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Antimicrobial Use of Native and Enzymatically Degraded Chitosans for Seafood Applications

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ANTIMICROBIAL USE OF NATIVE AND ENZYMATICALLY DEGRADED CHITOSANS FOR SEAFOOD APPLICATIONS

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An Abstract of the Thesis Presented
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Millions of pounds of crab and shrimp shell waste are created every year by the seafood industry. This waste is very resistant to biodegradation and disposal is problematic. Crustacean shells are composed mainly of chitin that can be converted to chitosan by deacetylation. Many current shelf life extension and pathogen inhibition methods employ the use of various chemical preservatives. Chitosan, an abundant natural polysaccharide, possesses antimicrobial as well as functional properties that may be useful in improving quality of stored foods. These properties may be influenced by a number of factors that must be studied before determining the most useful food applications of chitosan.

The objectives of this research were to: 1) study the efficacy of powdered chitosan and chitosan dips in enhancing the refrigerated shelf life of minced salmon trim and salmon fillets; 2) determine the feasibility of degrading chitosan with commercially available enzymes, alpha amylase and bromelain; and 3)

study the effects of native and enzymatically degraded (with alpha amylase) chitosans in vitro, in two different media, against *Pseudomonas aeruginosa* and *Listeria innocua*.

The first study examined the effects of 1% high (HMW) and low molecular weight (LMW) chitosan dips (prepared in 1% acetic acid) applied to salmon fillets as well as powdered HMW and LMW chitosan mixed into salmon trim. The effects were measured over the course of two weeks of refrigerated storage and the analyses included: total aerobic plate counts (APC), total volatile base nitrogen (TVBN), and pH. The results of this study indicated that HMW chitosan dips could effectively reduce aerobic plate counts and reduce TVBN values of salmon fillets and thereby extend refrigerated shelf life. Mixing powdered native chitosan in salmon trim resulted in no significant differences among treatments.

The second study examined the ability of two common enzymes, alpha amylase and bromelain, to degrade chitosan solutions to confer water solubility to the chitosan at neutral pH. Results of this study indicated that bromelain was ineffective, however alpha amylase was able to degrade chitosan solutions as was evidenced by reduced solution viscosity and increased water solubility at neutral pH.

The third study examined the antimicrobial effects of different concentrations of alpha amylase degraded (for 10 minutes, 1hour and 24 hours) and native chitosans against *Listeria innocua* and *Pseudomonas aeruginosa* in nutrient (NB) and trypticase soy broth (TSB) over 4 days at 4°C. Chitosan treatments effectively reduced *Listeria* counts in TSB by at least one log, but only

significantly so on day three. The 24 hour degraded chitosan and lower percentage chitosan treatments were the least effective. No significant reductions in *Pseudomonas* counts were observed in TSB for any treatment. In NB all the chitosan treatments had significantly ($p<0.05$) lower (up to 3.4 logs) *Pseudomonas* counts than both controls on all four days. Log reductions increased with increasing chitosan percent. Ten minute degraded chitosan had slightly greater log reductions compared to other treatments. The results of this experiment indicated that the antimicrobial effectiveness of chitosan depended on the nutrient matrix, chitosan concentration, degradation time, and bacteria type.

The results of this research indicate that chitosan must be solubilized to act as an antimicrobial agent in salmon. Chitosan can be degraded successfully by alpha amylase, producing a water soluble chitosan that may have a use in a product such as minced salmon trim. In vitro antimicrobial action of the chitosan decreased with increasing degree of degradation and depended greatly on the type of media used, indicating that many factors may influence the effectiveness of chitosan in a real food system. Future research should be done to determine if the enzymatically produced water soluble chitosan can act as an antimicrobial agent when incorporated into salmon trim or when used as a dip on salmon fillets.

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INTRODUCTION

Consumer Preferences for Fresh Fish

Buying patterns for fresh fish vary from one region of the country to the other. There are many variables that affect consumer buying patterns (Hadlett and Raab, 1990). Some of these factors include cost, availability, objection to the smell, and fish quality. Quality can be an issue for those people who live in remote parts of the country and have to have their fish shipped in. The longer the supply chain, the more chance there is for temperature abuse, which can cause many quality problems with fresh fish.

Hadlett and Raab (1990) reported that 96 percent of the respondents to their consumer buying pattern survey considered freshness to be very important. Two thirds of the respondents thought it was difficult to determine the freshness of fish by appearance and the same number of persons said they would buy fish more often if they knew it was fresh. Quality was the most important factor when purchasing fresh fish.

In a study conducted by Peavey *et al.* (1994), consumer attitudes toward fresh fish were studied utilizing a survey. Freshness was the paramount concern as well as appearance and smell when consumers were purchasing fresh seafood with color of the fillet considered important as well. The survey results indicated that consumers would not buy a fillet that was gray or yellow, for white-fleshed fish, or a fillet of salmon that was "neon-red" or "funny-red." Texture and smell were also deemed as important to fresh fish consumers. Results indicated

that the texture of the fish should be firm and no detectable "fishy smell" should be present.

Fish Spoilage

Following death, all fish pass through the following stages: rigor mortis, post rigor, autolysis, and degradation caused by bacteria (Ehira and Uchiyama, 1986). The pH of the flesh first falls as glycogen stores are converted to lactic acid and then it rises as nucleotide compounds are broken down and bacteria produce basic waste products such as ammonia. Adenosine triphosphate (ATP) is catabolized during the spoilage process to the following components : Adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate (IMP), inosine (I), hypoxanthine (Hx), xanthine and uric acid (Ashie *et al.*, 1996). Initial breakdown of ATP to IMP results in a good, "fresh fish" flavor component in the flesh. Further breakdown of IMP to the other catabolic by-products results in detrimental changes in texture, color, odor and flavor (Haard, 1992).

During storage of seafood, the enzymes produced by the bacterial flora begin to break down the nitrogen rich nucleotides in the fish muscle (Jay, 2000a). The result of this is production of volatile compounds. These volatile compounds affect the flavor and odor of the fish. The major compound that is affected in marine fish is trimethylamine oxide (TMAO), an osmoregulatory compound most common in cold-water, white fish species such as cod (Regenstein and Regenstein, 1991). When TMAO breaks down it is first converted to

trimethylamine (TMA) and then to di-methylamine and finally to formaldehyde. Lipids in the fish can react with the TMA to produce off-odors and a fishy smell.

Many factors influence fish shelf life including temperature, handling stress (Erikson *et al.*, 1997), storage conditions (Ashie *et al.*, 1996) and starvation of the fish prior to capture (Einen and Thomassen, 1998). Spoilage of fish is the result of microbial action and natural enzyme processes within the flesh (Ashie *et al.*, 1996). Natural microbial flora exist on the surface of fish. Whole fish spoil more slowly than fish that have undergone processing, gutting and filleting. Bacteria can migrate from the gut of the fish into the flesh or may migrate through cuts in the flesh or through the lateral line, a process that occurs more quickly with temperature abuse. Once fish are processed, the bacterial load on the surface of the fish is distributed to the inside of the fish by knives or other processing equipment.

There are a few common classes of bacteria responsible for fish spoilