red} vs concentration data was extrapolated to zero concentration to acquire $[\eta]$. The Mark Houwink equation was used to calculate the viscosity-average molecular weight (M_v) of CH in solution based on the relationship between molecular weight and $[\eta]$.

Mark Houwink equation: $[\eta] = K(M_v)^a$ \qquad (3.6)

Where *K* and *a* are constants for various solute-solvent systems at different temperature, ionic strength, pH, degree of deacetylation (DD), and molecular weight range. The values *K* and *a* for this analysis were 199.0 * 10^{-5} and 0.59, respectively (Kasaai, 2007). The M_v of CH-TPP particles after processing with sonication and ultra-shearing was not analyzed because the *K* and *a* values were not available.

The CH particles in CH solution and CH-TPP nanoparticles in CH-TPP solution were analyzed for DD before and after processing with sonication and ultra-shearing. CH and CH-TPP solutions were prepared according to the method described in section 3.3.1. The solutions were then freeze dried (Heto PowerDry LL3000, Laurel, Maryland) for 48 h. DD of CH and CH-TPP were determined using the colloid titration method described by Toei and Kohara (1976). Freeze dried CH or CH-TPP (0.5 g) was dissolved in formic acid (5% v/v). CH or CH-TPP in formic acid solution (1 g) was mixed with 29 mL DW in an erlenmeyer flask. Three drops of 0.1% w/v

toluidine blue indicator (Sigma Aldrich, St. Louis, MO) were added and the solution was titrated

with n/400 potassium polyvinyl sulfate (PVSK), obtained from Wako Pure Chemical Industries,

Ltd., Japan, until a pink color was visible. A single deacetylated amino group of CH or CH-TPP

reacts with one molecule of PVSK. The molar ratio of deacetylated amino groups in the CH or

CH-TPP molecule was used to calculate DD based on the volume of PVSK solution that was

utilized. The following equation was used to calculate DD.

$$DD~(\%) = \left[\frac{X / 161}{X / 161 + Y / 203} \right] * 100 \qquad (3.7)$$

Where X (amount of glucosamine in molecule) = $1 / 400 * 1 / 1000 * f * 161 * V$

Y (amount of N-acetylglucosamine in molecule) = $0.5 * 1/100 - X$

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

3.3.4. Rheological properties of chitosan solutions

The viscosity, flow behavior, and viscoelastic properties of CH and CH-TPP solutions

were analyzed using an AR 2000 ex rheometer (TA Instrument, New Castle, DE) with fitted

plate geometry (a metal plate of 40 mm in diameter), using Universal Analysis software. A 200

µm gap between the metal plate and the rheometer plate was used to determine the flow behavior

of CH solutions. The shear stress was measured at shear rates from 0 to 1000 s^{-1}. The flow

behavior was modeled by the Power Law model using Eq. 3.8.

$$\sigma = K\dot{\gamma}^n \qquad (3.8)$$

Where σ is shear stress (Pa), K is the consistency index (Pa.s^n), $\dot{\gamma}$ is shear rate (s^{-1}), and n is the

flow behavior index. A plot of log σ versus log $\dot{\gamma}$ was produced from logarithms of σ and $\dot{\gamma}$. A

scatter plot was constructed and the magnitude of log K (y intercept) and n (slope) was

determined. At a shear rate of 1000 s^{-1}, the viscosity of CH and CH-TPP solutions was obtained.

The elastic modulus (G′) and viscous modulus (G″) of the CH and CH-TPP solutions was determined using frequency sweep tests. Frequencies between 0.001 and 0.75 Hz were used, as well as a 200 μm gap between plates. For the flow behavior and frequency sweep tests, the solutions were analyzed at 25 and 5 °C.

3.3.5. Particle size and total reducing sugar content of chitosan solutions

Particle size of CH particles and CH-TPP nanoparticles was determined by dynamic light scattering using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd.,Worcestershire, U.K.). Approximately 0.5 mL of sample was placed in spectrophotometer cuvettes for the analysis. Total reducing sugar (TRS) content of CH and CH-TPP solutions, before and after processing with sonication and ultra-shearing, was determined according to the Schales method with slight modification (Imoto & Yagishita, 1971). A reagent solution was prepared by dissolving 0.5 g of reagent grade potassium ferricyanide (Fisher Science Education, Nazareth, PA) in 1 L of 0.5 M sodium carbonate (Sigma Aldrich, St. Louis, MO). Subsequently, 1.5 mL of sample solution was mixed with 2 mL of the reagent solution in a capped test tube. After mixing, the tube cap was loosened and the sample was heated in boiling water for 15 min. After heating, the sample solution was cooled and centrifuged at 10000 rpm for 10 min with an Eppendorf 5417C centrifuge (Beckmann Instruments Inc., Westbury, NY). The absorbance was measured at 420 nm using a spectrophotometer (Genesys 20, Thermo Fisher Scientific, Fair Lawn, NJ). D-glucosamine, obtained from City Chemicals, LLC. (West Haven, CT), was used as the reference compound. A standard curve was constructed using concentrations of glucosamine from 0-0.13 mg/mL. The TRS content of the treatment solutions was expressed as mg glucosamine/g CH.

3.3.6. Deconvolution microscopy images of fluorescently labeled chitosan particles in solution and cross-sections of vacuum tumbled shrimp

CH was fluorescently labeled according to the method described by Qaqish and Amiji (1999) with some modifications. One gram of CH was dissolved in 99 mL of 0.1 M AA for 1 h. To the solution, 100 mL of methanol (EMD Chemicals Inc., Gibbstown, NJ) was added slowly and with continuous stirring to obtain CH-methanol solution. Fluorescein isothiocyanate F7250 isomer 1 (FITC) from Sigma Aldrich (St. Louis, MO) was dissolved in methanol at 1 mg/mL and then added slowly to the CH-methanol solution. The mixture was incubated in the dark at room temperature for 1 h to allow the primary amine group of D-glucosamine residue to react with the isothiocyanate group of FITC. After incubation, 0.1 M NaOH was used to precipitate the FITC labeled CH (FITC-CH). The precipitate was washed with DW and separated by centrifuging until the supernatant contained no detectable fluorescence, as measured with a Wallac Victor^2 microplate reader (PerkinElmer Inc., Shelton, CT). The FITC-CH was then freeze dried for 48 h and ground in a commercial blender to obtain FITC-CH powder. Solutions of 0.5% FITC-CH powder in 1% AA and 0.5% FITC-CH powder in 1% AA with the addition of 0.167% TPP (FITC-CH-TPP) were prepared as described in section 3.3.1, except FITC-CH was used instead of CH.

Peeled fresh white shrimp meat was tumbled with FITC-CH and FITC-CH-TPP solutions and cryogenically frozen as described in section 3.3.2. Prior to microscopy analysis, cross-sections of frozen shrimp were cut 2-3 mm thick with a double sided razor blade using a cryostat (Leica Microsystems, Wetzlar, Germany). The samples were transferred to microscopy slides and fixed using ethyl alcohol. Then they were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) with the addition of a cover slip. FITC labeled CH and CH-TPP particles, embedded in the outer and inner muscle tissue of the shrimp, were analyzed

with a Leica DM RXA2 fluorescent microscope (Leica Microsystems, Wetzlar, Germany), using deconvolution software. Micrographs of FITC labeled CH and CH-TPP particles in solution, as well as in shrimp samples, were captured using a green fluorescent protein (GFP) filter.

3.3.7. pH and free radical scavenging activity of treatment solutions

The pH of treatment solutions (section 3.3.1) after processing with sonication and ultra-shearing was measured with a VWR Symphony SB70P pH meter (VWR Scientific, Singapore). Free radical scavenging activity of treatment solutions was determined according to a method described by Kim and Thomas (2007). 2,2-diphenyl-1-picrylhydrazyl (DPPH) was obtained from Sigma Aldrich (St. Louis, MO) and a 0.2 mM concentration was prepared. Then 1 mL of each treatment solution was separately added into 1 mL of DPPH in a test tube. A blank was prepared by adding 1 mL of ethyl alcohol into 1 mL DPPH. The mixtures were vortexed and incubated for 30 min at room temperature in the dark. The absorbance of the solutions was read at 517 nm with a spectrophotometer. The DPPH radical scavenging capacities of the treatment solutions were determined from the difference in absorbance between the samples and blank. The results were expressed as % DPPH inhibition.

3.3.8. Proximate composition of fresh white shrimp

Fresh, medium size (36/40) head-on white shrimp (*Litopenaeus setiferus*) were obtained from a local seafood store in Baton Rouge, LA. The shrimp were placed on ice and transported to the Food Processing Pilot Plant, Louisiana State University Agricultural Center. They were stored at 4 °C in the cold processing room before they were manually de-headed and de-shelled. Peeled, fresh white shrimp meat was analyzed for moisture, lipid, protein, and ash content. The moisture content was determined according to AOAC (1995).

Lipid content was analyzed according to the Bligh and Dyer (1959) method with some modifications. Fresh shrimp samples (100 g) were homogenized in a Waring 51BL32 commercial blender (Torrington, Connecticut) for 2 min with 100 mL chloroform (Macron Fine Chemicals, Avantor Performance Materials, inc., Center Valley, PA) and 200 mL methanol (Fisher Scientific, Fair Lawn, NJ). The mixture was blended for another 30 sec after the addition of 100 mL chloroform. Subsequently, 100 mL of DW was added and the mixture was blended for 30 sec. A Coors No. 3 Büchner funnel was used to filter the homogenate through Whatman No. 1 filter paper, with suction from a Büchi V-700 vacuum pump, (Flawil, Switzerland) into a Büchner flask. Lipids in the tissue residue were obtained by blending the filter paper and residue with 100 mL chloroform for 30 sec. The mixture was filtered with Whatman No. 1 filter paper through the Büchner funnel. The blender and the residue in the Büchner funnel were rinsed with 50 mL chloroform. The solution in Büchner flask was poured into a 500 mL separatory funnel, allowing layers of water, methanol, and chloroform containing lipids to form. The chloroform layer was evaporated in a round-bottom flask at 50 ºC using a rotary evaporator (Rotavapor RE121, Büchi Labortechnik AG, Flawil, Switzerland). Remaining solvent was removed by drying the round-bottom flask in a forced draft oven at 105 ºC for 2 h. Lipid content was determined gravimetrically.

Protein content was determined using the Dumas combustion method with a Leco TruSpec nitrogen analyzer (Leco Corporation, St. Joseph, Michigan) at the Soil Testing and Plant Analysis Laboratory, Louisiana State University Agricultural Center. A protein to nitrogen conversion factor of 6.25 was applied. Ash content was identified with a Thermolyne Type 6000 muffle furnace (Thermo Scientific, Lawrence, KS) at 549 ºC (AOAC, 1999).

3.3.9. Microbial counts of thawed shrimp

Aerobic plate counts (APC), yeast and mold counts (YMC), and total coliform counts (TCC) were quantified using 3M Petrifilms (3M Microbiology, St. Paul, MN) according to manufacturer's instructions with some modification. Whole thawed shrimp samples were placed in 18 oz Whirl-Pak bags, from Weber Scientific (Nasco, Fort Atkinson, WI), and diluted to 10^{-1} concentration with 0.85% NaCl solution. The shrimp samples in the Whirl-Pak bags were homogenized using a Stomacher lab-blender type 400 (Tekman Co., Cincinnati, Ohio) for 2 min. Duplicated serial dilutions of shrimp samples in 0.85% NaCl solution were produced and separately plated on Petrifilms for APC, YMC, and TCC. Using a flat surface, 1 mL of shrimp dilution was placed on the bottom film and the inoculums were covered with the top film and spread using the provided 3M plastic spreaders. APC Petrifilms were incubated for 48 h at 35 °C and the red colonies were counted. For YMC Petrifilms, the blue-green colonies were counted after incubation for 72 h at 22 °C, while TCC Petrifilms were incubated for 24 h at 35 °C and counted for red gas forming colonies. APC, YMC, and TCC were done using the naked eye.

3.3.10. pH and moisture content of thawed shrimp

The pH of the shrimp was analyzed according to the method described by Sundararajan et al. (2011) with some modifications. Ten grams of shrimp sample was homogenized in a Waring 51BL32 commercial blender (Torrington, Connecticut) with 40 mL DW for 15 sec. The mixture was then poured into a 100 mL beaker and sonicated for 1 min at 4 °C with amplitude of 82% and pulser set at 2 sec. The pH of the homogenized sample was measured with a pH meter. The moisture content of the thawed shrimp samples was determined according to AOAC (1995).

3.3.11. Lipid oxidation of thawed shrimp

The degree of lipid oxidation present in shrimp during frozen storage was determined by TBARS (thiobarbituric-acid-reactive-substances) analysis according to the method described by Lemon (1975) with slight modification. All of the reagents utilized for the TBARS analysis were obtained from Sigma Aldrich (St. Louis, MO). Thiobarbituric acid (TBA) solution (0.02 M) was prepared by dissolving 1.4415 g TBA in 500 mL DW. An extraction solution was prepared by dissolving 7.5% trichloroacetic acid, 0.1% propyl gallate, and 0.1% ethylenediaminetetraacetic acid in 500 mL DW. The solutions were covered in aluminum foil and mixed overnight. Fifteen grams of thawed shrimp was homogenized with 30 mL extraction solution in a commercial blender for 30 sec and filtered using Whatman No. 1 filter paper. In a capped test tube, 5 mL of filtrate was added into 5 mL of TBA solution and then vortexed. The tube cap was loosened and the tube placed in boiling water for 40 min. After heating, the sample solution was cooled and centrifuged at 10000 rpm for 10 min. The absorbance of the solution was read at 530 nm with a spectrophotometer. TBARS values were determined from a standard curve of 1,1,3,3 tetraethoxypropane in concentrations of 0.0-0.01 μmoles/mL. The malondialdehyde (MDA) content was expressed as mg MDA/kg sample.

3.3.12. Color and texture of thawed shrimp

The surface color of thawed shrimp was measured in triplicate with a chroma meter LABSCAN XE (Hunterlab, VA) fitted with a pulsed xenon lamp and an aperture diameter of 13 mm. Whole shrimp were placed in a sample tray and fitted into the aperture to record the surface color. CIELAB color scales were used to express the results. The $L*$ values correlate with the degree of lightness to darkness, $a*$ values assess the degree of redness to greenness, and the $b*$ values measure the extent of yellowness to blueness.

Texture of the thawed shrimp was measured according to the method described by Thongphitak, Limsuwan, Chuchird, Raksakulthai, and Pansawat (2007). An Instron Universal testing device (Model 5544, Norwood, MA) equipped with a Warner-Blatzer blade and a 25-kg load cell was used. The instrumental settings and operations were determined using Bluehill Materials testing software (Bluehill 3, version 3.13, 2010, Instron). The thawed shrimp was cut transversely at the third abdominal segment with a speed of 2 mm per second and distance of 25 mm to determine the maximum cutting force (N).

3.3.13. Statistical analysis

Data was statistically analyzed using SAS software version 9.4 (SAS Institute Inc., Cary, NC). Mean values of triplicate analysis were reported with their standard deviations. To determine differences between treatments and within treatments during the storage time, Analysis of Variance (ANOVA) and Tukey's studentized range test was used at a significance level of $P \leq 0.05$.

3.4. Results and discussion

3.4.1. Molecular weight and degree of deacetylation of chitosan

The viscosity-average molecular weight (M_v) of chitosan (CH) decreased significantly from 338.10±12.86 to 296.95±5.65 kDa after processing with sonication and ultra-shearing (Table 3.1). The degree of deacetylation (DD) of CH after processing (64.55±0.89%) was significantly lower than before processing (70.86±1.30%). Also, chitosan-sodium tripolyphosphate (CH-TPP) had the lowest DD (19.98±0.54%) ($P \leq 0.05$). Our results are in agreement with No, Kim, and Meyers (1999) who reported that increasing the time that CH was treated with techniques such as ultrasonication, heating, grinding, autoclaving, and ozonation could decrease its viscosity. The viscosity of CH is associated with its molecular weight, exhibiting higher molecular weights with increasing viscosities and lower molecular weights

with decreasing viscosities. Baxter, Zivanovic, and Weiss (2005) reported that the intrinsic viscosity of CH could be reduced exponentially with increased sonication time. The rates of intrinsic viscosity decrease were linearly correlated with increased ultrasonic intensity.

Table 3.1. Molecular weight and degree of deacetylation of chitosan before and after processing with sonication and ultra-shearing.

Sample	M_v (kDa)	DD (%)
CH/BP	338.10 ± 12.86^a	70.86 ± 1.30^a
CH/AP	296.95 ± 5.65^b	64.55 ± 0.89^b
CH-TPP/AP	-	19.98 ± 0.54^c

[a,b]Means±SD with different letters within a column indicate significant difference ($P \leq 0.05$). CH = chitosan, CH-TPP = chitosan-sodium tripolyphosphate, BP = before processing with sonication and ultra-shearing, and AP = after processing with sonication and ultra-shearing.

Our results were consistent with the findings of Savitri, Juliastuti, Handaratri, Sumarno, and Roesyadi (2014), who observed a reduction in molecular weight and DD after sonicating CH using various acetic acid (AA) concentrations (0.2-1% v/v). The lower hydration energies of GlcN-GlcN glucosidic bonds, compared to glucosamine and acetyl group interactions, promoted more efficient degradation of the polymer chain. Sonication may have led to the breakage of glucosamine units instead of N-glucosamine units. This left CH with a higher concentration of GlcNAc and lower DD. Moreover, CH-TPP had the lowest DD, likely due to the electrostatic mechanism of CH-TPP nanoparticle formation. CH nanoparticles are produced by interactions between the positively charged amine groups of CH and the negatively charged groups of sodium tripolyphosphate (TPP) (Azeredo, 2013). The amine groups that were bound to TPP in acidic media may not have been detected during the DD analysis, resulting in considerably lower DD compared to CH before and after processing.

3.4.2. Rheological properties of chitosan solutions

At 25 and 5 °C, CH and CH-TPP solutions exhibited shear-thinning (pseudoplastic) characteristics, as made apparent by their flow behavior index values (n), which were less than 1 (Table 3.2) (Bjorn, Karlsson, Svensson, Ejlertsson, & de La Monja, 2012). In addition, our results show that K (consistency index) and apparent viscosity values of CH and CH-TPP treatment solutions were not significantly different between treatment solutions at the same temperature (25 and 5 °C) or within each treatment solution at different temperatures (25 and 5 °C) within their respective analysis.

Table 3.2. Flow characteristics of chitosan solutions at 25 and 5 °C.

Solution	n	K (x10^{-3} Pa s^n)	Apparent viscosity (x10^{-3} Pa s)
At 25 °C			
CH/AP	0.97 ± 0.01^a	6.21 ± 0.21^a	4.41 ± 0.49^{ab}
CH-TPP/AP	0.94 ± 0.02^a	5.73 ± 0.04^a	3.74 ± 0.48^a
At 5 °C			
CH/AP	0.96 ± 0.01^a	6.64 ± 0.84^a	5.54 ± 0.41^b
CH-TPP/AP	0.93 ± 0.01^a	6.02 ± 0.15^a	4.98 ± 0.24^{ab}

[a,b]Means±SD with different letters within a column indicate significant difference ($P \leq 0.05$). CH = chitosan, CH-TPP = chitosan-sodium tripolyphosphate, K = consistency index, n = flow behavior index, and AP = after processing with sonication and ultra-shearing.

Sathivel et al. (2007) observed pseudoplastic behavior in a 1% CH in 2% lactic acid solution at 5-25 °C. Also, Abdou, Osheba, and Sorour (2012) reported pseudoplastic behavior for a CH solution and CH-TPP nanoparticle solution that were used as edible coating solutions for fish fingers during frozen storage at -18 °C. The rheological properties of CH solutions yield practical information for process optimization. According to Hwang and Shin (2000), flow behavior of CH solutions has two distinct viscosity regions: Newtonian and power-law flow

regions. At low shear rates, the Newtonian flow region can be observed through the maintenance of constant viscosity. This occurs because the effect of exerted shear force on the rate of intermolecular disentanglements is similar to that of newly formed entanglements. When the shear rate increases, leading to lower viscosity, the power-law flow region can be observed due to a higher rate of disentanglements than entanglements. Solutions with higher CH concentrations are more pseudoplastic, resulting from the restricted movement of individual polymer chains and a higher number of entanglements.

CH and CH-TPP solutions were both shown to have higher loss modulus (G″) than storage modulus (G′) at 25 and 5 °C, which is typical of fluid-like solutions (Figure 3.3). Arancibia et al. (2015) reported higher G″ than G′ for a 1% CH in 0.15 M lactic acid solution at temperatures below 45 °C. As the CH and CH-TPP solutions exhibited fluid-like behavior, they may be good for processing.

3.4.3. Particle size and total reducing sugar content of chitosan solutions

Particle size of CH in solution was significantly higher than that of CH-TPP in solution (Table 3.3). Processing with sonication and ultra-shearing could reduce particle size of CH in solution from 3428.50±198.70 to 1469.50±67.18 nm and CH-TPP in solution from 312.90±12.93 to 135.83±3.73 nm (P ≤ 0.05). The total reducing sugar (TRS) content of CH solution before processing was higher than that of CH-TPP solution before processing and the TRS content of CH solution after processing was greater than that of CH-TPP solution after processing (P ≤ 0.05). Processing with sonication and ultra-shearing could increase the TRS content of CH solution from 20.93±1.71 to 44.65±0.63 mg glucosamine/g CH and the TRS content of CH-TPP solution from 14.30±0.76 to 29.57±1.97 mg glucosamine/g CH (P ≤ 0.05).
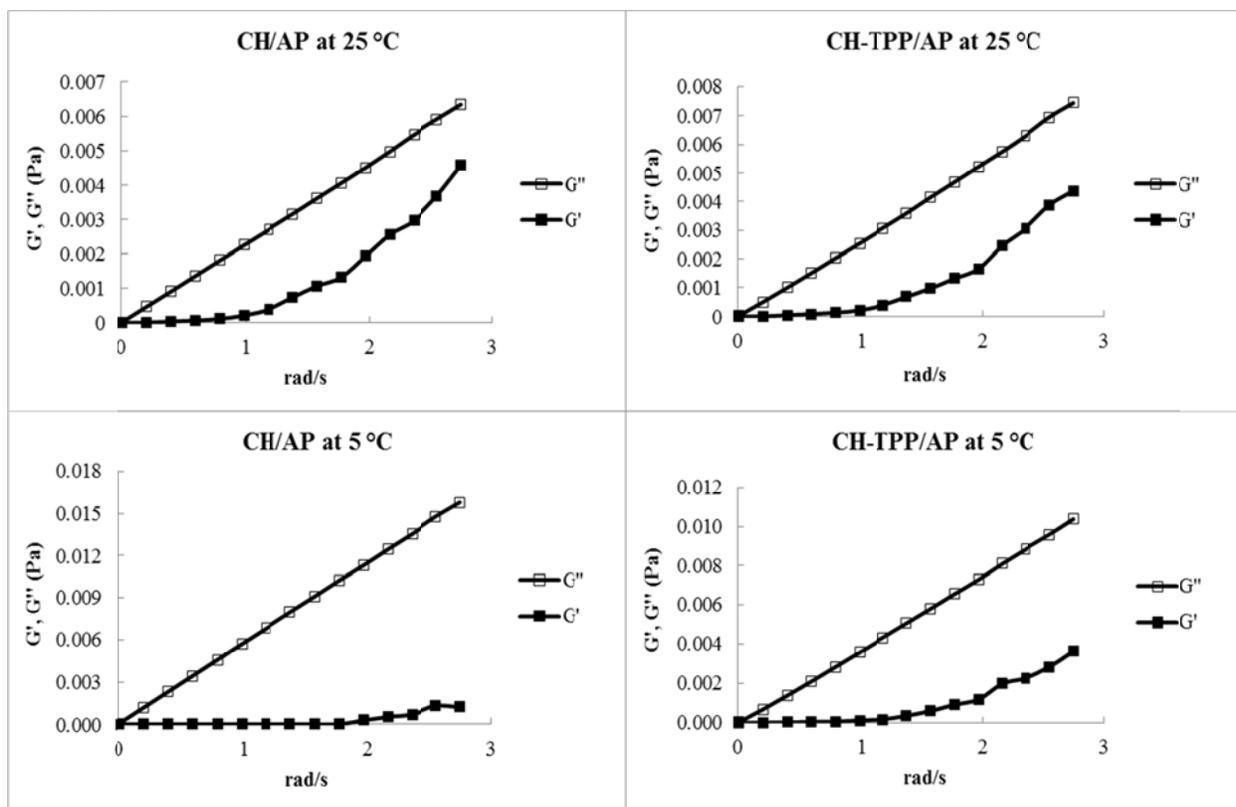
Figure 3.3. Frequency sweeps of chitosan solutions at 25 and 5 °C: storage modulus (G′) and loss modulus (G″) vs. angular frequency (rad/s). CH = chitosan, CH-TPP = chitosan-sodium tripolyphosphate, and AP = after processing with sonication and ultra-shearing.

Table 3.3. Particle size of chitosan particles in solution and total reducing sugar content of chitosan solutions before and after processing with sonication and ultra-shearing.

Solution	Particle size (nm)	TRS (mg glucosamine/g CH)
CH/BP	3428.50±198.70[a]	20.93±1.71[c]
CH/AP	1469.50±67.18[b]	44.65±0.63[a]
CH-TPP/BP	312.90±12.93[c]	14.30±0.76[d]
CH-TPP/AP	135.83±3.73[d]	29.57±1.97[b]

[a-d]Means±SD with different letters within a column indicate significant difference (P ≤ 0.05). CH = chitosan, CH-TPP = chitosan-sodium tripolyphosphate, BP = before processing with sonication and ultra-shearing, and AP = after processing with sonication and ultra-shearing.

Nanoparticles are particulate dispersions or solid particles within the size range of 10-1000 nm (V. J. Mohanraj & Y. Chen, 2007). CH-TPP nanoparticles are spontaneously formed

when TPP is mixed with CH in acidic medium due to the molecular linkages between phosphate and amino groups (Gan et al., 2005). CH molecule degradation by ultrasonic radiation is largely caused by the cavitation effect. Cavitation contributes vibration wave energy, shear stress at the cavitation interphase, and localized high pressure and temperature (Gronroos et al., 2001). CH is degraded concurrently by cavitation throughout the entirety of the solution (Tsaih, Tseng, & Chen, 2004). Cavitation can assist in the reduction of CH particle sizes and it can break down nanoparticle clusters to obtain fine particles (Lan, Yang, & Li, 2004). During ultrasonic radiation, the amount of cavitation produced is proportional to input energy. However, processing CH with shearing mainly degrades the polymer by entanglement and stretch between the entangled molecules using shear force. During shearing, the polymer is sheared in the same direction as shearing flow. The tearing mechanism during shearing requires that the shear stress applied to a polymer exceed the polymer's specific critical value and the length of the polymer should be enough for entanglement and stretching to occur. The cavitation effect can generate smaller CH particles than the tearing effect (Tsai, Bai, & Chen, 2008). Although, in our study it was clearly observed that processing a CH-TPP solution with sonication and ultra-shearing could aid in breaking down the gels that were formed during ionotropic gelation of CH and TPP more effectively than sonication alone.

Sonication has been shown to increase formation of shorter chain molecules and reducing sugars in starch (Tomasik & Zaranyika, 1995). Also, Bhattacharyya, Datta, and Bhattacharjee (2012) reported that sonicating sugarcane bagasse at 80% amplitude and 0.8 cycle for 10 min after dilute acid hydrolysis could increase TRS extraction by around 25%. In CH, bonds with higher hydration energies are less efficiently degraded. GlcN-GlcN glucosidic bonds have a lower hydration energy (0.65-0.67 kcal/mol) than GlcN-GlcNAc (0.74-0.75 kcal/mol), GlcNAc-

GlcN (0.75-0.76 kcal/mol), or GlcNAc-GlcNAc (0.85 kcal/mol). Because of the high degradation rate of GlcN-GlcN glucosidic bonds during sonication, greater amounts of GlcN units may have been degraded and dissolved in the solution, thus reducing glucosamine chain length and increasing the quantity of TRS (Savitri et al., 2014). Lower TRS content in CH-TPP solutions before and after processing, compared to CH solutions at the same conditions, may be linked to decreased amine group availability following ionotropic gelation of CH and TPP.

3.4.4. Deconvolution microscopy images of fluorescently labeled chitosan particles in solution and cross-sections of vacuum tumbled shrimp

Micrographs of fluorescein isothiocyanate F7250 isomer 1 (FITC) labeled CH and CH-TPP particles in 1% acetic acid (AA) solution are shown in Figure 3.4. Particle sizes of CH and CH-TPP particles (as shown in Table 3.3) were similar with those observed in micrographs of FITC labeled CH and CH-TPP particles in solution. The FITC labeled CH and CH-TPP particles appeared to be generally spherical with no agglomeration.
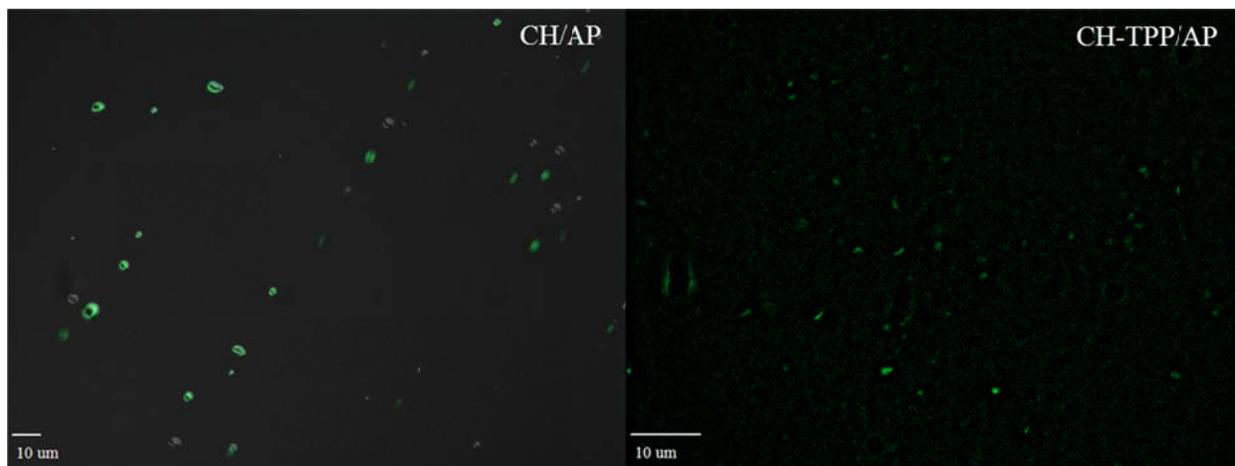
Figure 3.4. Micrographs of FITC labeled particles of CH and CH-TPP in solution at 40x and 100x magnification, respectively. CH = chitosan, CH-TPP = chitosan-sodium tripolyphosphate, and AP = after processing with sonication and ultra-shearing.

It was observed that FITC labeled CH and CH-TPP particles could penetrate inside of and attach to shrimp muscle tissue (Figure 3.5). Although, the majority of FITC labeled CH and

CH-TPP particles were accumulated at the outer layers of the shrimp muscle tissue. The inner shrimp muscle tissue layers had a lower quantity of FITC labeled CH and CH-TPP particles and diminished particle agglomeration compared to the outer layers.
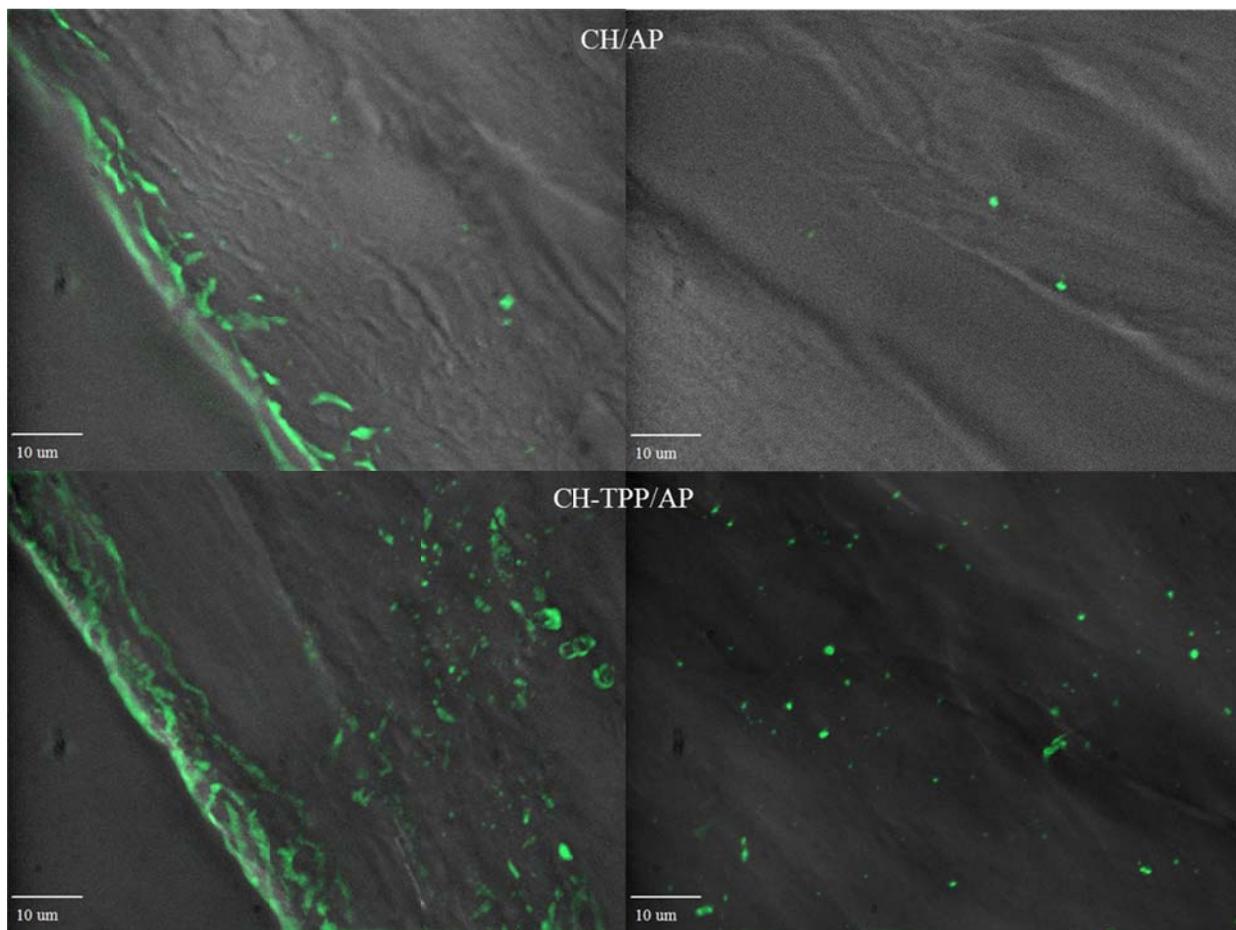
Figure 3.5. Micrographs of FITC labeled particles of CH and CH-TPP in cross-sections of the outer (left) and inner (right) shrimp muscle tissue at 100x magnification. CH = chitosan, CH-TPP = chitosan-sodium tripolyphosphate, and AP = after processing with sonication and ultra-shearing.

It appeared as though there were a greater quantity of CH-TPP particles that could penetrate into the shrimp, compared to CH particles. The bioadhesive properties of CH were clearly demonstrated in these micrographs. These attributes greatly benefit the functionality of CH as a biodegradable compound by possibly leading to decreased moisture transfer, lower

oxygen permeability, and lower surface dehydration in meat during frozen storage (Debeaufort, Quezada-Gallo, & Voilley, 1998).

Particle size is an important aspect of particle uptake and intracellular trafficking within tissues (Gan et al., 2005). Nanoparticles generally have higher intracellular uptake compared to microparticles. Due to their small size and relative mobility, they are available to a greater number of biological targets (Mohanraj & Chen, 2007). The sub-cellular and sub-micron size of CH-TPP nanoparticles allows for deep penetration into tissues through fine capillaries and fenestration in the epithelial lining (Gan et al., 2005). Desai, Labhasetwar, Walter, Levy, and Amidon (1997) reported that the uptake for nanoparticles (100 nm) was 2.5 fold higher than microparticles (1 μm) and 6 fold higher than microparticles (10 μm) in a Caco-2 cell line. In an *ex vivo* canine carotid artery model, smaller nanoparticles (~100 nm) showed more than three times greater arterial uptake compared to larger nanoparticles (~275 nm). This was because the smaller nanoparticles could penetrate through the sub-mucosal layers, unlike the larger nanoparticles which were generally localized in the epithelial lining (Desai, Labhasetwar, Amidon, & Levy, 1996; Song, Labhasetwar, Cui, Underwood, & Levy, 1998).

In addition to lowering the particle size of CH and CH-TPP by processing with sonication and ultra-shearing, the utilization of vacuum tumbling can play an important role in enhancing the uptake of CH and CH-TPP particles by shrimp meat. Tumbling has been reported to promote higher brine uptake by meat. The mechanism of this process may be related to structural component damage, which can result in higher swelling potential (Cheng & Sun, 2008; Theno, Siegel, & Schmidt, 1978). It has been shown that gases can dissolve when coming into contact with meat (Bruce, Wolfe, Jones, & Price, 1996; Gros, Dussap, & Brient, 1996). The use of vacuum can promote greater exchange between occluded internal gases in food and the external

soaking medium (Collignan, Bohuon, Deumier, & Poligne, 2001; Fito, 1994; Fito, Chiralt, Barat, Spiess, & Behsnilian, 2001; Lenart & Flink, 1984; Raoult-Wack, 1994; Rastogi & Raghavarao, 1996; Roa, Tapia, & Millan, 2001). Changes in pressure can also have an effect on capillary uptake (Chiralt et al., 2001). The hydrodynamic mechanism (HDM) is a mass transfer mechanism that occurs during solid-liquid interactions. According to the HDM, vacuum allows for the internal gas in a food product to expand and flow out, while promoting the transfer of liquid into the new void spaces (capillary penetration). After atmospheric pressure is regained, the residual gas in the pores compresses and results in greater infiltration of liquid into the pores (Fito, 1994; Fito & Pastor, 1994).

Porosity may possibly be enhanced during vacuum processing due to the likely breakdown of meat tissues near areas of weaker cohesion, such as along muscle fibers around the perimysium (Deumier et al., 2003). Vacuum tumbling was found to be effective in decreasing cooking losses, as well as improving tenderness and water hold capacity of hams compared to controls that were not tumbled (Rejt et al., 1978). Solomon, Norton, and Schmidt (1980) reported that tumbling ham muscles with applied vacuum could provide greater cure (11.16% sodium chloride, 1.67% TPP, 2.22% sucrose, 0.087% sodium nitrite, and 84.89% deionized water) absorption and binding functionality than tumbling alone. Although vacuum could facilitate higher cure absorption, there was an unequal distribution of marinade within whole muscles as measured by the sodium chloride content at three different depths. Sodium chloride content was found to be higher in exterior muscle layers, showing a decrease in concentration from outer to inner depths of the muscle.

3.4.5. pH and free radical scavenging activity of treatment solutions

The pH values of CH, CH-TPP, TPP, and AA solutions after processing with sonication and ultra-shearing, as well as for DW, were 3.60±0.01, 3.89±0.00, 3.38±0.01, 2.85±0.01, and 6.14±0.01, respectively. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical inhibition was used as a measure of the antioxidant capacity of treatment solutions. Before processing, CH solution had the highest DPPH inhibition (11.95±0.69%), followed by CH-TPP solution (9.29±0.57%) (P ≤ 0.05). DPPH inhibition using TPP solution (6.78±0.19%) was not significantly different from that of AA solution (6.64±0.26%), although both were lower than the values of CH and CH-TPP solutions (P ≤ 0.05). CH after processing had significantly higher DPPH inhibition (18.08±0.84%) compared to other treatment solutions (CH-TPP, TPP, and AA) after processing (Table 3.4). The unprocessed distilled water (DW) control solution had the lowest DPPH inhibition (1.06±0.19%) among treatment solutions before or after processing (P ≤ 0.05), indicating very little antioxidant capacity.

Table 3.4. Free radical scavenging activity of treatment solutions.

Solution	DPPH inhibition (%)	
	BP	AP
CH	11.95±0.69^{aB}	18.08±0.84^{aA}
CH-TPP	9.29±0.57^{bA}	9.22±0.39^{bA}
TPP	6.78±0.19^{cA}	7.18±0.50^{cA}
AA	6.64±0.26^{cA}	6.39±0.51^{cA}
DW	1.06±0.19^{d}	-

[a-d]Means±SD with different letters within a column indicate significant difference (P ≤ 0.05). [A,B]Means±SD with different letters within a row indicate significant difference (P ≤ 0.05). CH = chitosan, CH-TPP = chitosan-sodium tripolyphosphate, TPP = sodium tripolyphosphate, AA = acetic acid, DW = distilled water, BP = before processing with sonication and ultra-shearing, and AP = after processing with sonication and ultra-shearing.

The free radical scavenging activity of CH solution increased after processing, unlike that of other treatment solutions, which were not significantly different after processing within their respective treatment. Free radical scavenging activity of AA and TPP solutions is likely attributed to the H-abstraction reaction (Denisov & Shestakov, 2013). Hydrogen atoms in the carboxylic group of AA may bind to DPPH radicals, contributing to higher scavenging activity. CH solution had higher DPPH inhibition after processing because the antioxidant activity of CH is related to its molecular weight, exhibiting greater activities at lower molecular weights (Dash, Chiellini, Ottenbrite, & Chiellini, 2011). It was reported by Kim and Thomas (2007) that CH could scavenge DPPH radicals, showing varying activities at different molecular weights (30, 90, and 120 kDa). The highest scavenging activity was demonstrated by 30 kDa CH, possibly because lower molecular weight CH could have higher mobility than 120 kDa CH, decreasing the possibility of inter and intra molecular bonding between high molecular weight CH molecules and perhaps enhancing the exposure of amino groups. There are three modes of action for free radical scavenging activity by CH: (1) the hydroxyl groups in the polysaccharide unit can react with radicals by the H-abstraction reaction, (2) the active hydrogen ions in free amino groups can react with radicals to form stable macromolecule radicals, and (3) the amino groups can form ammonium (NH_3^+) groups by absorbing hydrion from the solution, then reacting with radicals through addition reaction (Xie, Xu, & Liu, 2001).

In addition, higher scavenging activities at greater CH concentrations may be due to the availability of an increased number of amine groups (Kim & Thomas, 2007). This may explain why, in our study, CH-TPP solutions had lower free radical scavenging activities than CH solutions before and after processing. Many of the amine groups in CH-TPP solution were likely bound to TPP groups during ionotropic gelation, thus decreasing the quantity of amine groups

available to react with DPPH radicals. Also, the particle size of CH particles was reduced to a much larger extent after processing than the sizes of CH-TPP particles (Table 3.3), which were already nano-scale immediately after they were formed.

3.4.6. Proximate composition of fresh white shrimp

Fresh white shrimp (*Litopenaeus setiferus*) had a proximate composition (g/100 g) of 77.88±0.18 moisture, 19.27±0.12 protein, 1.48±0.00 ash, and 1.20±0.14 lipids (Table 3.5). Our results are similar to those reported by Solval et al. (2014) and Sundararajan et al. (2011). The fatty acids found in white shrimp are mainly polyunsaturated and contain 12.8±1.4% Eicosapentaenoic acid C20-5 ω-3 (EPA) and 9.8±2.6% Docosahexaenoic acid C22-6 ω-3 (DHA) (Bottino, Gennity, Lilly, Simmons, & Finne, 1980). This may make shrimp susceptible to oxidation during frozen storage.

Table 3.5. Proximate composition of white shrimp (*Litopenaeus setiferus*).

Component	Content (g/100 g)
Moisture	77.88±0.18
Total lipids	1.20±0.14
Protein	19.27±0.12
Ash	1.48±0.00

3.4.7. Microbial counts of thawed shrimp

Throughout the entire storage time, aerobic plate counts (APC) of CH and CH-TPP treatments were significantly lower compared to the other treatments (Figure 3.6). TPP had similar APC to AA and both treatments had lower APC than DW and untreated shrimp (NT) (P ≤ 0.05). All treatments, within their respective treatment, showed lower APC from day 0 to day 60 of storage and were not significantly different from day 60 to day 120 (P ≤ 0.05). Throughout the entire storage time, similar yeast and mold counts (YMC) were observed between treatments

(P ≤ 0.05). The YMC for all treatments, within their respective treatment, increased significantly from day 0 to day 60, but were not significantly different from day 60 to day 120 (Figure 3.7). In our study, the total coliform counts (TCC) in the shrimp samples were too few to count at any storage time.
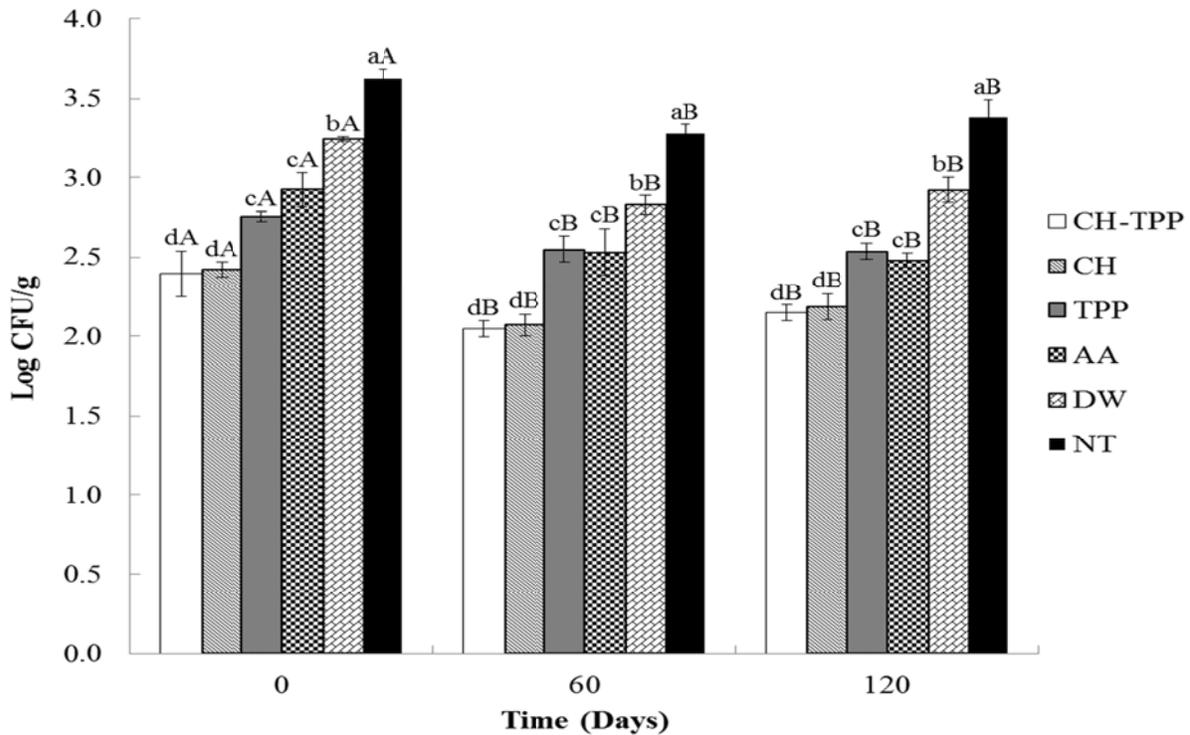
Figure 3.6. Aerobic plate counts of thawed shrimp during storage at -20 °C. [a-d]Means±SD with different letters between treatments at the same storage times indicate significant difference (P ≤ 0.05). [A,B]Means±SD with different letters within treatments at different storage times indicate significant difference (P ≤ 0.05). CH = chitosan, CH-TPP = chitosan-sodium tripolyphosphate, TPP = sodium tripolyphosphate, AA = acetic acid, DW = distilled water, and NT = no treatment.

AA solutions have been reported to provide significant bacteriostatic and bactericidal effects when applied to a variety of meat products using different concentrations of AA, with or without the addition of other antimicrobial compounds (Bell, Cutter, & Sumner, 1997; Surve, Sherikar, Bhilegaonkar, & Karkare, 1991; Taher, Khaton, Fakir, Hasnat, & Rahman, 2013). CH has been shown to have higher antimicrobial activity at lower pH values, as well as with increasing temperatures (Raafat & Sahl, 2009).

The interaction between protonated amine groups of CH and the negatively charged surface components of various bacteria and fungi may play an extensive role in the antimicrobial mechanisms of CH (Raafat & Sahl, 2009). The lethal action of CH on microorganisms can be described in six steps: (1) adsorption onto the bacterial cell surface, (2) diffusion through the cell wall, (3) adsorption onto the cytoplasmic membrane, (4) disruption of the cytoplasmic membrane, (5) leakage of the cytoplasmic constituents, and (6) death of the cell (Kong, Chen, Xing, & Park, 2010). CH-TPP nanoparticles have a greater surface area per unit volume and higher charge density than CH. Both of these factors greatly contribute to their interaction with anionic bacterial cell membranes (Azeredo, 2013).
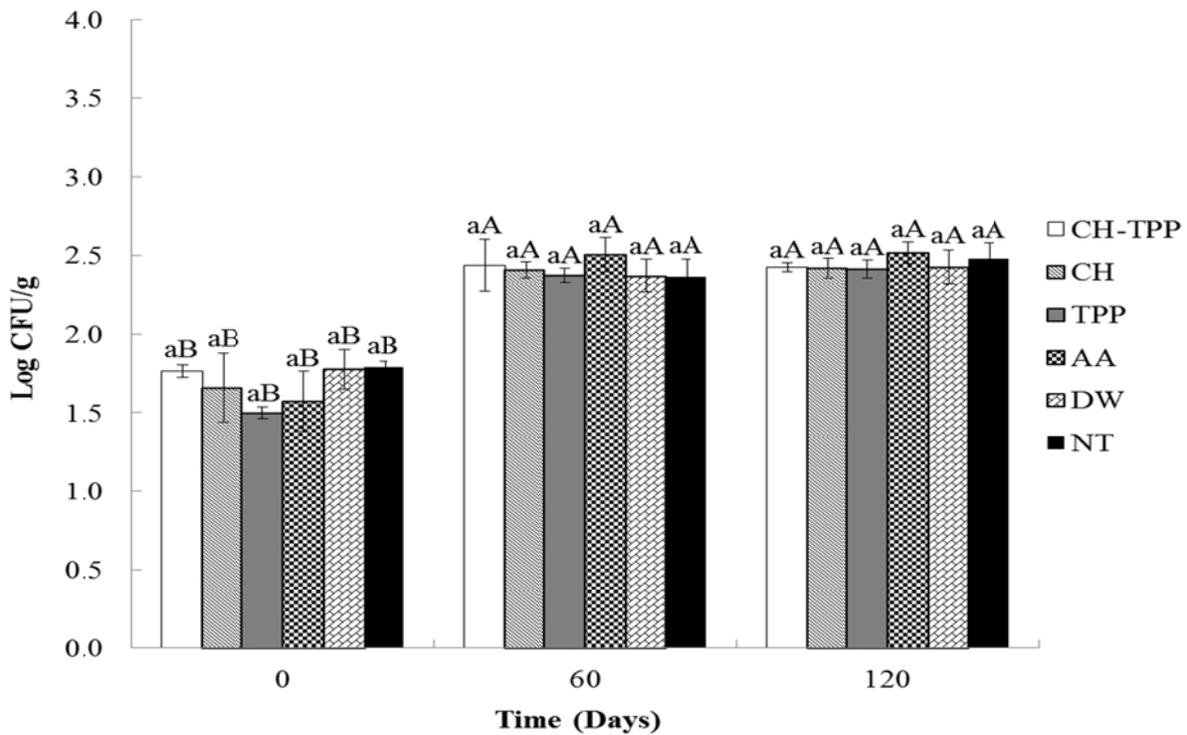
Figure 3.7. Yeast and mold counts of thawed shrimp during storage at -20 °C. [a]Means±SD with same letters between treatments at the same storage times are not significantly different (P ≤ 0.05). [A,B]Means±SD with different letters within treatments at different storage times indicate significant difference (P ≤ 0.05). CH = chitosan, CH-TPP = chitosan-sodium tripolyphosphate, TPP = sodium tripolyphosphate, AA = acetic acid, DW = distilled water, and NT = no treatment.

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Abdou et al. (2012) observed the effects of CH and CH-TPP nanoparticle edible coatings on the microbial counts of fish fingers during frozen storage at -18 °C for 6 months. Fish fingers coated with a CH solution (2, 2.8, or 4%) or CH-TPP solution (2, 2.8, or 4%) had lower psychrophilic bacteria, coliform bacteria, proteolytic bacteria, and total bacterial counts during the storage time than uncoated fish fingers and fish fingers coated with a commercial edible coating. They also reported that total bacterial counts decreased for uncoated fish fingers and those coated with a commercial edible coating for the first 2 months of storage, after which they slowly increased. The total bacterial counts of fish fingers coated with 2% CH and 2% CH-TPP solutions decreased for the first 4 months of storage, then increased slightly. Total bacterial counts of fish fingers coated with 2.8 or 4% CH solution, as well as those coated with 2.8 or 4% CH-TPP solution decreased for the first 5 months, after which a slight increase was observed. Furthermore, they attributed the initial reductions in total bacterial counts for the first few months of all treatments during frozen storage to inter and extra cellular ice crystal formation during the freezing process. These ice crystals may have caused irreversible damage to the outer and cytoplasmic membranes of bacteria (Uljas & Ingham, 1999). The rate of microbial death is highest during the initial freezing process and it gradually declines over time in frozen storage (Adams & Moss, 1995). Pertaining to YMC, mold spores and gram-positive bacteria possess a greater resistance to low temperatures than gram negative bacteria (Fellows, 2009). Tsai et al. (2002) found that CH was more effective at inhibiting bacteria than fungi. According to Allan and Hadwiger (1979), CH was ineffective at inhibiting fungi that contained chitin or CH in their cell walls.

3.4.8. pH and moisture content of thawed shrimp

CH and TPP had similar pH values initially, while other treatments were significantly different (P ≤ 0.05). At 120 days of storage, there were significant differences in pH values between all treatments. The pH values for all treatments, within their respective treatment, increased significantly every 30 days over the 120 day storage duration (Figure 3.8).
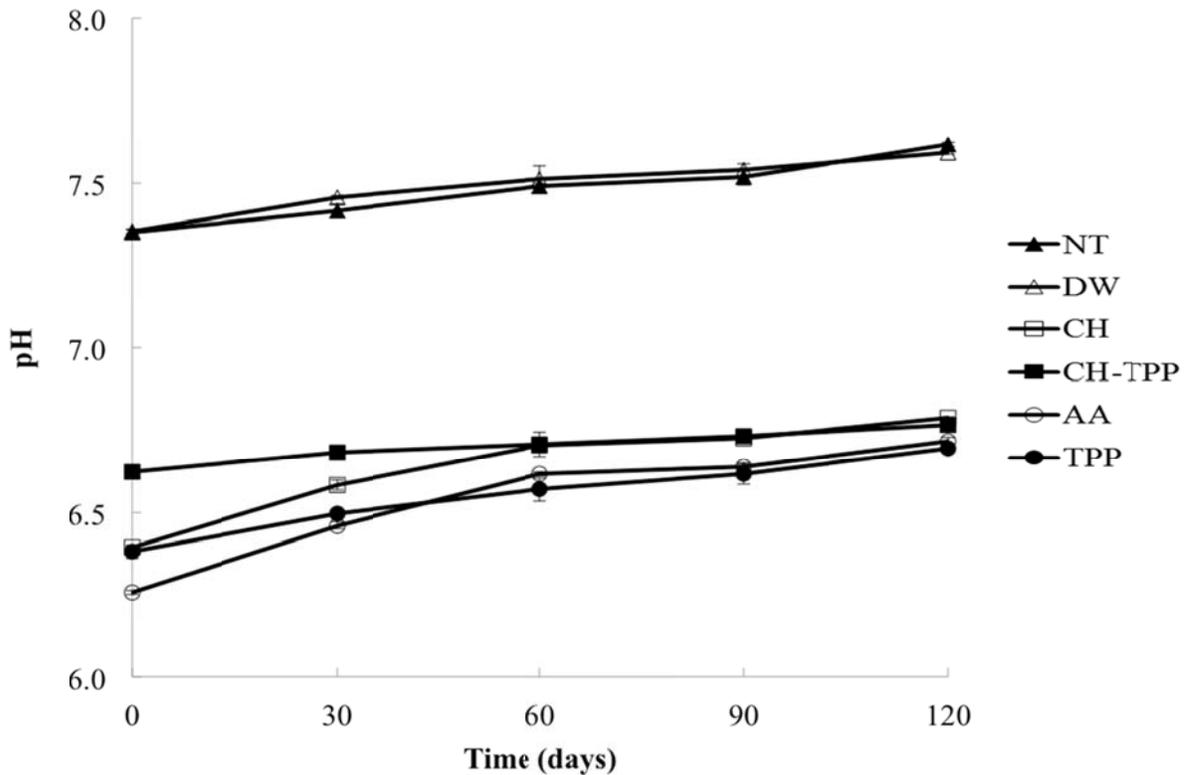
Figure 3.8. pH of thawed shrimp during storage at -20 °C. CH = chitosan, CH-TPP = chitosan-sodium tripolyphosphate, TPP = sodium tripolyphosphate, AA = acetic acid, DW = distilled water, and NT = no treatment.

Elevated shrimp pH during storage has been reported in various studies (Goswami, Ravindra, & Nayak, 2001; Solval et al., 2014; Sundararajan et al., 2011). The pH of shrimp can serve as an indicator of physicochemical changes (Riaz & Qadri, 1990). An increase in shrimp pH during storage is the result of biochemical reactions (Shamshad, Riaz, Zuberi, & Qadri, 1990). Accumulation of indoles, trimethylamines, and total volatile bases has been linked to

higher pH values (Mendes, Goncalves, Pestana, & Pestana, 2005; Zeng, Thorarinsdottir, & Olafsdottir, 2005). According to Goswami et al. (2001), pH values below 7.2 correspond with prime quality shrimp and a pH between 7.7 and 7.95 is an indicator of poor, but acceptable quality shrimp. Shrimp is classified as spoiled at pH above 7.95.

The initial moisture contents of all treatments were not significantly different besides NT, which was significantly lower than DW (Figure 3.9). At 120 days of storage, NT had significantly lower moisture than the other treatments. Moisture contents between CH, CH-TPP, and DW treatments were not significantly different, but they were higher than AA ($P \leq 0.05$). The DW treatment had significantly higher moisture than TPP, which was similar to AA ($P \leq 0.05$). Within their respective treatment, the moisture contents of DW, AA, and NT decreased significantly from day 0 to day 120 of storage, while CH, CH-TPP, and TPP showed no significant difference. Over time, seafood in frozen storage undergoes surface dehydration, resulting in decreased water holding capacity of tissues (Shenouda, 1980).

Also, the water holding capacity of meat is dependent on muscle pH. The isoelectric point of myofibrillar proteins (actin and myosin) is around 5.1. As the pH of muscle approaches this value, it experiences lower water holding capacity. At the isoelectric point, there would be minimum net charges on the protein, which would decrease the space between filaments for water binding (Alvarado & McKee, 2007). CH coating solutions can serve as moisture-sacrificing agents instead of moisture barriers. Application of these coatings can delay moisture losses in a food product until the moisture contained within the CH coating undergoes sublimation (Kester & Fennema, 1986). TPP is a commonly used functional food additive that promotes moisture retention in seafood (Goncalves & Ribeiro, 2008a).

Figure 3.9. Moisture content of thawed shrimp during storage at -20 °C. CH = chitosan, CH-TPP = chitosan-sodium tripolyphosphate, TPP = sodium tripolyphosphate, AA = acetic acid, DW = distilled water, and NT = no treatment.

3.4.9. Lipid oxidation of thawed shrimp

Initially, TBARS (thiobarbituric-acid-reactive-substances) of the CH treatment were significantly lower than the other treatments, with the exception of CH-TPP, to which it was not significantly different (Figure 3.10). The CH-TPP treatment had similar TBARS values to TPP and DW, although it was lower than AA and NT (P ≤ 0.05). TBARS of TPP, DW, and AA treatments were not significantly different. NT had the highest TBARS compared to other treatments (P ≤ 0.05). At 120 days of storage, CH and CH-TPP treatments had the lowest TBARS, followed by DW, TPP, AA, and NT (P ≤ 0.05). Within their respective treatment, the TBARS of all treatments increased significantly from day 0 to day 120 of storage. The TBARS

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of every treatment in our study did not exceed the maximum level that would indicate good

seafood quality (5 mg MDA/kg sample), as reported by Ibrahim Sallam (2007).

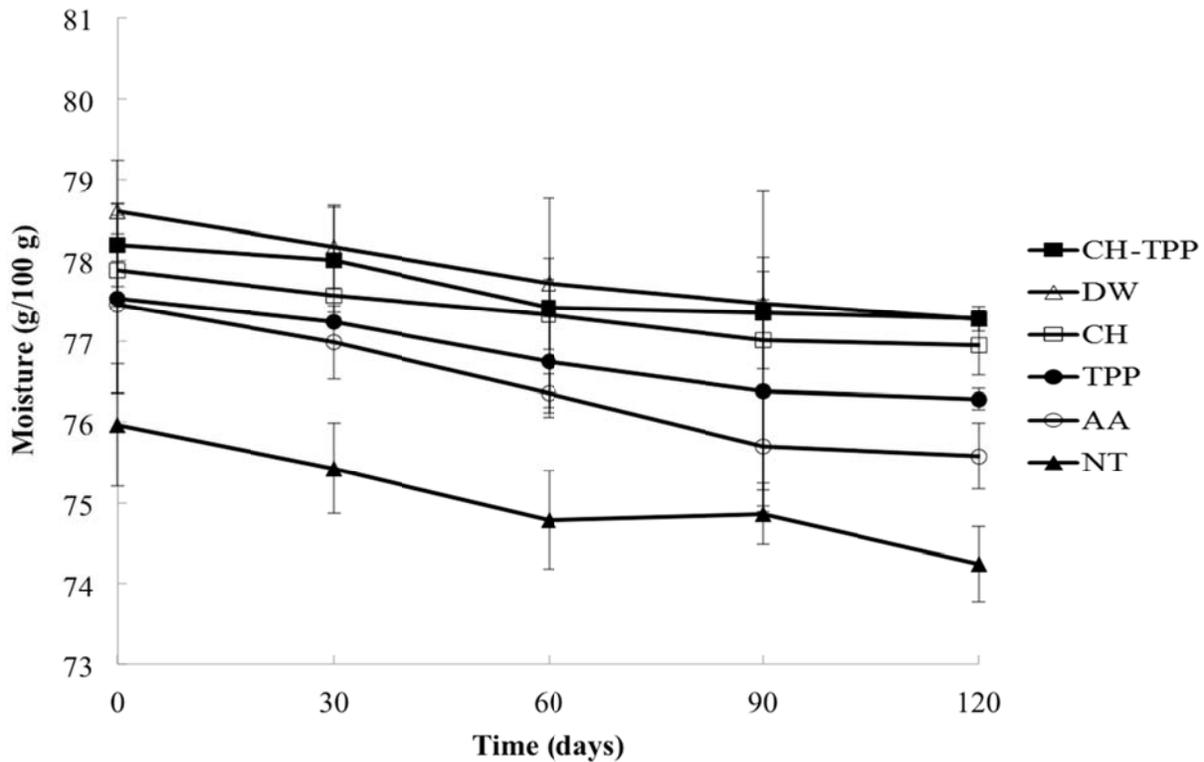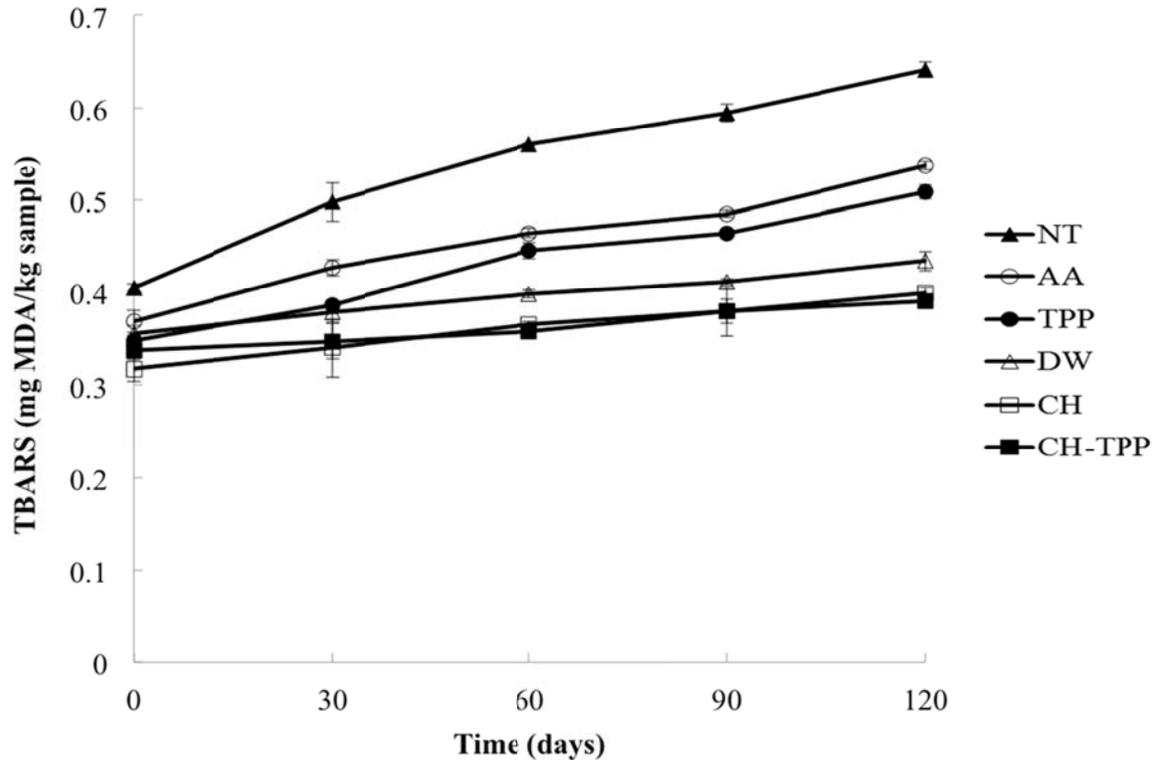Figure 3.10. TBARS of thawed shrimp during storage at -20 °C. CH = chitosan, CH-TPP = chitosan-sodium tripolyphosphate, TPP = sodium tripolyphosphate, AA = acetic acid, DW = distilled water, and NT = no treatment.

The use of edible coatings for meat, poultry, or fish can decrease oxygen transfer, provide

antioxidant activity, and add nutritional value, depending on the type and concentration of

coating that is applied (Gennadios, Hanna, & Kurth, 1997). Oxidation of the unsaturated lipids in

seafood during storage can lead to degradation of texture, flavor, and odor. Metal ions, such as

Fe^{2+}, hemin, Cu^{2+}, and Fe^{3+} can act as catalysts for oxidation. Water can have antioxidant effects

at very low levels by lowering the activity of metal catalysts and promoting recombination of

free radicals (Flick, Hong, & Knobl, 1992). According to Jacobsen, Timm, and Meyer (2001),

decreasing pH values of fish oil enriched mayonnaise yielded a pro-oxidative effect as a result of

greater metal ion catalytic activity. This may explain why TPP and AA treatments had higher TBARS than DW. Also, the oxidative stability during refrigerated storage of protein isolates extracted from Atlantic croaker (*Micropogonias undulates*) using acid-aided and alkali-aided processes was investigated by Kristinsson and Liang (2006). Acid-aided isolates showed low oxidative stability and had higher TBARS than the alkali-aided isolates, ground croaker muscle, and surimi that was formed from the same fish.

In addition to its free radical scavenging activity, CH can effectively chelate metal ions (Lin & Chou, 2004). Yang and Zall (1984) reported that CH had a higher metal chelating ability than other natural polymers procured from seafood wastes. (Dash et al., 2011). The sorption capacity of CH-TPP nanoparticles for lead ions was examined by Qi and Xu (2004). They found that the maximum sorption capacity of CH-TPP nanoparticles was high (around 395 mg/g). The amine and tripolyphosphoric groups of CH-TPP nanoparticles could facilitate lead ion chelation, with higher activities observed above pH 6.5, the pKa value of CH's amino group. Kamil, Jeon, and Shahidi (2002) evaluated the antioxidative activity of CH with different viscosities (14 cP, 57 cP, and 360 cP) in cooked comminuted herring. All CH treated herring samples showed lower peroxide values and total volatile aldehyes compared to untreated samples. The low viscosity CH (14 cP) was found to have the greatest antioxidative effect. The antioxidant effect of CH's with different viscosities in cooked comminuted fish model systems could be affected by the molecular weight of the polymer, which may be able to regulate chelation of metal ions. The NH_3^+ groups of CH are able to increase hydrodynamic volume by extended chain conformation through intramolecular electric repulsive forces. This may explain why lower viscosity (low molecular weight) CH displayed superior chelation properties, compared to high viscosity CH. In a study conducted by Sathivel et al. (2007), it was found that glazing skinless pink salmon filets

with a 1% w/w CH solution delayed lipid oxidation and elicited a higher thaw yield than in the non-glazed control after frozen storage for eight months.

3.4.10. Color and texture of thawed shrimp

The initial L^* value of NT was significantly lower than the other treatments (Table 3.6). At 120 days of storage, there were no significant differences between treatments. From day 0 to day 120, only the L^* value of NT increased significantly within its respective treatment. Moisture migration and recrystallization of ice crystals in food can cause drip loss (Zaritzky, 2000). Due to the significant decrease in moisture content of NT during storage time (as shown in Figure 3.9), the increase in L^* value may be related to drip loss. According to Woelfel, Owens, Hirschler, Martinez-Dawson, and Sams (2002), higher L^* values in broiler meat were correlated with greater drip loss.

There were no significant differences in a^* values between treatments at the initial storage period. At 120 days of storage, a^* values between CH, CH-TPP, TPP, and AA treatments were not significantly different, although only those of CH and CH-TPP were lower than DW and NT ($P \leq 0.05$). NT had lower a^* values than the other treatments ($P \leq 0.05$). Within their respective treatment, from day 0 to day 120, a^* values of CH, CH-TPP, and AA were not significantly different, however TPP, DW, and AA values decreased ($P \leq 0.05$). Lipid oxidation in foods occurs during frozen storage over time. This can lead to color fading, possibly due to the degradation of astaxanthin, a carotenoid pigment found in crustaceans (Erickson & Hung, 2012).

Initially, there were no significant differences in b^* values between treatments. At 120 days of storage, the b^* value of CH treatment was similar to CH-TPP, both of which were lower than TPP, AA, and NT ($P \leq 0.05$). The b^* value of CH treatment was also similar to DW. Additionally, the value of DW treatment was similar to TPP and lower than AA, while TPP was

similar to AA (P ≤ 0.05). NT had the highest b^* value among all treatments (P ≤ 0.05). Within their respective treatment, CH and CH-TPP were the only treatments to not experience a significant increase in their b^* values over the duration of storage.

Table 3.6. Color of thawed shrimp during storage at -20 °C.

	Treatment	Time (days)				
		0	30	60	90	120
L^*	CH-TPP	57.58±1.37[aA]	54.29±2.72[aA]	57.11±3.05[aA]	57.76±0.98[aA]	56.45±0.95[aA]
	CH	54.80±0.45[aA]	53.39±2.23[aA]	55.28±0.96[aA]	55.58±1.87[aA]	55.39±1.43[aA]
	TPP	58.35±0.77[aA]	57.99±3.29[aA]	58.57±1.10[aA]	57.18±2.71[aA]	56.22±1.74[aA]
	AA	56.00±0.74[aA]	56.03±3.25[aA]	57.35±2.05[aA]	54.50±2.39[aA]	55.52±1.11[aA]
	DW	56.62±0.98[aA]	55.88±2.26[aA]	56.08±1.85[aA]	55.84±1.29[aA]	56.13±0.67[aA]
	NT	48.92±1.19[bB]	53.23±0.21[aA]	53.19±1.16[aA]	52.96±0.40[aA]	53.38±1.04[aA]
a^*	CH-TPP	2.65±0.42[aA]	2.55±0.28[aA]	2.22±0.55[aA]	2.16±0.19[aA]	2.09±0.05[aA]
	CH	2.63±0.54[aA]	2.35±0.59[aA]	2.16±0.36[aA]	2.19±0.72[aA]	2.04±0.05[aA]
	TPP	2.73±0.11[aA]	2.42±0.20[aA]	2.41±0.13[aA]	2.11±0.11[aA]	1.90±0.23[abB]
	AA	2.86±0.73[aA]	2.70±0.13[aA]	2.39±0.16[aA]	2.05±0.22[aA]	1.85±0.20[abA]
	DW	2.71±0.01[aA]	2.50±0.37[aA]	2.26±0.37[aA]	1.88±0.20[aA]	1.63±0.16[bB]
	NT	2.82±0.13[aA]	2.35±0.18[aA]	1.81±0.28[aB]	1.49±0.17[aBC]	0.95±0.08[cC]
b^*	CH-TPP	4.00±0.76[aA]	4.17±0.14[cA]	4.39±0.33[cA]	4.68±0.51[bA]	4.95±0.29[eA]
	CH	4.12±0.25[aA]	4.34±0.17[bcA]	4.69±0.45[bcA]	5.01±0.14[bA]	5.63±0.17[deA]
	TPP	4.60±0.54[aC]	5.17±0.51[abBC]	5.99±0.67[abcABC]	6.47±0.28[aAB]	6.78±0.30[bcA]
	AA	4.85±0.68[aB]	5.60±0.04[aB]	6.22±0.61[abAB]	6.59±0.30[aAB]	7.26±0.60[bA]
	DW	4.21±0.35[aB]	4.55±0.13[bcB]	4.77±0.55[bB]	5.30±0.35[bA]	6.19±0.48[dcA]
	NT	4.62±0.45[aC]	5.66±0.01[aB]	6.80±0.67[aB]	7.54±0.35[aAB]	8.66±0.24[aA]

L^*, a^*, and b^* are the degree of lightness to darkness, redness to greenness, and yellowness to blueness, respectively. [a-e]Means±SD with different letters within a column indicate significant difference (P ≤ 0.05). [A-C]Means±SD with different letters within a row indicate significant difference (P ≤ 0.05). CH = chitosan, CH-TPP = chitosan-sodium tripolyphosphate, TPP = sodium tripolyphosphate, AA = acetic acid, DW = distilled water, and NT = no treatment.

Tsironi, Dermesonlouoglou, Giannakourou, and Taoukis (2009) modeled the changes in b^* values of shrimp during frozen storage using a zero order reaction. It was suggested that the b^* values of shrimp could be used a quality index with low values indicating higher quality. During frozen storage, the yellowing of fish flesh has been associated with migration of

carotenoids to the subcutaneous fat layer, as well as lipid oxidation and the carbonyl-amine reaction (Lakshmanan, 2000).

Throughout the entire storage time, similar cutting force values were observed between treatments (Figure 3.11) (P ≤ 0.05). The cutting force values for all treatments, within their respective treatment, were not significantly different at any storage time. Slow freezing of seafood may result in moisture loss due to the puncturing of muscle by large ice crystals, leading to undesirable texture changes (Shenouda, 1980). Rapid freezing causes smaller ice crystal formation in plant and animal tissues compared to slow freezing, resulting in less structural damage to cell walls (Rahman, 2007).
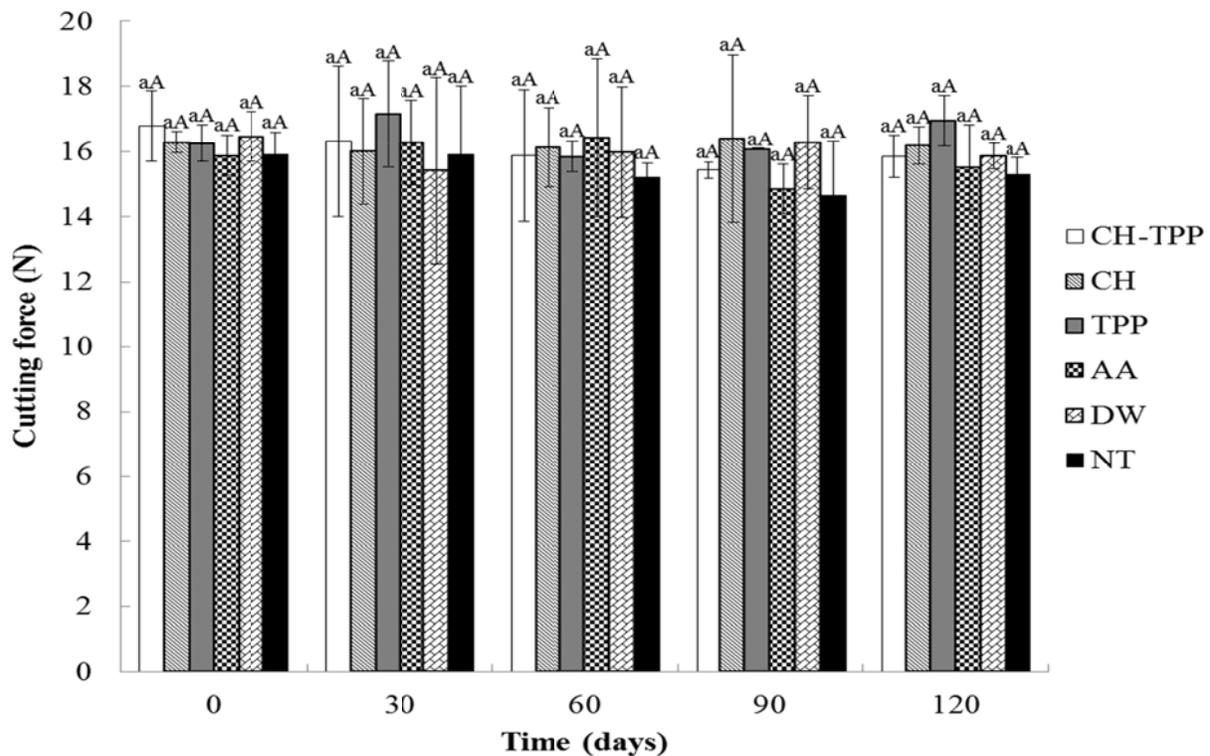
Figure 3.11. Cutting force of thawed shrimp during storage at -20 °C. [a]Means±SD with same letters between treatments at the same storage times are not significantly different (P ≤ 0.05). [A]Means±SD with same letters within treatments at different storage times are not significantly different (P ≤ 0.05). CH = chitosan, CH-TPP = chitosan-sodium tripolyphosphate, TPP = sodium tripolyphosphate, AA = acetic acid, DW = distilled water, and NT = no treatment.

Protein denaturation can cause changes in muscle texture that lead to quality loss (Zaritzky, 2000). Seafood that is rapidly frozen experiences lower protein denaturation rates than slow frozen products because of less time spent in the zone where a majority of ice crystal formation occurs (-1 to -2 °C) (Johnston, 1994). Shrimp that are quickly frozen and stored at low temperatures experience little drip loss compared to slowly frozen shrimp (Goncalves & Ribeiro, 2008b). Drip loss can alter product appearance, texture, juiciness, and incur nutrient losses (Zaritzky, 2000). In our study, the use of cryogenic freezing with liquid N_2 and storage at a low temperature (-20 °C) likely contributed to higher textural quality in all shrimp samples.

3.5. Conclusion

Processing with sonication and ultra-shearing could reduce the molecular weight of chitosan (CH) and particle sizes of CH and chitosan-sodium tripolyphosphate (CH-TPP). The particles in CH-TPP solution were nano-scale, while CH solution contained microparticles. CH and CH-TPP solutions exhibited fluid-like behavior, which may be good for processing. They also had higher free radical scavenging activities than the other treatment solutions. Vacuum tumbling with CH and CH-TPP solutions facilitated particle penetration into the shrimp. The particles were able to adhere onto shrimp muscle tissue, localizing mainly at the outer layers. Aerobic plate counts (APC) of CH and CH-TPP treated shrimp were lower compared to other treatments during the entire storage time. CH and CH-TPP were shown to be ineffective against yeasts and molds at the concentrations used. CH and CH-TPP treatments could preserve color, texture, and moisture content of shrimp during the entire storage time. In addition, CH and CH-TPP treatments had the highest reduction in lipid oxidation compared to other treatments at 120 days of storage at -20 °C. This study showed that a CH or CH-TPP solution, combined with

vacuum tumbling, can be effective at reducing APC and lipid oxidation in shrimp during frozen

storage, while maintaining desired physicochemical properties.

3.6. References

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CHAPTER 4. EFFECTS OF WATER-SOLUBLE CHITOSAN ON THE QUALITY CHARACTERISTICS OF CRYOGENICALLY FROZEN SHRIMP

4.1. Abstract

Chitosan (CH) is a polysaccharide present in the exoskeleton of crustaceans and cell walls of fungi. It can be dissolved in acids and forms relatively high viscosity solutions. CH can be partially hydrolyzed to reduce its molecular weight and achieve water solubility. Water-soluble chitosan (WSC) has been shown to possess antioxidant, antimicrobial, and antitumor activities and it may have greater application potential than CH in food due to its superior water solubility at neutral pH values. CH penetration into shrimp may be facilitated by vacuum tumbling with a low viscosity WSC solution. It is expected that this would reduce lipid oxidation and microbial loads. WSC was produced by enzymatic hydrolysis. Two treatment solutions were prepared: (1) a 0.1 M acetic acid (AA) solution and (2) a WSC in distilled water (DW) solution. Fresh shrimp meat was separately vacuum tumbled with the solutions, cryogenically frozen, and evaluated for quality characteristics under frozen storage. Fresh shrimp meat tumbled with DW and fresh shrimp meat without tumbling (NT) were used as controls. Shrimp treated with WSC had lower aerobic plate counts (APC) and yeast and mold counts (YMC) compared to other treatments after 120 days of storage at -20 °C. WSC treated shrimp retained their color, texture, and moisture contents. Additionally, WSC produced the highest reduction in lipid oxidation compared to other treatments. This study showed that a WSC solution, combined with vacuum tumbling, can be effective at reducing APC, YMC, and lipid oxidation in shrimp during frozen storage, while maintaining desired physicochemical properties.

Keywords: water-soluble chitosan, shrimp, freezing, vacuum tumbling

4.2. Introduction

CH is a non-toxic biopolymer composed of β-(1-4)-linked 2-amino-2-deoxy-glucopyranose and 2-acetamido-2-deoxy-β-D-glucopyranose. It has good biodegradability, immunostimulating effects, and high mucoadhesive properties, allowing for usage in films, tablets, matrices, as well as the formulation of micro and nano particles (Rampino, Borgogna, Blasi, Bellich, & Cesaro, 2013). One of the main advantages of CH is its antimicrobial properties. The mode of action for these properties is related to electrostatic binding of positively charged CH to cationic bacterial cell surfaces, allowing for the disruption of bacterial metabolism. At low molecular weights (less than ~ 5 kDa) CH can adsorb to DNA molecules, blocking the transcription of RNA from DNA (Benhabiles et al., 2012). CH also possesses antioxidant activities that allow for free radical scavenging (Xie, Xu, & Liu, 2001) and chelation of metal ion catalysts (Yen, Yang, & Mau, 2008). Although CH has vast potential in many fields, because of its water insolubility at neutral pH values, uses may still be limited in some areas (Ilyina, Tatarinova, & Varlamov, 1999; Xie et al., 2001), such as in food, health, agriculture (Roncal, Oviedo, de Armentia, Fernandez, & Villaran, 2007), and biomedical applications (Snyman, Hamman, Kotze, Rollings, & Kotze, 2002). In addition, the large particle size (Qi, Xu, Jiang, Hu, & Zou, 2004) and high viscosity (Jo, Lee, Lee, & Byun, 2001) of CH in solution may limit its penetration into shrimp muscle tissues.

Reduction of particle size to sub-micron levels can allow for deep penetration into tissues through fine capillaries and fenestration in the epithelial lining (Gan, Wang, Cochrane, & McCarron, 2005). CH can be partially hydrolyzed to achieve water solubility by obtaining shorter chain lengths with more free amino groups in D-glucosamine units as a result of a decrease in molecular weight (Qin et al., 2003). Chemical or enzymatic methods may be used to

prepare low molecular weight CH (Akiyama, Kawazu, & Kobayashi, 1995). Unlike enzymatic methods, the chemical procedure involves harsh hydrolysis conditions, low product yields, and chemical modifications, such as formation of carboxyl groups and deamination (Qin, Du, & Xiao, 2002). Enzymatic hydrolysis may be suitable for large scale production of low molecular weight CH which retains its native biological properties due to the higher specificity of enzymes and use of milder conditions (Yalpani & Pantaleone, 1994). Water-soluble low molecular weight CH has been shown to have significant biological activities, including antimicrobial (No, Park, Lee, & Meyers, 2002; Vishu, Varadaraj, Gowda, & Tharanathan, 2005; Zheng & Zhu, 2003) and antitumor activity (Qin, Du, Xiao, Li, & Gao, 2002; Seo et al., 2000), as well as the ability to slow the progression of diabetes mellitus (Kondo, Nakatani, Hayashi, & Ito, 2000). It also has potential as a DNA delivery system (Richardson, Kolbe, & Duncan, 1999). Water-soluble chitosan (WSC) has a wide array of antioxidant properties, including DPPH, hydrogen peroxide, and superoxide anion radical scavenging activities, as well as Cu^{2+} ion chelating ability (Lin & Chou, 2004). Additionally, WSC can scavenge hydroxyl radicals (Xie, Xu, Wang, & Liu, 2002).

Vacuum tumbling can improve tenderness, decrease cooking losses, and enhance the water holding capacity of meat (Rejt, Kubicka, & Pisula, 1978). It also facilitates mass transfer in meat by increasing solution uptake through the pores (Deumier, Trystram, Collignan, Guedider, & Bohuon, 2003). CH penetration into shrimp may be facilitated by vacuum tumbling with a low viscosity WSC solution. The objective of this study was to evaluate the effects of vacuum tumbling with a WSC solution on the quality characteristics of cryogenically frozen shrimp.

4.3. Materials and methods

4.3.1. Preparation of water-soluble chitosan and treatment solutions

Water-soluble chitosan (WSC) was prepared by enzymatic hydrolysis of chitosan (CH) with chitosanase from *Streptomyces* sp. N174 (EMD Millipore Corp., Billerica, MA) using an enzyme concentration of 100 U/mL according to the method described by Kuroiwa et al. (2009) with some modifications. Medium molecular weight CH was purchased from Sigma Aldrich (St. Louis, MO). Reagent-grade acetic acid (AA) was obtained from EMD Chemicals Inc. (Gibbstown, NJ). WSC was prepared using the process shown in Figure 4.1.
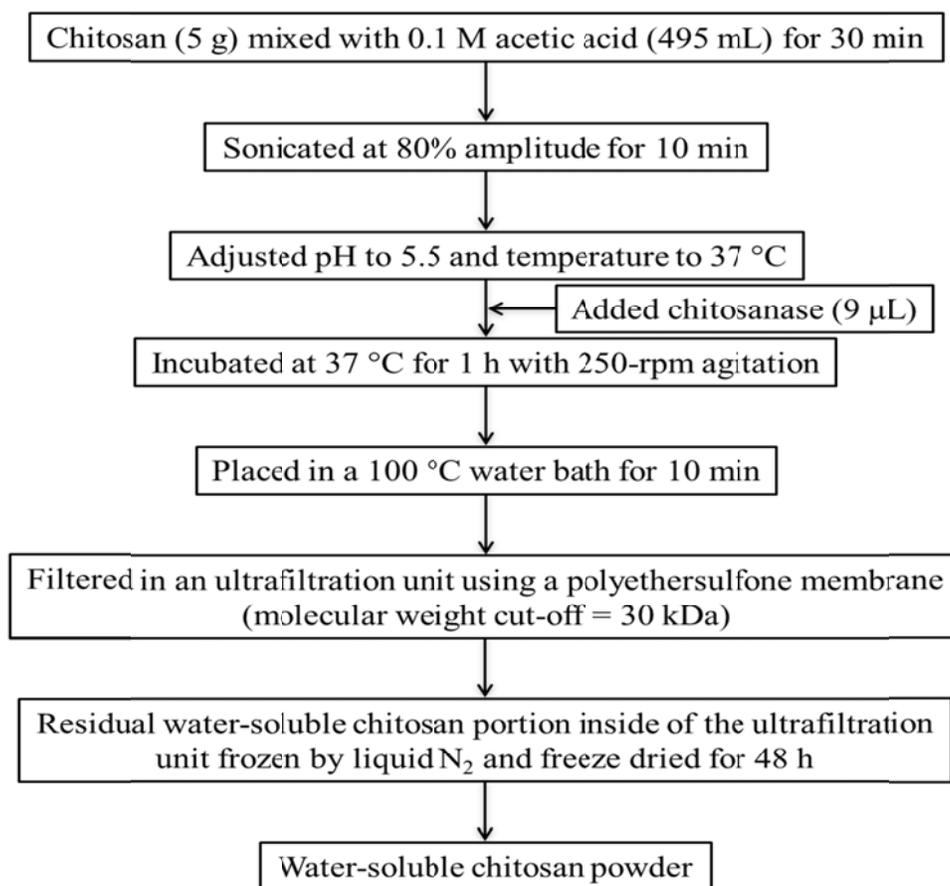
Figure 4.1. Flowchart of water-soluble chitosan preparation.

CH (5 g) was dissolved in 495 mL of 0.1 M AA for 30 min at room temperature. The CH solution was then sonicated for 10 min in an ice bath at 4 °C with amplitude of 80% and pulser set to 2 sec using an ultrasonic processor (Model WU-04711-70, Cole-Parmer Inc., Vernon, IL, USA) fitted with a 22 mm tip diameter. The pH of the CH solution was measured with a VWR Symphony SB70P pH meter (VWR Scientific, Singapore) and adjusted to 5.5 using NaOH. The CH solution was poured into a 1 L reactor and heated to 37 °C prior to the addition of 9 µL chitosanase. The reactor was then placed in an incubator shaker (Lab line incubator shaker model 3525, Fisher Scientific Inc., Pittsburgh, PA), where enzymatic hydrolysis proceeded for 1 h at 37 °C, with constant stirring at 250 rpm. After one hour, the reaction was terminated by placing the mixture in a 100 °C water bath for 10 min.

The hydrolyzed CH solution was cooled and then filtered in an ultrafiltration unit (Model 8400, Millipore Co., Bedford, MA) using a polyethersulfone membrane (Millipore Co., Bedford, MA) with a molecular weight cut-off of 30 kDa to separate WSC from low molecular weight water-soluble chitosan (LMWWSC). A S46725 Barnstead/Thermolyne Cimarec 2 magnetic stirring table (Barnstead/ Thermolyne, Dueuque, IA) was used to contain the ultrafiltration unit and a nitrogen pressurization system operating at 14 psi was utilized. After filtration, the residual WSC portion inside of the ultrafiltration unit was frozen with liquid nitrogen and dried in a freeze dryer (Heto PowerDry LL3000, Laurel, Maryland) for 48 h. After drying, the WSC was ground in a Waring 51BL32 commercial blender (Torrington, Connecticut) to obtain WSC powder. Two treatment solutions were prepared for this study: (1) a 0.1 M AA solution in distilled water (DW), and (2) a 0.5% WSC solution in DW. The solutions were mixed for 30 min at room temperature. DW was compared with other treatment solutions for pH analysis and free radical scavenging activity.

4.3.2. Vacuum tumbling with treatment solutions and freezing of shrimp

The shrimp processing and storage methods are shown in Figure 4.2. Peeled fresh white shrimp meat was separately tumbled with treatment solutions (WSC, AA, or DW) at a 1:1 ratio by weight in a 2270 g capacity Reveo MariVac vacuum tumbler (Eastman Outdoors, Flushing, MI) in a cold processing room (4 °C) for 10 min.
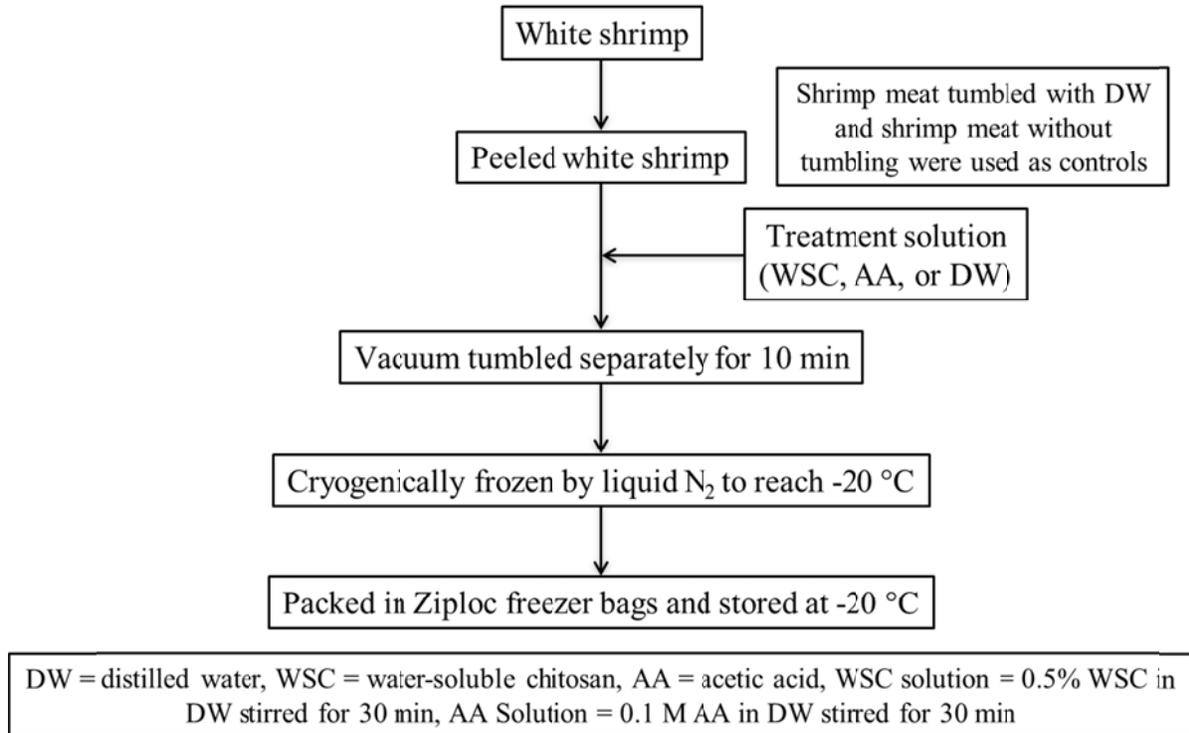
Figure 4.2. Flow diagram of shrimp processing and storage.

Fresh shrimp meat tumbled with DW, as well as fresh shrimp meat without tumbling were used as controls. After tumbling, excess solution was allowed to drain for 30 sec. The shrimp were then frozen with liquid nitrogen using a cabinet type cryogenic freezer (Air Liquide, Houston, TX, USA). During cryogenic freezing, thermocouples attached to a data logger (Comark, Comark Ltd. Stevenage, Herts, UK) were embedded into the middle of the second abdominal segment of the shrimp to record temperature changes until a core temperature of -20 °C was reached. Frozen shrimp samples were packed into one quart Ziploc freezer bags (SC

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Johnson, Racine, WI) and stored at -20 °C for 120 days, undergoing physicochemical analysis

every 30 days and microbiological analysis in 60 day intervals. Frozen shrimp was thawed at 4

°C for 22 h prior to analysis.

4.3.3. Molecular weight, degree of deacetylation, and water solubility of chitosan

CH (5 g) was dissolved in 495 mL of 0.5 M AA/0.5 M sodium acetate (NaAc) for 30 min

to produce CH in AA/NaAc solution. The CH in AA/NaAc solution, as well as WSC in

AA/NaAc solution, prepared by dissolving 2.5 g WSC in 497.5 mL of 0.5 M AA/0.5 M NaAc,

was analyzed for intrinsic viscosity $[\eta]$. To remove insoluble materials the solutions were passed

through Whatman #4 filter paper. Then 7 mL of known concentrations (0.1-0.5%) of the

solutions were individually placed in a Cannon-Fenske routine viscometer (Cannon-Fenske, No.

100). The flow time of the solutions through the viscometer capillary in a 25 °C constant-

temperature water bath were recorded as seconds in order to calculate $[\eta]$.

$$\eta_{rel} (\text{Relative viscosity}) = \frac{t_{solution(efflux\ time\ of\ solution)}}{t_{solvent\ (efflux\ time\ of\ solvent)}} \quad\quad (4.1)$$

$$\eta_{sp} (\text{Specific viscosity}) = \eta_{rel} - 1 \quad\quad (4.2)$$

$$\eta_{inh} (\text{Inherent viscosity}) = \frac{(Ln\ \eta_{rel})}{C} \quad\quad (4.3)$$

$$\eta_{red} (\text{Reduced viscosity}) = \frac{\eta_{sp}}{C} \quad\quad (4.4)$$

$$[\eta] = \lim_{c \to 0} \frac{\eta_{sp}}{c} \equiv \lim_{c \to 0} c^{-1} \ln \eta_{rel} \quad\quad (4.5)$$

Where C = Concentration of CH or WSC in AA/NaAc solution (g/dL, %)

η_{inh} and η_{red} were plotted on a graph and $[\eta]$ (dL/g) was obtained from the common intercept of

both plots on the ordinate at C = 0. η_{red} vs concentration data was extrapolated to zero

concentration to acquire $[\eta]$. The Mark Houwink equation was used to calculate the viscosity-

average molecular weight (M_v) of CH or WSC in solution based on the relationship between molecular weight and $[\eta]$.

Mark Houwink equation: $[\eta] = K(M_v)^a$ (4.6)

Where K and a are constants for various solute-solvent systems at different temperature, ionic strength, pH, degree of deacetylation (DD), and molecular weight range. The values K and a for this analysis were $199.0 * 10^{-5}$ and 0.59, respectively (Kasaai, 2007).

The CH particles in CH solution before processing with sonication and the WSC particles in WSC solution were analyzed for DD. CH and WSC solutions were prepared according to the method described in section 4.3.1. The solutions were then freeze dried for 48 h. DD of CH and WSC was determined using the colloid titration method described by Toei and Kohara (1976). Freeze dried CH or WSC (0.5 g) was dissolved in formic acid (5% v/v). CH or WSC in formic acid solution (1 g) was mixed with 29 mL DW in an erlenmeyer flask. Three drops of 0.1% w/v toluidine blue indicator (Sigma Aldrich, St. Louis, MO) were added and the solution was titrated with n/400 potassium polyvinyl sulfate (PVSK), obtained from Wako Pure Chemical Industries, Ltd., Japan, until a pink color was visible. A single deacetylated amino group of CH or WSC reacts with one molecule of PVSK. The molar ratio of deacetylated amino groups in the CH or WSC molecule was used to calculate DD based on the volume of PVSK solution that was utilized. The following equation was used to calculate DD.

$$DD\ (\%) = \left[\frac{X/161}{X/161 + Y/203} \right] * 100 \qquad (4.7)$$

Where X (amount of glucosamine in molecule) $= 1/400 * 1/1000 * f * 161 * V$

Y (amount of N-acetylglucosamine in molecule) $= 0.5 * 1/100 - X$

V: Titrated volume of n/400 PVSK; f: Factor of PVSK solution $= 1.01$

The water solubility of CH and WSC was determined according to the method described by Cano-Chauca, Stringheta, Ramos, and Cal-Vidal (2005) with some modifications. CH or WSC (0.5 g) was mixed with 50 mL DW for 24 h at 150 rpm and 25 °C using an incubator shaker. The mixture was centrifuged using a Beckman Coulter Allegra 25R (Beckman Coulter, Inc., Schaumburg, IL) at 5000 rpm for 5 min. An aliquot of the supernatant (25 mL) was transferred into a pre-weighed aluminum dish and oven-dried for 24 h at 105 °C. The weight difference was used to calculate solubility (%).

4.3.4. Rheological properties of water-soluble chitosan solution

The viscosity, flow behavior, and viscoelastic properties of WSC solution was analyzed using an AR 2000 ex rheometer (TA Instrument, New Castle, DE) with fitted plate geometry (a metal plate of 40 mm in diameter), using Universal Analysis software. A 200 μm gap between the metal plate and the rheometer plate was used to determine the flow behavior of WSC solution. The shear stress was measured at shear rates from 0 to 1000 s^{-1}. The flow behavior was modeled by the Power Law model using Eq. 4.8.

$$\sigma = K\dot{\gamma}^n \qquad (4.8)$$

Where σ is shear stress (Pa), K is the consistency index (Pa.s^n), $\dot{\gamma}$ is shear rate (s^{-1}), and n is the flow behavior index. A plot of log σ versus log $\dot{\gamma}$ was produced from logarithms of σ and $\dot{\gamma}$. A scatter plot was constructed and the magnitude of log K (y intercept) and n (slope) was determined. At a shear rate of 1000 s^{-1}, the viscosity of WSC solution was obtained. The elastic modulus (G′) and viscous modulus (G″) of WSC solution was determined using frequency sweep tests. Frequencies between 0.001 and 0.75 Hz were used, as well as a 200 μm gap between plates. For the flow behavior and frequency sweep tests, the solutions were analyzed at 25 and 5 °C.

4.3.5. Particle size and total reducing sugar content of chitosan solutions

Particle size of CH and WSC particles in solution was determined by dynamic light scattering using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd.,Worcestershire, U.K.). Approximately 0.5 mL of CH or WSC solution was placed in spectrophotometer cuvettes for the analysis. Total reducing sugar (TRS) content of CH solution before and after enzymatic hydrolysis, as well as the WSC and LMWWSC portions obtained after ultrafiltration, were determined according to the Schales method with slight modification (Imoto & Yagishita, 1971). A reagent solution was prepared by dissolving 0.5 g of reagent grade potassium ferricyanide (Fisher Science Education, Nazareth, PA) in 1 L of 0.5 M sodium carbonate (Sigma Aldrich, St. Louis, MO). Subsequently, 1.5 mL of sample solution was mixed with 2 mL of the reagent solution in a capped test tube. After mixing, the tube cap was loosened and the sample was heated in boiling water for 15 min. After heating, the sample solution was cooled and centrifuged at 10000 rpm for 10 min with an Eppendorf 5417C centrifuge (Beckmann Instruments Inc., Westbury, NY). The absorbance was measured at 420 nm using a spectrophotometer (Genesys 20, Thermo Fisher Scientific, Fair Lawn, NJ). D-glucosamine, obtained from City Chemicals, LLC. (West Haven, CT), was used as the reference compound. A standard curve was constructed using concentrations of glucosamine from 0-0.13 mg/mL. The TRS content of the treatment solutions was expressed as mg glucosamine/g CH.

4.3.6. Deconvolution microscopy images of fluorescently labeled chitosan particles in solution and cross-sections of vacuum tumbled shrimp

WSC was fluorescently labeled according to the method described by Qaqish and Amiji (1999) with some modifications. One gram of WSC was dissolved in 99 mL of 0.1 M AA for 1 h. To the solution, 100 mL of methanol (EMD Chemicals Inc., Gibbstown, NJ) was added slowly and with continuous stirring to obtain WSC-methanol solution. Fluorescein isothiocyanate F7250

isomer 1 (FITC) from Sigma Aldrich (St. Louis, MO) was dissolved in methanol at 1 mg/mL and then added slowly to the WSC-methanol solution. The mixture was incubated in the dark at room temperature for 1 h to allow the primary amine group of D-glucosamine residue to react with the isothiocyanate group of FITC. After incubation, 0.1 M NaOH was used to precipitate the FITC labeled WSC (FITC-WSC). The precipitate was washed with DW and separated by centrifuging until the supernatant contained no detectable fluorescence, as measured with a Wallac Victor^2 microplate reader (PerkinElmer Inc., Shelton, CT). The FITC-WSC was then freeze dried for 48 h and ground in a commercial blender to obtain FITC-WSC powder. A solution of 0.5% FITC-WSC powder in 0.1 M AA was prepared.

Peeled fresh white shrimp meat was tumbled with FITC-WSC solution and cryogenically frozen as described in section 4.3.8. Prior to microscopy analysis, cross-sections of frozen shrimp were cut 2-3 mm thick with a double sided razor blade using a cryostat (Leica Microsystems, Wetzlar, Germany). The samples were transferred to microscopy slides and fixed using ethyl alcohol. Then they were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) with the addition of a cover slip. FITC labeled WSC particles, embedded in the outer and inner muscle tissue of the shrimp, were analyzed with a Leica DM RXA2 fluorescent microscope (Leica Microsystems, Wetzlar, Germany), using deconvolution software. Micrographs of FITC labeled particles in solution, as well as in shrimp samples, were captured using a green fluorescent protein (GFP) filter.

4.3.7. pH and free radical scavenging activity of treatment solutions

The pH of treatment solutions (section 4.3.1) was measured with a pH meter (VWR Scientific, Singapore). Free radical scavenging activity of treatment solutions was determined according to a method described by Kim and Thomas (2007). 2,2-diphenyl-1-picrylhydrazyl

(DPPH) was obtained from Sigma Aldrich (St. Louis, MO) and a 0.2 mM concentration was prepared. Then 1 mL of each treatment solution was separately added into 1 mL of DPPH in a test tube. A blank was prepared by adding 1 mL of ethyl alcohol into 1 mL DPPH. The mixtures were vortexed and incubated for 30 min at room temperature in the dark. The absorbance of the solutions was read at 517 nm with a spectrophotometer. The DPPH radical scavenging capacities of the treatment solutions were determined from the difference in absorbance between the samples and blank. The results were expressed as % DPPH inhibition.

4.3.8. Microbial counts of thawed shrimp

Aerobic plate counts (APC), yeast and mold counts (YMC), and total coliform counts (TCC) were quantified using 3M Petrifilms (3M Microbiology, St. Paul, MN) according to manufacturer's instructions with some modification. Whole thawed shrimp samples were placed in 18 oz Whirl-Pak bags, from Weber Scientific (Nasco, Fort Atkinson, WI), and diluted to 10^{-1} concentration with 0.85% NaCl solution. The shrimp samples in the Whirl-Pak bags were homogenized using a Stomacher lab-blender type 400 (Tekman Co., Cincinnati, Ohio) for 2 min. Duplicated serial dilutions of shrimp samples in 0.85% NaCl solution were produced and separately plated on Petrifilms for APC, YMC, and TCC. Using a flat surface, 1 mL of shrimp dilution was placed on the bottom film and the inoculums were covered with the top film and spread using the provided 3M plastic spreaders. APC Petrifilms were incubated for 48 h at 35 °C and the red colonies were counted. For YMC Petrifilms, the blue-green colonies were counted after incubation for 72 h at 22 °C, while TCC Petrifilms were incubated for 24 h at 35 °C and counted for red gas forming colonies. APC, YMC, and TCC were done using the naked eye.

4.3.9. pH and moisture content of thawed shrimp

The pH of the shrimp was analyzed according to the method described by Sundararajan et al. (2011) with some modifications. Ten grams of shrimp sample was homogenized in a commercial blender with 40 mL DW for 15 sec. The mixture was then poured into a 100 mL beaker and sonicated for 1 min at 4 °C with amplitude of 82% and pulser set at 2 sec. The pH of the homogenized sample was measured with a pH meter. The moisture content of the thawed shrimp samples was determined according to AOAC (1995). Moisture content % was calculated as [(weight of initial sample-weight of dry sample)/initial weight of sample]*100.

4.3.10. Lipid oxidation of thawed shrimp

The degree of lipid oxidation present in shrimp during frozen storage was determined by TBARS (thiobarbituric-acid-reactive-substances) analysis according to the method described by Lemon (1975) with slight modification. All of the reagents utilized for the TBARS analysis were obtained from Sigma Aldrich (St. Louis, MO). Thiobarbituric acid (TBA) solution (0.02 M) was prepared by dissolving 1.4415 g TBA in 500 mL DW. An extraction solution was prepared by dissolving 7.5% trichloroacetic acid, 0.1% propyl gallate, and 0.1% ethylenediaminetetraacetic acid in 500 mL DW. The solutions were covered in aluminum foil and mixed overnight. Fifteen grams of thawed shrimp was homogenized with 30 mL extraction solution in a commercial blender for 30 sec and filtered using Whatman No. 1 filter paper. In a capped test tube, 5 mL of filtrate was added into 5 mL of TBA solution and then vortexed. The tube cap was loosened and the tube placed in boiling water for 40 min. After heating, the sample solution was cooled and centrifuged at 10000 rpm for 10 min. The absorbance of the solution was read at 530 nm with a spectrophotometer. TBARS values were determined from a standard curve of 1,1,3,3

tetraethoxypropane in concentrations of 0.0-0.01 μmoles/mL. The malondialdehyde (MDA) content was expressed as mg MDA/kg sample.

4.3.11. Color and texture of thawed shrimp

The surface color of thawed shrimp was measured in triplicate with a chroma meter LABSCAN XE (Hunterlab, VA) fitted with a pulsed xenon lamp and an aperture diameter of 13 mm. Whole shrimp were placed in a sample tray and fitted into the aperture to record the surface color. CIELAB color scales were used to express the results. The *L** values correlate with the degree of lightness to darkness, *a** values assess the degree of redness to greenness, and the *b** values measure the extent of yellowness to blueness. Texture of the thawed shrimp was measured according to the method described by Thongphitak, Limsuwan, Chuchird, Raksakulthai, and Pansawat (2007). An Instron Universal testing device (Model 5544, Norwood, MA) equipped with a Warner-Blatzer blade and a 25-kg load cell was used. The instrumental settings and operations were determined using Bluehill Materials testing software (Bluehill 3, version 3.13, 2010, Instron). The thawed shrimp was cut transversely at the third abdominal segment with a speed of 2 mm per second and distance of 25 mm to determine the maximum cutting force (N).

4.3.12 Statistical analysis

Data was statistically analyzed using SAS software version 9.4 (SAS Institute Inc., Cary, NC). Mean values of triplicate analysis were reported with their standard deviations. To determine differences between treatments and within treatments during the storage time, Analysis of Variance (ANOVA) and Tukey's studentized range test was used at a significance level of $P \leq 0.05$.

4.4. Results and discussion

4.4.1. Molecular weight, degree of deacetylation, and water solubility of chitosan

The viscosity-average molecular weight (M_v) of chitosan (CH) (375.23±33.61 kDa) was significantly higher than that of water-soluble chitosan (WSC) (115.57±6.91 kDa). The degree of deacetylation (DD) of WSC (61.18±1.19%) was significantly lower compared to CH (70.11±1.30%) (Table 4.1). Also, the water solubility of WSC (89.5±1.28%) was significantly higher than CH (0.95±0.06%).

Table 4.1. Molecular weight, degree of deacetylation, and water solubility of chitosan.

Sample	M_v (kDa)	DD (%)	Solubility (%)
CH	375.23±33.61[a]	70.11±1.30[a]	0.95±0.06[b]
WSC	115.57±6.91[b]	61.18±1.19[b]	89.5±1.28[a]

[a,b]Means±SD with different letters within a column indicate significant difference ($P \leq 0.05$). CH = chitosan and WSC = water-soluble chitosan.

Enzymatic hydrolysis of CH has been shown to yield CH with lower molecular weight and increased water solubility (Qin et al., 2003). Unlike enzymatic methods, the chemical procedure involves harsh hydrolysis conditions, low product yields, and chemical modifications, such as formation of carboxyl groups and deamination (Qin, Du, & Xiao, 2002). Enzymatic hydrolysis may be suitable for large scale production of low molecular weight chitosan which retains its native biological properties, due to the higher specificity of enzymes and use of milder conditions (Yalpani & Pantaleone, 1994).

CH with different molecular weights can be fractioned using an ultrafiltration membrane, making it possible to separate CH into low, medium, and high molecular weight fractions (Jeon, Park, & Kim, 2001). Jeon et al. (2001) observed that for significant inhibition of microorganisms, a CH molecular weight of around 10 kDa or greater was required, suggesting

that a minimum degree of polymerization is required for effective antimicrobial activity. As described in section 4.3.1, hydrolyzed CH was separated into WSC and low molecular weight water-soluble chitosan (LMWWSC) using an ultrafiltration unit and a membrane with a molecular weight cut-off of 30 kDa. The decrease in DD for WSC, compared to CH may have been due to filtering out the majority of monosaccharides and oligosaccharides in the solution, which typically have molecular weights of up to 10 kDa (Kim & Rajapakse, 2005; Xia, Liu, Zhang, & Chen, 2011). This may have reduced the number of available amine groups that could be detected during the DD analysis.

CH is water-insoluble because phase separation occurs in CH solutions with pH values of greater than 6.5, which is the approximate pKa value of CH's amino group. CH retains a positive charge in solutions that have pH values lower than around 6.5. The majority of the amino groups of CH should be protonated at pH values lower than 4. This contributes to enhanced swelling of the polymer network due to greater electrostatic repulsion between charged groups of the same sign at low pH values (Nystrom, Kjoniksen, & Iversen, 1999). CH can be partially hydrolyzed to achieve water solubility by obtaining shorter chain lengths with more free amino groups in D-glucosamine units as a result of a decrease in molecular weight (Qin et al., 2003). Lowering the molecular weight of CH alters the content of N-acetylglucosamine units, resulting in different CH conformations (Kubota, Tatsumoto, Sano, & Toya, 2000). Also, a decrease in intermolecular attraction forces, such as van der Waals forces and hydrogen bonds, can explain the high solubility of low molecular weight CH (Qin et al., 2003).

4.4.2. Rheological properties of water-soluble chitosan solution

At 25 and 5 °C, WSC solution exhibited shear-thinning (pseudoplastic) characteristics, as made apparent by its flow behavior index values (*n*), which were less than 1 (Table 4.2) (Bjorn,

Karlsson, Svensson, Ejlertsson, & de La Monja, 2012). In addition, our results show that K (consistency index) and apparent viscosity values of WSC solution were similar at both temperatures (25 and 5 °C) within their respective analysis (P ≤ 0.05).

Table 4.2. Flow characteristics of water-soluble chitosan solution at 25 and 5 °C.

Temperature	n	K ($\times 10^{-3}$ Pa s^n)	Apparent viscosity ($\times 10^{-3}$ Pa s)
25 °C	0.99±0.03[a]	3.93±0.39[a]	3.64±0.40[a]
5 °C	0.96±0.02[a]	5.66±0.43[a]	4.23±0.09[a]

[a]Means±SD with same letters within a column are not significantly different (P ≤ 0.05). K = consistency index and n = flow behavior index.

Nystrom et al. (1999) reported that at low concentrations and shear rates, WSC had Newtonian behavior, but when the shear rate increased, a shear thinning (pseudoplastic) phenomenon was observed. This occurred because at higher shear rates, shear forces can disrupt the polymer network, thus reducing the quantity of interchain bonds and lowering the resistance to flow and viscosity. Also, the hydrophobicity of CH had an effect on viscosity enhancement, showing greater viscosity enhancement with increasing hydrophobicity. Two distinct viscosity regions were reported by Hwang and Shin (2000) with regard to the flow behavior of CH solutions: Newtonian and power-law flow regions. The Newtonian flow region can be observed through the maintenance of a constant viscosity at low shear rates. This occurs because the effect of exerted shear force on the rate of intermolecular disentanglements is similar to that of newly formed entanglements. When the shear rate increases, the power-law flow region can be observed due to a higher rate of disentanglements than entanglements, leading to a lower viscosity. The pseudoplasticity of solutions with higher CH concentrations is the result of restricted movement of individual polymer chains and a higher number of entanglements.

WSC solution was shown to have higher loss modulus (G″) than storage modulus (G′) at

25 and 5 °C, which is typical of fluid-like solutions (Figure 4.3). A higher G″ than G′ was reported by Arancibia et al. (2015) for a 1% CH in 0.15 M lactic acid solution at temperatures below 45 °C. The rheological properties of CH solutions yield practical information for process optimization. Because WSC solution exhibited fluid-like behavior, it may be good for processing.
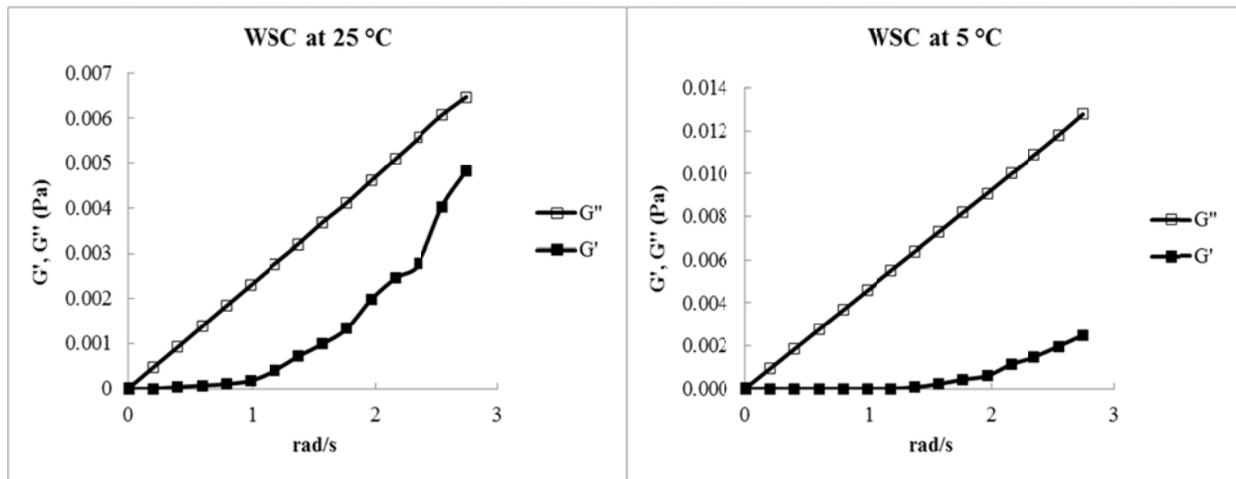

Figure 4.3. Frequency sweeps of water-soluble chitosan solution at 25 and 5 °C: storage modulus (G′) and loss modulus (G″) vs. angular frequency (rad/s). WSC = water-soluble chitosan.

4.4.3. Particle size and total reducing sugar content of chitosan solutions

Particle size of WSC solution (756.53±27.81 nm) was significantly lower than that of CH before enzymatic hydrolysis (CH/BH) solution particles (5066.00±154.15 nm) (Table 4.3). As the study was mainly focused on particle size in CH and WSC solution before and after enzymatic and ultrafiltration, particle size in CH solution after enzymatic hydrolysis (CH/AH) and LWMWSC were not examined. The total reducing sugar (TRS) content of CH after enzymatic hydrolysis (CH/AH) solution (189.10±3.23 mg glucosamine/g CH) was significantly higher compared to CH/BH solution (54.23±1.11 mg glucosamine/g CH). TRS content of LMWWSC solution (153.46±1.29 mg glucosamine/g CH) was greater than WSC solution (74.78±1.17 mg glucosamine/g CH) (P ≤ 0.05).

In our study, CH/BH was sonicated prior to enzymatic hydrolysis because at low acid concentrations (<1% v/v), complete dissolution of CH is unlikely. CH molecule degradation by ultrasonic radiation is largely caused by the cavitation effect. This can temporarily disperse aggregates, permanently break a polymer's weakest chemical linkage, and accelerate mass transport (Savitri, Juliastuti, Handaratri, Sumarno, & Roesyadi, 2014). Cavitation contributes vibration wave energy, shear stress at the cavitation interphase, and localized high pressure and temperature (Gronroos et al., 2001). CH is degraded concurrently by cavitation throughout the entirety of the solution (Tsaih, Tseng, & Chen, 2004).

In addition, it has been reported that lowering the particle size of several different polymer substrates has led to increased enzyme reaction rates (Dasari & Berson, 2007; Mooney, Mansfield, Touhy, & Saddler, 1998; Yeh, Huang, & Chen, 2010). In a study conducted by Yeh et al. (2010), reducing substrate (cellulose) particle size could improve affinity between cellulose and enzyme, thus increasing hydrolysis rate due to the enhanced particle surface area. The enzymatic hydrolysis process could effectively reduce the particle size of CH. The polymer chain length of CH can be shortened by class I enzymes, such as the chitosanase *Streptomyces* sp. N174, which can split both GlcN-GlcN and GlcNAc-GlcN linkages (Tremblay, Yamaguchi, Fukamizo, & Brzezinski, 2001). The TRS content of CH solutions can provide information about the polymer's level of hydrolysis (Jeon et al., 2001). An increase in TRS content from CH/BH solution to CH/AH solution was related to the cleavage of glycosidic linkages during enzymatic hydrolysis (Li et al., 2005). This may reduce glucosamine chain lengths and increase the quantity of TRS in solution. WSC solution had lower TRS content, compared to LMWWSC solution, because using a 30 kDa membrane could likely filter out the bulk of monosaccharides and

oligosaccharides in the ultrafiltration unit. These low molecular weight compounds can influence

TRS content to a greater extent than the longer chain polymers.

Table 4.3. Particle size of chitosan particles in solution and total reducing sugar content of chitosan solutions before and after enzymatic hydrolysis and ultrafiltration.

Solution	Particle size (nm)	TRS (mg glucosamine/g CH)
CH/BH	5066.00±154.15[a]	54.23±1.11[d]
CH/AH	-	189.10±3.23[a]
WSC	756.53±27.81[b]	74.78±1.17[c]
LMWWSC	-	153.46±1.29[b]

[a-d]Means±SD with different letters within a column indicate significant difference ($P \leq 0.05$). CH = chitosan, WSC = water-soluble chitosan, LMWWSC = low molecular weight water-soluble chitosan, BH = before enzymatic hydrolysis, and AH = after enzymatic hydrolysis.

4.4.4. Deconvolution microscopy images of fluorescently labeled chitosan particles in solution and cross-sections of vacuum tumbled shrimp

Micrographs of fluorescein isothiocyanate F7250 isomer 1 (FITC) labeled WSC particles in 0.1 M acetic acid (AA) solution are shown in Figure 4.4. The particle size of WSC particles, as shown in section 4.4.3, was similar with those observed in micrographs of FITC labeled WSC particles in solution. The FITC labeled WSC particles appeared to be generally spherical with no agglomeration. It was observed that FITC labeled WSC particles could penetrate inside of and attach to shrimp muscle tissue (Figure 4.5). Most of the FITC labeled WSC particles appeared to accumulate at the outer layers of the shrimp muscle tissue. The inner shrimp muscle tissue layers had a lower quantity of FITC labeled WSC particles and diminished particle agglomeration, compared to the outer layers. These micrographs display the bioadhesive properties of WSC. Penetration of WSC into shrimp muscle tissue may play a role in the retention of quality characteristics during frozen storage.
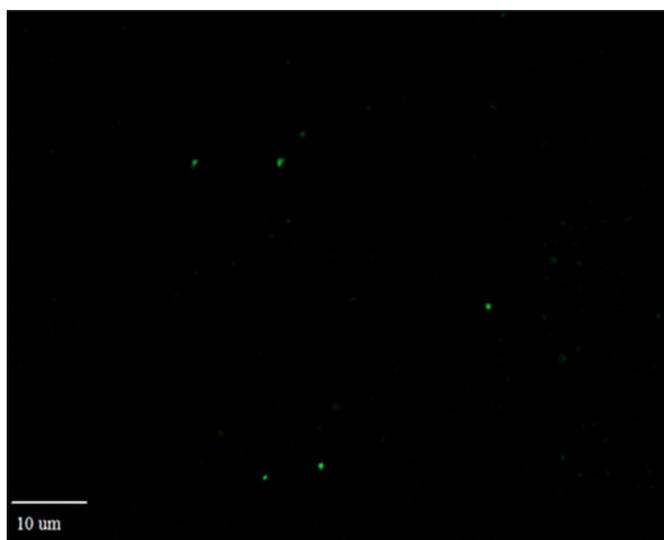
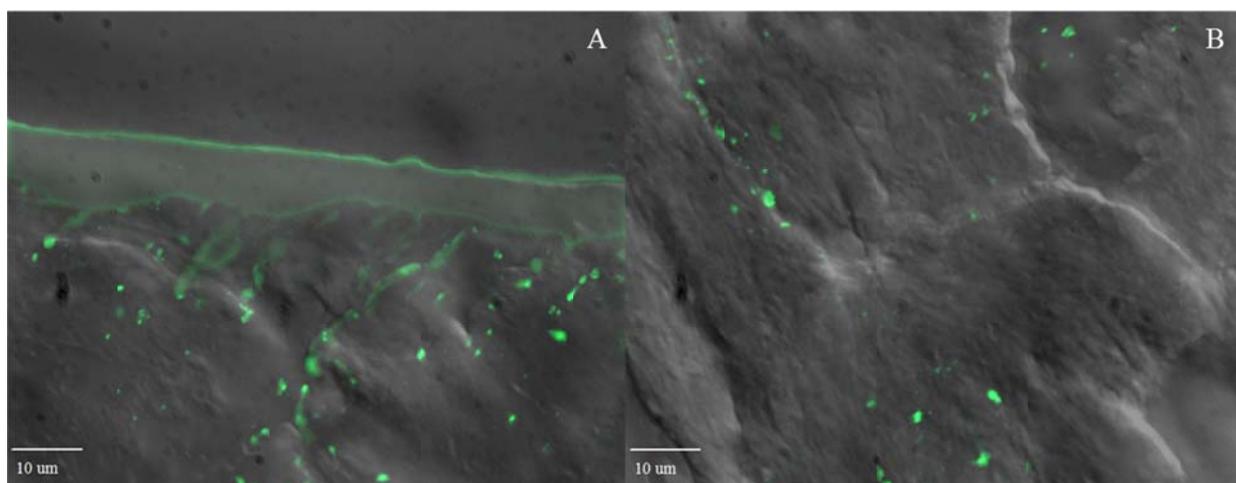Figure 4.4. Micrograph of FITC labeled water-soluble chitosan particles in solution at 100x magnification.

Figure 4.5. Micrographs of FITC labeled water-soluble chitosan particles in cross-sections of the outer (A) and inner (B) shrimp muscle tissue at 100x magnification.

Nanoparticles are particulate dispersions or solid particles that range from 10-1000 nm in size. In our study, the particle size of WSC solution was found to be 756.53 ± 27.8 nm (see Table 4.3). Particle size influences *in vivo* distribution, targeting ability, and the biological fate of nanoparticle systems. The intracellular uptake of nanoparticles is generally higher compared to microparticles. Due to their small size and relative mobility, they are available to a greater number of biological targets (Mohanraj & Chen, 2007). Particle size determines intracellular

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uptake and trafficking of particles, as well the pathways of uptake. The sub-cellular and sub-micron size of WSC may allow for deep penetration into tissues through fine capillaries and fenestration in the epithelial lining, similar to that which was reported for chitosan-sodium tripolyphosphate nanoparticles (Gan et al., 2005).

Tumbling has been reported to promote higher brine uptake by meat. The mechanism of this process may be related to structural component damage, which can result in higher swelling potential (Cheng & Sun, 2008; Theno, Siegel, & Schmidt, 1978). Vacuum is an important component of the vacuum tumbling process and its function is to prevent air from diffusing into the protein gel structure, ensuring that a tacky exudate is present on the surface of meat proteins after tumbling (Price & Schweigert, 1987). Vacuum promotes greater exchange between occluded internal gases in food and the external soaking medium (Collignan, Bohuon, Deumier, & Poligne, 2001; Fito, 1994; Fito, Chiralt, Barat, Spiess, & Behsnilian, 2001; Lenart & Flink, 1984; Raoult-Wack, 1994; Rastogi & Raghavarao, 1996; Roa, Tapia, & Millan, 2001). The vacuum tumbling process is influenced by the hydrodynamic mechanism (HDM), a mass transfer mechanism that occurs during solid-liquid interactions. According to the HDM, vacuum allows for the internal gas in a food product to expand and flow out, while promoting the transfer of liquid into the new void spaces (capillary penetration). After atmospheric pressure is regained, the residual gas in the pores compresses and results in greater infiltration of liquid into the pores (Fito, 1994; Fito & Pastor, 1994). It was shown by Deumier et al. (2003) that turkey meat, which was immersed in a 35% salt solution, had greater solution uptake during pulsed vacuum brining compared to atmospheric pressure brining. The likely breakdown of meat tissues near areas of weaker cohesion during vacuum processing, such as along muscle fibers around the perimysium, may have enhanced meat porosity. Alvarado and Sams (2004) observed that tumbling chicken

breast under vacuum with a solution containing 0.54% sodium chloride and 0.42% sodium tripolyphosphate could facilitate migration of sodium ions from the solution into the center of the muscle. After tumbling, it was found that the sodium ion distribution was largely centralized in the outer muscle layers.

4.4.5. pH and free radical scavenging activity of treatment solutions

The pH values of WSC, AA, and distilled water (DW) solutions were 5.57 ± 0.01, 2.95 ± 0.01, and 6.12 ± 0.01, respectively (Table 4.4). 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical inhibition was used as a measure of the antioxidant capacity of treatment solutions. WSC solution had significantly higher DPPH inhibition ($11.22\pm0.19\%$) than AA and DW solutions. AA solution had higher DPPH inhibition ($4.55\pm0.61\%$) than DW solution ($1.14\pm0.37\%$) ($P \leq 0.05$).

The free radical scavenging activity of AA solution is likely related to the H-abstraction reaction (Denisov & Shestakov, 2013). Hydrogen atoms in the carboxylic group of AA may bind to DPPH radicals, leading to higher scavenging activity. WSC has a wide array of antioxidant properties, including DPPH, hydrogen peroxide, and superoxide anion radical scavenging activities, as well as Cu^{2+} ion chelating ability (Lin & Chou, 2004). It was shown by Xie et al. (2001) that WSC can scavenge hydroxyl radicals ($\bullet OH$). WSC can scavenge $\bullet OH$ by the H-abstraction reaction, the formation of stable macromolecule radicals from the reaction between $\bullet OH$ and the active hydrogen atoms of residual free amino groups, and addition reaction by the absorption of hydrogen ions from solution and the subsequent formation of ammonium (NH_3^+) groups. According to a study conducted by Matsugo et al. (1998), WSC derivatives were able to inhibit the formation of thiobarbituric acid reactive substrates in *t*-butyl hydroperoxide and benzoyl peroxide induced lipid peroxidations as a result of their radical chain-breaking activity.

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Table 4.4. pH and free radical scavenging activity of treatment solutions.

Solution	pH	DPPH inhibition (%)
WSC	5.57 ± 0.01^{b}	11.22 ± 0.19^{a}
AA	2.95 ± 0.01^{c}	4.55 ± 0.61^{b}
DW	6.12 ± 0.01^{a}	1.14 ± 0.37^{c}

[a-c]Means±SD with different letters within a column indicate significant difference ($P \leq 0.05$). WSC = water-soluble chitosan, AA = acetic acid, and DW = distilled water.

4.4.6. Microbial counts of thawed shrimp

Initially, the aerobic plate counts (APC) of WSC, AA, and DW treatments were similar and significantly lower than that of untreated shrimp (NT) ($P \leq 0.05$). At 60 and 120 days of storage, WSC treatment had the lowest APC and NT had the highest (Figure 4.6) ($P \leq 0.05$). NT had significantly higher APC compared to the other treatments. The APC of AA and DW treatments were not significantly different. All treatments, within their respective treatment, showed lower APC from day 0 to day 60 of storage and were not significantly different from day 60 to day 120 ($P \leq 0.05$).

Yeast and mold counts (YMC) of all treatments were not significantly different at 0 and 60 days of storage (Figure 4.7). Although at day 120, YMC of WSC treatment was lower than the other treatments ($P \leq 0.05$). Within their respective treatment, YMC of all treatments increased significantly from day 0 to day 120, but unlike WSC, AA, and DW, the YMC of NT rose at each storage time ($P \leq 0.05$).
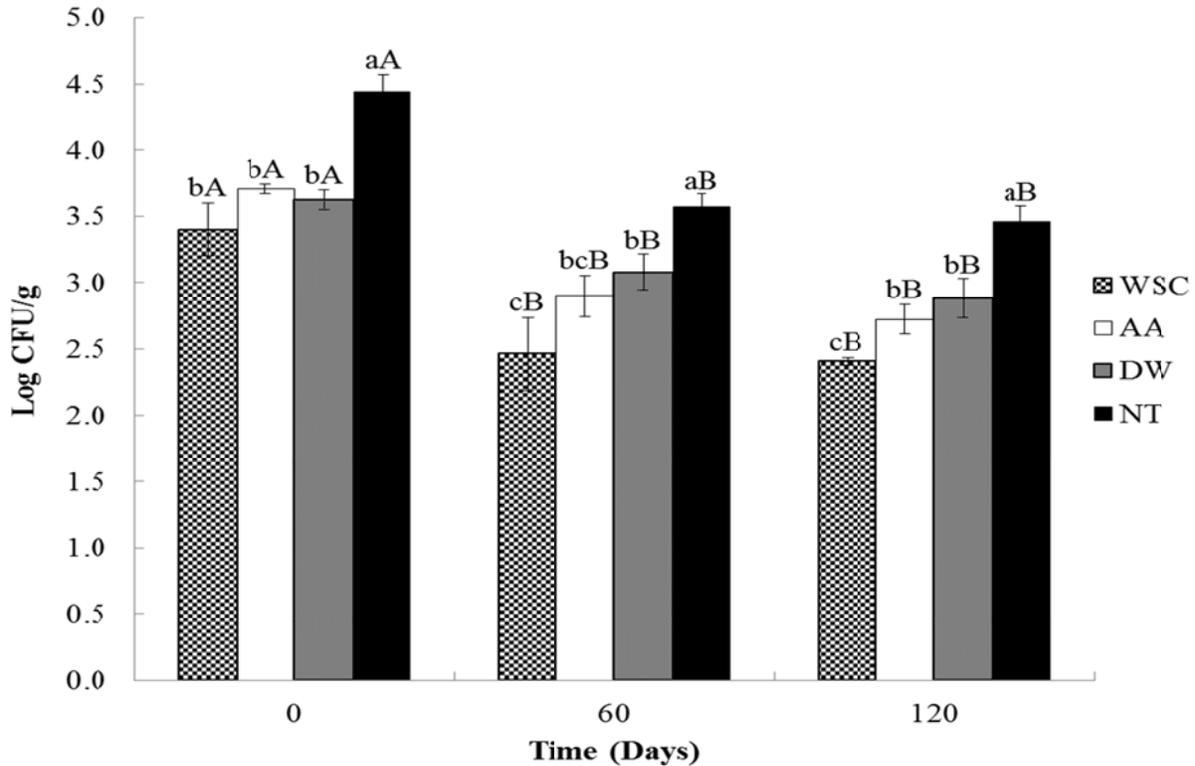
Figure 4.6. Aerobic plate counts of thawed shrimp during storage at -20 °C. [a-c]Means±SD with different letters between treatments at the same storage times indicate significant difference (P ≤ 0.05). [A,B]Means±SD with different letters within treatments at different storage times indicate significant difference (P ≤ 0.05). WSC = water-soluble chitosan, AA = acetic acid, DW = distilled water, and NT = no treatment.

A decrease in APC for all treatments from day 0 to day 60 of storage is likely related to inter and extra cellular ice crystal formation during the freezing process (Abdou, Osheba, & Sorour, 2012). These ice crystals may have caused irreversible damage to the outer and cytoplasmic membranes of bacteria (Uljas & Ingham, 1999). The rate of microbial death is highest during the initial freezing process and it gradually declines over time in frozen storage (Adams & Moss, 1995). With or without the addition of other antimicrobial compounds, AA solutions have been reported to provide significant bacteriostatic and bactericidal effects when applied to a variety of meat products at different concentrations (Bell, Cutter, & Sumner, 1997;

Surve, Sherikar, Bhilegaonkar, & Karkare, 1991; Taher, Khaton, Fakir, Hasnat, & Rahman, 2013).
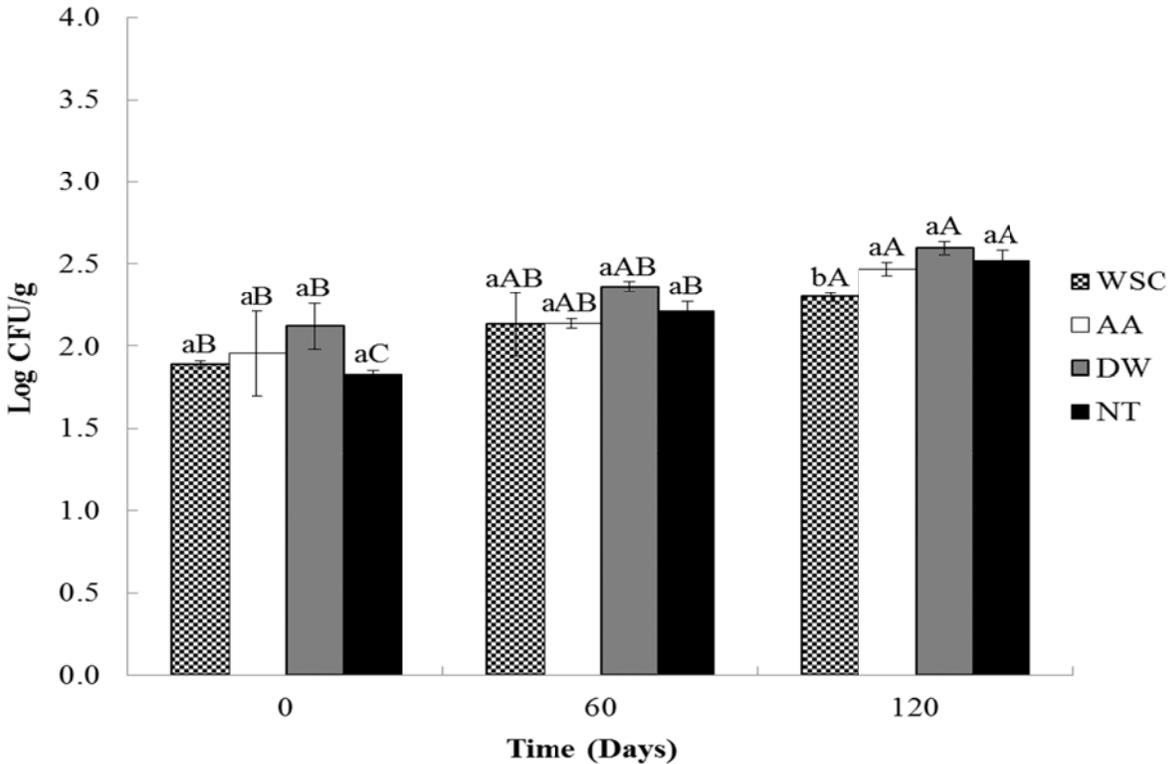

Figure 4.7. Yeast and mold counts of thawed shrimp during storage at -20 °C. [a,b]Means±SD with same letters between treatments at the same storage times are not significantly different (P ≤ 0.05). [A-C]Means±SD with different letters within treatments at different storage times indicate significant difference (P ≤ 0.05). WSC = water-soluble chitosan, AA = acetic acid, DW = distilled water, and NT = no treatment.

The antimicrobial mechanisms of CH may be related to the interaction between its protonated amine groups and the negatively charged surface components of various bacteria and fungi (Raafat & Sahl, 2009). At pH values below approximately 6.5, such as in WSC solution (Section 4.4.5), the amine groups of CH would be protonated (Nystrom et al., 1999). CH can inhibit microorganisms by adsorption onto the bacterial cell surface, diffusion through the cell wall, and adsorption onto the cytoplasmic membrane, leading to disruption of the cytoplasmic membrane, leakage of the cytoplasmic constituents, and cell death (Kong, Chen, Xing, & Park,

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2010). In another suggested mode of action, CH may be able to form a polymer membrane on the cell surface, preventing nutrients from reaching the cell (Zheng & Zhu, 2003). Diffused CH hydrolysis products could be able to interact with microbial DNA, resulting in inhibition of mRNA and protein synthesis (Sudarshan, Hoover, & Knorr, 1992). Xie et al. (2002) reported that WSC derivatives that were produced through etherification reaction and graft copolymerization showed greater antimicrobial activity against *Staphylococcus aureus* and E*scherichia coli* than either CH or hydroxypropyl CH. The mechanism of action may have been related to the amphiphilic structure and strong coordination capability of the WSC derivatives. Also, the addition of WSC (0.03%) to processed milk for reduction of microbial (bacterial and yeast) spoilage was reported by Ha and Lee (2001).

YMC for all treatments increased during storage time, possibly because mold spores and gram-positive bacteria possess a greater resistance to low temperatures than gram negative bacteria (Fellows, 2009). Tsai, Su, Chen, and Pan (2002) observed that CH was more effective at inhibiting bacteria than fungi, although in our study at 120 days of storage, WSC had significantly lower YMC than other treatments. The antimicrobial activity of CH is mainly contingent on molecular weight, concentration, DD, and type of bacteria (Jeon et al., 2001; No et al., 2002; Tsai et al., 2002; Zheng & Zhu, 2003). CH possesses antifungal properties that are executed through the suppression of sporulation and spore germination (Hernandez-Lauzardo et al., 2008). AA may have been less effective with longer storage time because its bacteriostatic effects are temporary and diminish over time as the pH gradually rises towards its normalcy (Surve et al., 1991). In addition, antimicrobial substances were not applied to DW and NT treatments, resulting in no YMC inhibition. We found that the total coliform counts (TCC) in the shrimp samples were too few to count at any storage time.

4.4.7. pH and moisture content of thawed shrimp

WSC and DW had similar pH values initially, while other treatments were significantly different (P ≤ 0.05). At 120 days of storage, there were significant differences in pH values between all treatments (Figure 4.8).
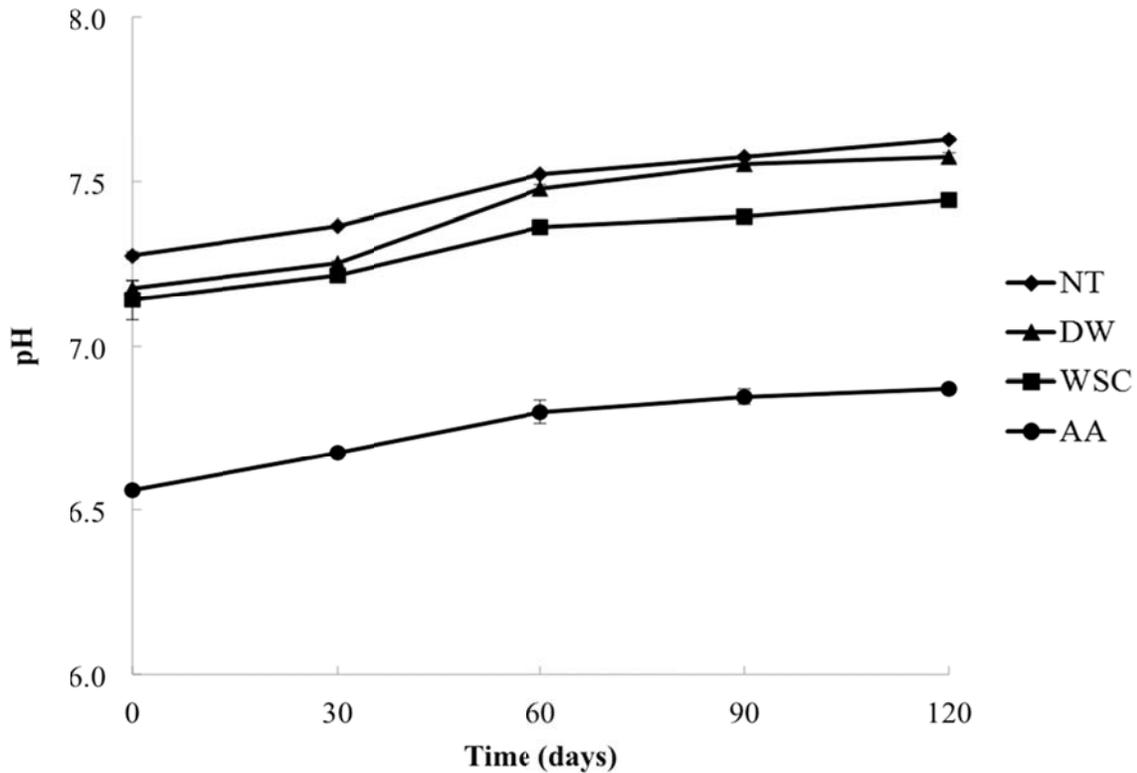

Figure 4.8. pH of thawed shrimp during storage at -20 °C. WSC = water-soluble chitosan, AA = acetic acid, DW = distilled water, and NT = no treatment.

The pH values for all treatments, within their respective treatment, increased significantly from day 0 to day 120 of storage. An increase in shrimp pH during storage has been reported in various studies (Goswami, Ravindra, & Nayak, 2001; Solval, Espinoza Rodezno, Moncada, Bankston, & Sathivel, 2014; Sundararajan et al., 2011). The pH of shrimp can serve as an indicator of physicochemical changes (Riaz & Qadri, 1990). Increasing shrimp pH during storage is the result of biochemical reactions (Shamshad, Riaz, Zuberi, & Qadri, 1990). Accumulation of indoles, trimethylamines, and total volatile bases has been linked to higher pH

values (Mendes, Goncalves, Pestana, & Pestana, 2005; Zeng, Thorarinsdottir, & Olafsdottir, 2005). Goswami et al. (2001) reported that pH values below 7.2 correspond with prime quality shrimp and a pH between 7.7 and 7.95 is an indicator of poor, but acceptable quality shrimp. Shrimp is considered spoiled at pH above 7.95.

The initial moisture content of NT was significantly lower than that of other treatments (Figure 4.9). At 120 days of storage, NT also had significantly lower moisture than the other treatments. Moisture contents between WSC, DW, and AA treatments were not significantly different after 0 and 120 days of storage. Within their respective treatment, the moisture contents of WSC, DW, and AA showed no significant difference from day 0 to day 120 of storage, however NT decreased ($P \leq 0.05$).
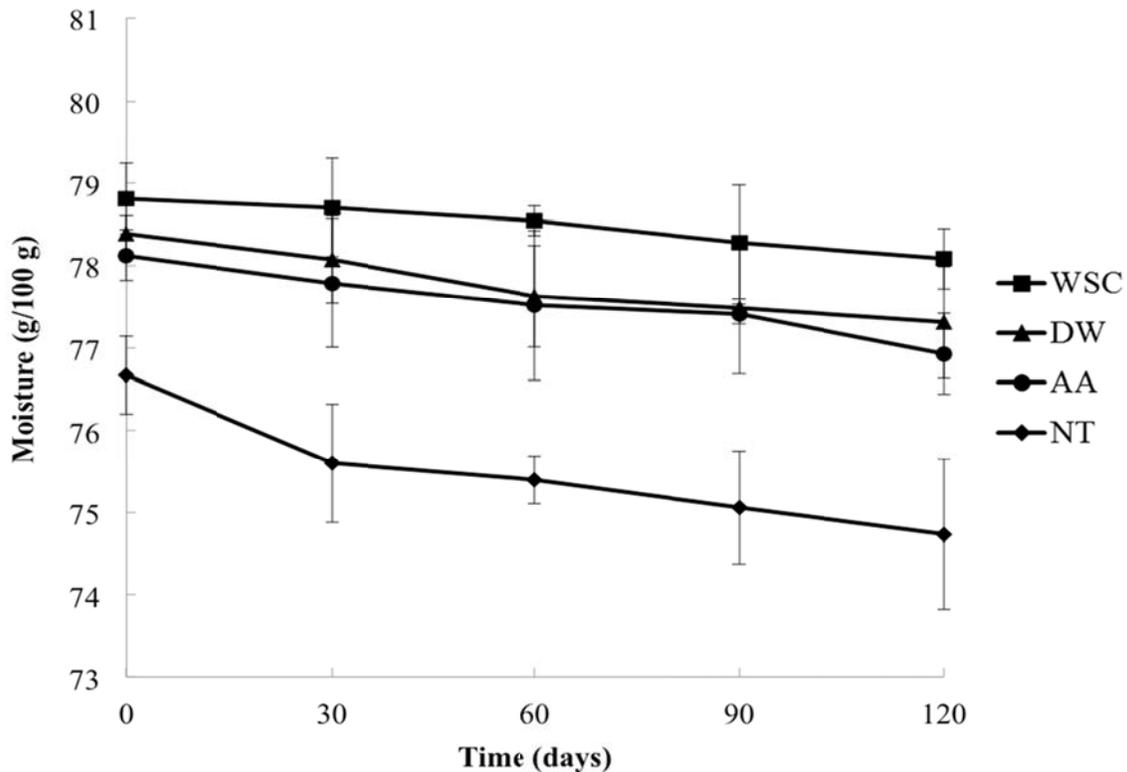

Figure 4.9. Moisture content of thawed shrimp during storage at -20 °C. WSC = water-soluble chitosan, AA = acetic acid, DW = distilled water, and NT = no treatment.

According to Rejt et al. (1978), vacuum tumbling was found to be effective in decreasing cooking losses, as well as improving tenderness and water hold capacity of hams compared to controls that were not tumbled. Tumbling can increase water and acid transport between meat and solution likely due to a greater transfer surface area resulting from meat surface destructuring. This can lead to accelerated marinade penetration and diffusion (Ghavimi, Rogers, Althen, & Ammerman, 1986; Katsaras & Budras, 1993). Seafood in frozen storage undergoes surface dehydration over time, resulting in decreased water holding capacity of tissues (Shenouda, 1980). The application of edible films and coatings can decrease moisture transfer in frozen foods, lower oxygen permeability, and limit surface dehydration in fresh or frozen meats (Debeaufort, Quezada-Gallo, & Voilley, 1998).

4.4.8. Lipid oxidation of thawed shrimp

Initially, there were no significant differences in TBARS (thiobarbituric-acid-reactive-substances) values between AA, DW, and NT treatments (Figure 4.10). TBARS of WSC were similar to DW (P ≤ 0.05). AA and NT had significantly higher TBARS than WSC. At 120 days of storage, NT had the highest TBARS, followed by AA, DW, and WSC (P ≤ 0.05). Within their respective treatment, the TBARS of all treatments increased significantly from day 0 to day 120. Ibrahim Sallam (2007) reported that the maximum TBARS values that indicate good seafood quality are 5 mg MDA/kg sample, which none of the treatments in our study exceeded.

Lipid oxidation occurs through enzymatic and non-enzymatic processes (Zaritzky, 2000). In frozen seafood, the major type of oxidation present is typically autooxidation, in addition to some enzymatically derived oxidation (Schultz, 1962). Metal ions, such as Fe^{2+}, hemin, Cu^{2+}, and Fe^{3+} act as catalysts for oxidation. Jacobsen, Timm, and Meyer (2001) observed greater metal ion catalytic activity in fish oil enriched mayonnaise after lowering the pH, which led to a

pro-oxidative effect. Also, the oxidative stability of protein isolates extracted from Atlantic croaker (*Micropogonias undulates*) using acid-aided and alkali-aided processes during refrigerated storage, was investigated by Kristinsson and Liang (2006). Acid-aided isolates had higher TBARS than the alkali-aided isolates, ground croaker muscle, and surimi that was formed from the same fish. This may explain why the AA treatment had higher TBARS than WSC and DW.
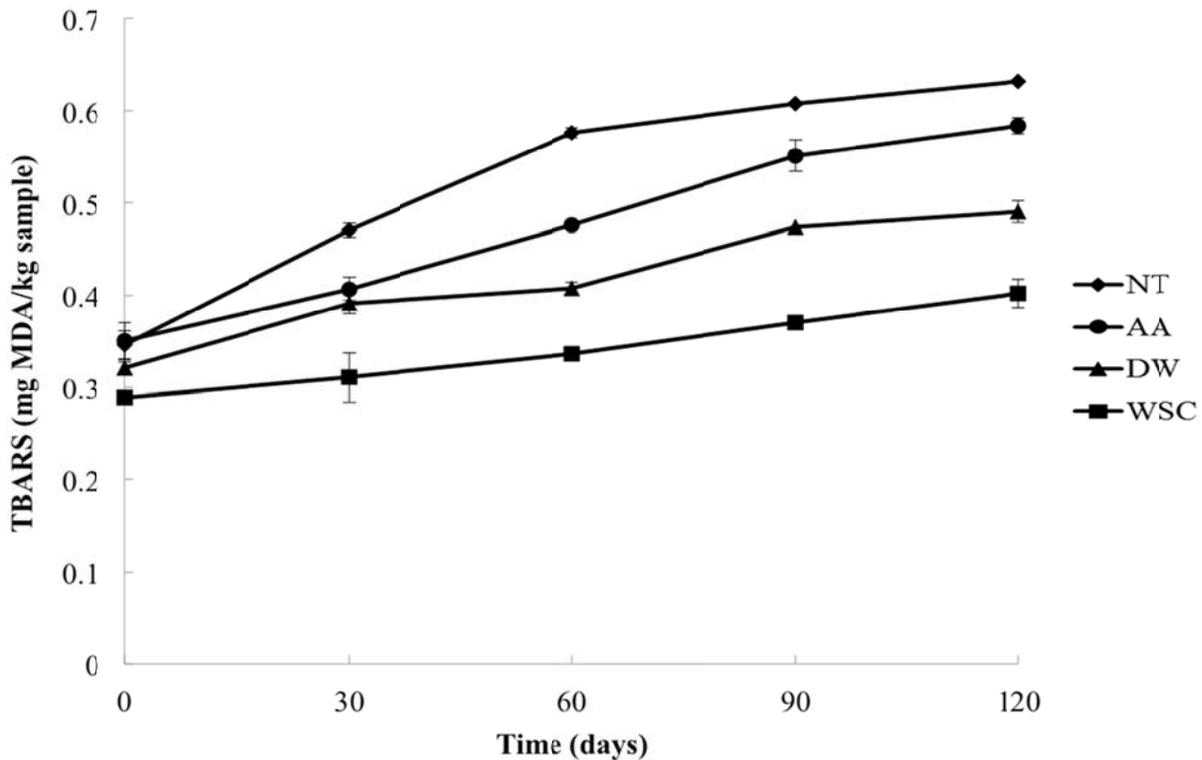
Figure 4.10. TBARS of thawed shrimp during storage at -20 °C. WSC = water-soluble chitosan, AA = acetic acid, DW = distilled water, and NT = no treatment.

WSC possesses strong antioxidant activity due to its free amino groups. It can chelate metal ions and scavenge DPPH, hydrogen peroxide, and superoxide anion radicals (Lin & Chou, 2004), as well as hydroxyl radicals (Xie et al., 2002). The antioxidant activity of CH is related to its molecular weight, exhibiting greater activity at lower molecular weights (Dash, Chiellini, Ottenbrite, & Chiellini, 2011). Kim and Thomas (2007) reported that of the CH's with different

molecular weights (30, 90, and 120 kDa) that were tested for DPPH free radical scavenging activity, 30 kDa CH was the most effective. This occurred because the 30 kDa CH had greater mobility than the higher molecular weight CH, thus decreasing the possibility of inter and intramolecular bonding between high molecular weight CH molecules and perhaps enhancing the exposure of amino groups. CH has also been successfully applied as a glazing solution to preserve the quality of seafood during frozen storage (Abdou et al., 2012; Sathivel, 2005; Solval et al., 2014). In a study conducted by Sathivel, Liu, Huang, and Prinyawiwatkul (2007), it was found that glazing skinless pink salmon filets with a 1% w/w CH solution delayed lipid oxidation and elicited higher thaw yield than in the non-glazed control after eight months of frozen storage.

4.4.9. Color and texture of thawed shrimp

The initial L^* value of NT was significantly lower than the other treatments (Table 4.5). At 120 days of storage, there were no significant differences between treatments. There was no significant difference in L^* values of any treatment from day 0 to day 120, within their respective treatment. Nowsad, Kanoh, and Niwa (2000) reported that water-washing minced broiler meat with 0.1% NaCl solution could increase the L^* value.

There were no significant differences in a^* values between treatments at the initial storage period. At 120 days of storage, a^* values between WSC, AA, and DW treatments were not significantly different, although AA was also similar to NT, which had significantly lower values than the other treatments ($P \leq 0.05$). Within their respective treatment, from day 0 to day 120, a^* values of WSC and DW were not significantly different, however the values of AA and NT decreased from day 1 to day 120 ($P \leq 0.05$). During frozen storage, color change in products can occur due to pigment oxidation, lipolysis, and freezer burn (Hui, 2006). These factors can produce color fading, possibly due to degradation of astaxanthin, a carotenoid pigment found in

crustaceans (Erickson & Hung, 2012). Initially, there were no significant differences in *b** values between treatments. At 120 days of storage, the *b** values of these pairs of treatments were not significantly different: WSC and DW, AA and DW, AA and NT. The WSC treatment had a significantly lower *b** value than AA and NT. Within their respective treatment from day 0 to day 120, the *b** values of WSC and DW were not significantly different, but those of AA and DW increased (P ≤ 0.05). Tsironi, Dermesonlouoglou, Giannakourou, and Taoukis (2009) suggested that the *b** values of shrimp could be used a quality index.

Table 4.5. Color of thawed shrimp during storage at -20 °C.

	Treatment	Time (days)				
		0	30	60	90	120
L*	WSC	53.22±0.63[aA]	54.95±0.50[aA]	55.30±2.17[aA]	54.78±1.82[aA]	54.83±2.31[aA]
	AA	52.02±0.21[aA]	54.54±2.55[aA]	55.36±2.12[aA]	52.68±0.34[abA]	53.28±0.37[aA]
	DW	51.94±1.05[aA]	53.00±0.46[aA]	53.01±0.96[aA]	53.15±1.71[abA]	54.19±2.21[aA]
	NT	48.86±0.86[bA]	51.86±2.85[aA]	52.09±1.32[aA]	50.18±0.86[bA]	51.84±1.13[aA]
a*	WSC	2.42±0.29[aA]	2.26±0.12[aA]	2.18±0.48[aA]	2.15±0.52[aA]	2.07±0.11[aA]
	AA	2.61±0.24[aA]	2.61±0.11[aA]	2.41±0.15[aA]	2.15±0.10[aB]	1.9±0.16[abB]
	DW	2.68±0.13[aA]	2.73±0.52[aA]	2.60±0.39[aA]	2.22±0.29[aA]	2.08±0.09[aA]
	NT	2.55±0.34[aA]	2.56±0.33[aA]	2.04±0.27[aAB]	1.90±0.11[aAB]	1.52±0.20[bB]
b*	WSC	4.63±0.34[aA]	4.48±0.43[bA]	4.57±0.09[bA]	5.06±0.38[aA]	5.14±0.21[cA]
	AA	5.04±0.29[aB]	5.23±0.10[abB]	5.58±0.59[abAB]	5.90±0.24[aAB]	6.52±0.36[abA]
	DW	4.45±0.54[aA]	4.61±0.06[bA]	4.77±0.29[bA]	5.24±0.65[aA]	5.92±0.23[bcA]
	NT	4.52±0.57[aB]	6.24±0.33[aAB]	6.41±0.43[aAB]	6.63±0.54[aA]	7.32±0.66[aA]

L*, *a**, and *b** are the degree of lightness to darkness, redness to greenness, and yellowness to blueness, respectively. [a-c]Means±SD with different letters within a column indicate significant difference (P ≤ 0.05). [A,B]Means±SD with different letters within a row indicate significant difference (P ≤ 0.05). WSC = water-soluble chitosan, AA = acetic acid, DW = distilled water, and NT = no treatment.

The changes in *b** values of shrimp were modeled using a zero order reaction during frozen storage. Low *b** values were correlated with higher shrimp quality. During frozen storage,

the yellowing of fish flesh has been associated with the migration of carotenoids to subcutaneous fat layers, lipid oxidation, and the carbonyl-amine reaction (Lakshmanan, 2000).

Throughout the entire storage time, similar cutting force values were observed between treatments (Figure 4.11) ($P \leq 0.05$). The cutting force values for all treatments, within their respective treatment, were not significantly different at any storage time.
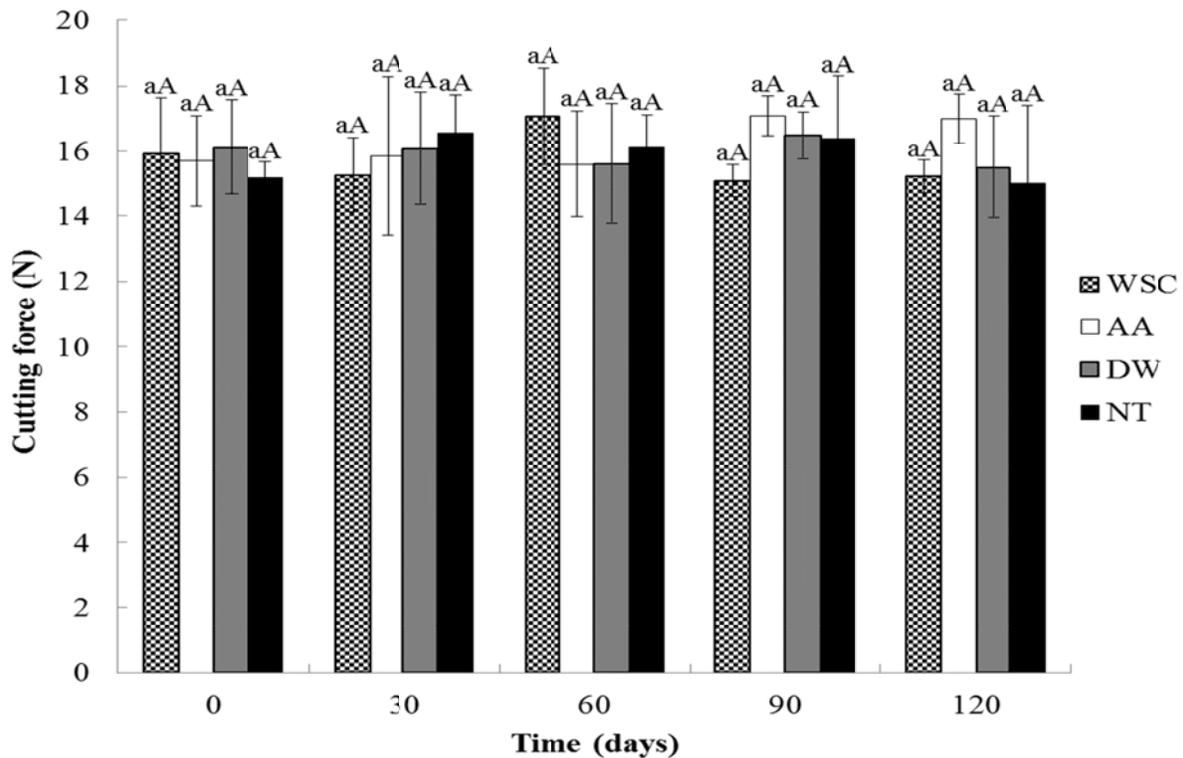
Figure 4.11. Cutting force of thawed shrimp during storage at -20 °C. [a]Means±SD with same letters between treatments at the same storage times are not significantly different ($P \leq 0.05$). [A]Means±SD with same letters within treatments at different storage times are not significantly different ($P \leq 0.05$). WSC = water-soluble chitosan, AA = acetic acid, DW = distilled water, and NT = no treatment.

In our study, application of liquid nitrogen for fast freezing of shrimp, in addition to the use of low storage temperatures (-20 °C), provided high textural quality and may have minimized protein denaturation. Protein denaturation caused by freezing is typically the result of a number of factors, such as dehydration, oxidation, salt concentration, ice formation,

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recrystallization, and cellular metabolite activity (Zaritzky, 2000). Slow freezing of seafood may result in moisture loss due to the puncturing of muscle by large ice crystals, resulting in undesirable changes in texture (Shenouda, 1980). Rapid freezing causes smaller ice crystal formation in plant and animal tissues compared to slow freezing, leading to less structural damage in their cell walls (Rahman, 2007). Protein denaturation can cause changes in muscle texture that are accompanied by quality loss (Zaritzky, 2000). Seafood that is rapidly frozen experiences lower protein denaturation rates than that which is slowly frozen because there is less time spent in the zone where a majority of ice crystal formation occurs (-1 to -2 °C) (Johnston, 1994). Moreover, shrimp that are quickly frozen and stored at low temperatures have much lower amounts of drip loss compared to slowly frozen shrimp (Goncalves & Ribeiro, 2008). Drip loss can alter product appearance, texture, juiciness, and incur nutrient losses (Zaritzky, 2000).

4.5. Conclusion

Ultrasonication, with subsequent enzymatic hydrolysis, could break down the chitosan (CH) polymer chain and reduce particle size to nano-scale, producing water-soluble chitosan (WSC). Enzymatic hydrolysis increased the total reducing sugar content by shortening glucosamine chain lengths through the cleavage of CH's glycosidic linkages. WSC had higher water solubility and lower molecular weight compared to CH. WSC solution exhibited fluid-like behavior. WSC had greater free radical scavenging activity than acetic acid (AA) and distilled water (DW). Vacuum tumbling with WSC solution facilitated penetration of particles into the shrimp. The particles were able to adhere onto shrimp muscle tissue, localizing mainly at the outer layers. Aerobic plate counts (APC) and yeast and mold counts (YMC) of WSC treated shrimp were lower compared to other treatments after 120 days of storage. WSC could preserve

color, texture, and moisture content of shrimp during the entire storage time. Additionally, WSC had the highest reduction in lipid oxidation compared to other treatments after 120 days of storage at -20 °C. This study showed that a WSC solution, combined with vacuum tumbling, can be effective at reducing APC, YMC, and lipid oxidation in shrimp during frozen storage, while maintaining desired physicochemical properties.

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CHAPTER 5. CONCLUSIONS

Shrimp were vacuum tumbled with chitosan (CH), chitosan-sodium tripolyphosphate (CH-TPP), and water-soluble chitosan (WSC) solutions, then cryogenically frozen and evaluated for quality retention during frozen storage. Ionotropic gelation and enzymatic hydrolysis could produce nano-scale CH-TPP and WSC particles, respectively, while CH particles were micro-scale after processing with sonication and ultra-shearing. The three solutions exhibited fluid-like behavior and they had higher free radical scavenging activities than the other treatment solutions. Vacuum tumbling with CH, CH-TPP, and WSC solutions facilitated particle penetration into the shrimp. The particles were able to adhere onto shrimp muscle tissue, localizing mainly at the outer layers. Aerobic plate counts (APC) of CH, CH-TPP, and WSC treated shrimp were lower compared to other treatments after 120 days of storage. However, the WSC treatment also decreased yeast and mold counts (YMC) in shrimp, while CH and CH-TPP were ineffective. CH, CH-TPP, and WSC treatments could preserve color, texture, and moisture content of shrimp during the entire storage time. In addition, CH, CH-TPP, and WSC treatments had a higher reduction in lipid oxidation than other treatments after 120 days of storage at -20 °C. This study showed that CH, CH-TPP, and WSC solutions, combined with vacuum tumbling, can be effective at reducing APC and lipid oxidation in shrimp during frozen storage, while maintaining desired physicochemical properties. As WSC can be dissolved in water, it may have greater application potential in foods than CH or CH-TPP.

APPENDIX A: AEROBIC PLATE COUNTS OF SHRIMP VACUUM TUMBLED WITH CHITOSAN NANOPARTICLE SOLUTION AND STORED AT REFRIGERATED TEMPERATURE

Materials and methods

Treatment solutions were prepared according to the method described in section 3.3.1. Peeled, fresh white shrimp meat was separately tumbled with treatment solutions (acetic acid (AA), chitosan (CH), sodium tripolyphosphate (TPP), chitosan-sodium tripolyphosphate (CH-TPP), and distilled water (DW)) at a 1:1 ratio by weight in a vacuum tumbler in a cold processing room (4 °C) for 10 min. Fresh shrimp meat tumbled with DW, as well as fresh shrimp meat without tumbling were used as controls. After tumbling, excess solution was allowed to drain for 30 sec. Samples were allowed to dry under a laminar hood for 7 min. They were packed into one quart Ziploc freezer bags and stored at 4 °C for 24 days, undergoing microbiological analysis at 0, 6, 12, and 24 days of frozen storage. APC were quantified using Petrifilms according to manufacturer's instructions with some modifications. Whole thawed shrimp were placed in 18 oz Whirl-Pak bags and diluted to 10^{-1} concentration with 0.85% NaCl solution. The samples in the Whirl-Pak bags were homogenized using a lab-blender for 2 min. Duplicated serial dilutions of samples in 0.85% NaCl solution were produced and separately plated on Petrifilms for APC. Using a flat surface, 1 mL of shrimp dilution was placed on the bottom film and the inoculums were covered with the top film and spread using the provided plastic spreaders. APC Petrifilms were incubated for 48 h at 35 °C and the red colonies were counted.

Results and discussion

Initially, NT had significantly higher APC (4.70±0.05 log CFU/g) than other treatments (Figure A1). At 24 days of storage, CH had significantly lower APC (3.39±0.26 log CFU/g) compared to the other treatments. CH-TPP had lower APC (5.00±0.06 log CFU/g) than every treatment, with the exception of CH ($P \leq 0.05$). The APC of TPP (5.91±0.06 log CFU/g) was

similar to AA (5.98±0.09 log CFU/g) and both treatments had lower APC than DW (7.60±0.08 log CFU/g) and NT (6.88±0.24 log CFU/g), but higher APC than CH and CH-TPP (P ≤ 0.05). DW had significantly higher APC compared to the other treatments and it exceeded the upper acceptability limit for viable counts in fresh fish (10^7 CFU/g) recommended by ICMSF (1986), while NT was near the limit. According to Parallel Food Testing in the European Union (1995), a European consumer study reported that fish was assumed "to not be in a good enough condition to be stored for long" when total plate counts reached 10^6 CFU/g. Within their respective treatment, the APC of DW and NT increased at each storage time, while APC of DW, TPP, and AA only increased from day 12 to day 24 (P ≤ 0.05). CH was the only treatment to have similar APC throughout the entire storage time, within its respective treatment (P ≤ 0.05).
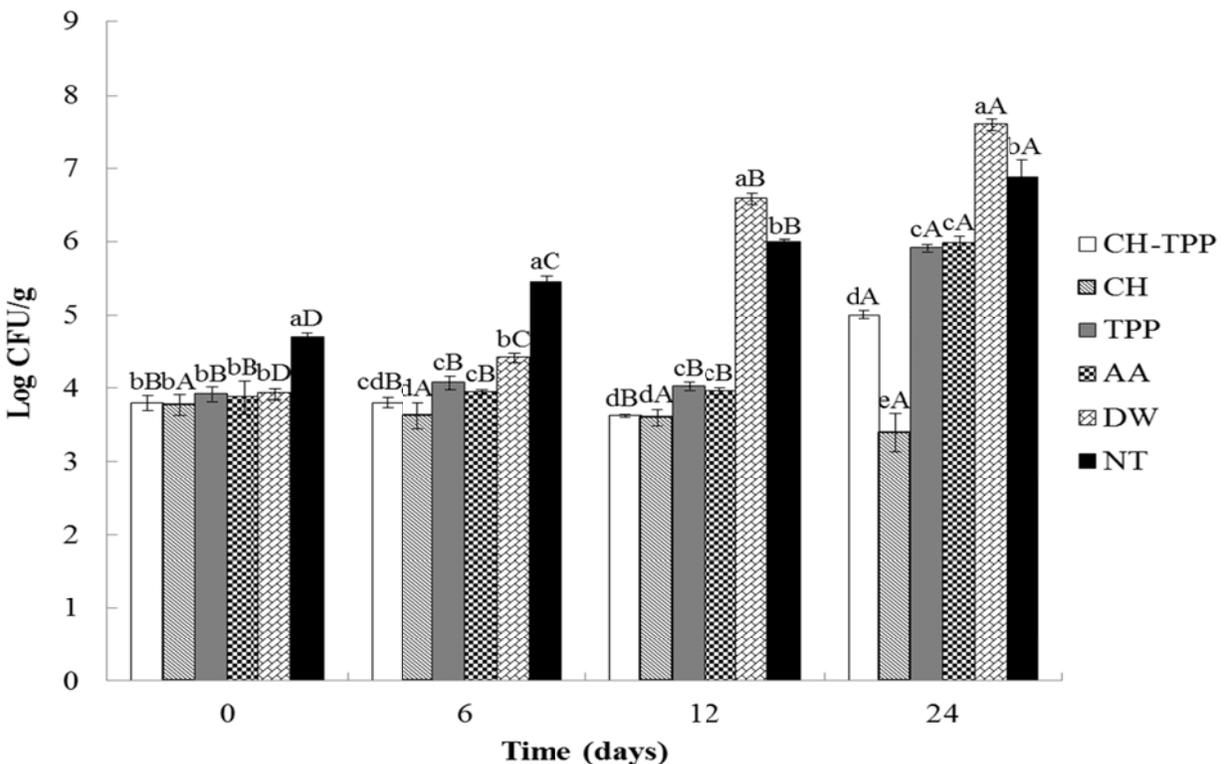

Figure A1. Aerobic plate counts of shrimp during storage at 4 °C. [a-e]Means±SD with different letters between treatments at the same storage times indicate significant difference (P ≤ 0.05). [A-D]Means±SD with different letters within treatments at different storage times indicate significant difference (P ≤ 0.05). CH = chitosan, CH-TPP = chitosan-sodium tripolyphosphate, TPP = sodium tripolyphosphate, AA = acetic acid, DW = distilled water, and NT = no treatment.

DW surpassed NT in APC at 24 days of storage, possibly because the DW treatment may have higher water activity than NT shrimp. Food products with higher water activities are subject to a greater rate of microbial growth over time (Rahman, 2007). According to Surve, Sherikar, Bhilegaonkar, and Karkare (1991) the bacteriostatic effect of AA is temporary and diminishes over time as the pH gradually rises towards its normalcy.

According to a study conducted by Jarry et al. (2001) on the antimicrobial activity of CH against different microorganisms, it was shown that bacteria that were separated from the CH solution by membrane filtration could grow rapidly. The bacteriostatic activity of CH may be linked to its ability to bind to microbial cells, negating any further activity against unbound microorganisms (Rhoades & Roller, 2000). CH's amine groups are positively charged in acidic medium, creating a large charge density (Gan, Wang, Cochrane, & McCarron, 2005). The ability of CH's NH_3^+ groups to bind to negatively charged cell walls of bacteria, resulting in impairment of essential bacterial activities, has been a commonly suggested mode of action (Raafat & Sahl, 2009). In another proposed mode of action, CH may be able to form a polymer membrane on the cell surface, preventing nutrients from reaching the cell (Zheng & Zhu, 2003). The smaller particle size and larger surface area of CH and CH-TPP particles after processing with sonication and ultra-shearing may be able to improve antimicrobial activity. The reduction in particle size could allow for more compact adsorption onto the surface of bacteria cells, leading to membrane disruption, leakage of intracellular components, and subsequent cell death (Qi, Xu, Jiang, Hu, & Zou, 2004).

As shown in Figure A1, vacuum tumbling shrimp with CH and CH-TPP solutions could facilitate solution absorption and effective CH adhesion was observed at the outer and inner layers of shrimp tissue. Deumier (2006) reported that vacuum tumbling deboned chicken legs in

a 1-5% lactic acid solution for 1-10 min could improve microbiological quality. Processing with higher tumbling speeds resulted in greater decontamination of chicken legs, reducing total viable counts and Enterobacteriaceae. The relative contributions of acid-soaking, continuous vacuum, pulsed vacuum, and tumbling on mass variation in whole deboned chicken legs treated for 5 min with a 1% lactic acid solution were 5, 10, 16, and 68%, respectively. At 2% lactic acid concentration, the relative contributions of acid-soaking, continuous vacuum, pulsed vacuum, and tumbling were 19, 13, 30, and 38%, respectively.

The bioactive free amine groups of CH are also an important determinant of CH-TPP nanoparticle antimicrobial activity (Azeredo, 2013). CH-TPP nanoparticles may possess less free amine groups than CH due to the electrostatic interaction between amine groups and TPP. Also, in a study conducted by Rampino, Borgogna, Blasi, Bellich, and Cesaro (2013), it was shown that CH-TPP nanoparticles stored in a moist environment for 1 month showed an increase in size. This likely occurred because of particle aggregation and the interaction between free polymer chains and the particle network, leading to reorganization of intermolecular entanglements, swelling, and syneresis, due to TPP that can facilitate an influx of water by osmosis. The moist environment provided by fresh white shrimp (77.88±0.18 g/100 g water) may lead to CH-TPP nanoparticle degradation over long term refrigerated storage.

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APPENDIX B: AEROBIC PLATE COUNTS OF SHRIMP VACUUM TUMBLED WITH WATER-SOLUBLE CHITOSAN SOLUTION AND STORED AT REFRIGERATED TEMPERATURE

Materials and methods

Treatment solutions were prepared according to the method described in section 4.3.1. Peeled, fresh white shrimp meat was separately tumbled with treatment solutions (water-soluble chitosan (WSC), acetic acid (AA), and distilled water (DW)) at a 1:1 ratio by weight in a vacuum tumbler in a cold processing room (4 °C) for 10 min. Fresh shrimp meat tumbled with DW, as well as fresh shrimp meat without tumbling (NT) were used as controls. After tumbling, excess solution was allowed to drain for 30 sec. Samples were allowed to dry under a laminar hood for 7 min. They were packed into one quart Ziploc freezer bags and stored at 4 °C for 24 days, undergoing microbiological analysis at 0, 6, 12, and 24 days of frozen storage. APC were quantified using Petrifilms according to manufacturer's instructions with some modifications. Whole thawed shrimp were placed in 18 oz Whirl-Pak bags and diluted to 10^{-1} concentration with 0.85% NaCl solution. The samples in the Whirl-Pak bags were homogenized using a lab-blender for 2 min. Duplicated serial dilutions of samples in 0.85% NaCl solution were produced and separately plated on Petrifilms for APC. Using a flat surface, 1 mL of shrimp dilution was placed on the bottom film and the inoculums were covered with the top film and spread using the provided plastic spreaders. APC Petrifilms were incubated for 48 h at 35 °C and the red colonies were counted.

Results and discussion

Initially, NT had significantly higher APC (4.72±0.08 log CFU/g) than other treatments (Figure B1). At 24 days of storage, AA had significantly lower APC (6.24±0.15 log CFU/g) compared to the other treatments. APC of WSC treatment (7.41±0.03 log CFU/g) was lower than DW (8.15±0.15 log CFU/g) and NT (7.84±0.12 log CFU/g) ($P \leq 0.05$). DW had significantly

higher APC than the other treatments. Within their respective treatment, the APC of WSC, DW,

and NT increased at each storage time, while APC of AA increased from day 0 to day 6 and day

12 to day 24 (P ≤ 0.05). The DW treatment surpassed NT in APC at 24 days of storage because

DW may have higher water activity than NT. It has been shown that food products with higher

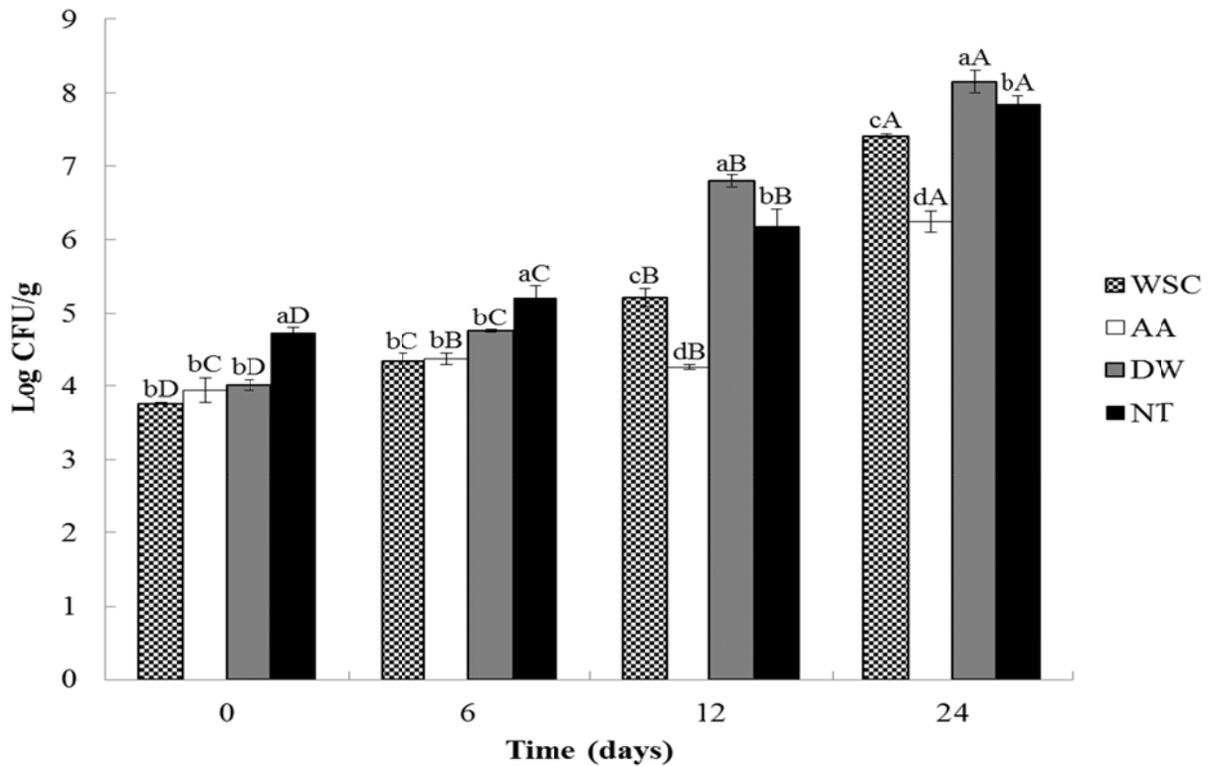water activities are subject to a greater rate of microbial growth over time (Rahman, 2007).

Figure B1. Aerobic plate counts of shrimp during storage at 4 °C. [a-d]Means±SD with different letters between treatments at the same storage times indicate significant difference (P ≤ 0.05). [A-D]Means±SD with different letters within treatments at different storage times indicate significant difference (P ≤ 0.05). WSC = water-soluble chitosan, AA = acetic acid, DW = distilled water, and NT = no treatment.

The antimicrobial mechanism of AA is related to hydrogen ion infiltration into the cell

and subsequent alteration of pH balance, which may lead to cell injury or death. Additionally, the

undissociated form of AA was also shown to be an important factor that inhibits growth of many

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pathogenic and spoilage microorganism (Adams & Hall, 1988; Lindgren, 1993). Adams and Hall (1988) reported that undissociated acid molecules can penetrate into bacterial plasma membranes. Release of hydrogen ions and conjugate bases by acid dissociation occurs due to the higher intracellular pH of the cytoplasm, resulting in interruption of the membrane protein-motive force. This inactivates the energy yielding and transport process that is dependent upon it, leading to the inhibition of cell growth. According to Surve, Sherikar, Bhilegaonkar, and Karkare (1991), the bacteriostatic effect of AA is temporary, especially at low concentrations, and lessens over time simultaneously with a gradual rise in pH towards normalcy.

The AA treatment had higher antimicrobial activity compared to WSC at 24 days of storage, likely because there were a greater number of hydrogen ions in AA solution (0.1 M) compared to WSC particles in solution (0.5%) that could interact with microorganisms. The ability of CH's NH_3^+ groups to bind to negatively charged cell walls of bacteria, resulting in impairment of essential bacterial activities, has been a commonly suggested mode of action (Raafat & Sahl, 2009). Liu, Du, Wang, and Sun (2004) found irregular or broken down cell membranes in *E. coli* and *S. aureus* treated with a CH solution, indicating that the cell membrane can be destroyed by CH. Also, CH may be able to form a polymer membrane on the cell surface, preventing nutrients from reaching the cell (Zheng & Zhu, 2003). Je and Kim (2006) stated that the positive charges of WSC can facilitate binding to anionic lipopolysaccharides on bacterial membranes by electrostatic interaction, hindering nutrient flows and leading to bacterial death. They also found that leakage of cytoplasmic α-galactosidase from the cell membrane was dependent on the dose of WSC applied and the treatment time. Because of this possible nutrient depletion mechanism, in our study, WSC particles may have required a longer time to inhibit bacteria so they could be more synergistic with frozen storage, rather than refrigerated storage.

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This is especially true when using lower concentrations of WSC during refrigerated storage, due to the higher microbial loads and faster spoilage times associated with this storage method, compared to frozen storage. According to a study conducted by Jarry et al. (2001) on the antimicrobial activity of CH against different microorganisms, it was shown that bacteria which were separated from the CH solution by membrane filtration could grow rapidly. The bacteriostatic activity of CH may be linked to its ability to bind to microbial cells, negating any further activity against unbound microorganisms (Rhoades & Roller, 2000). The amine groups of CH are positively charged in acidic medium, creating a large charge density (Gan, Wang, Cochrane, & McCarron, 2005). Higher positive charge density in CH is associated with strong electrostatic interactions (Kong et al., 2008). WSC dissolved in water may not have as high of a charge density as CH in AA, which can possibly decrease its capability to bind to microorganisms.

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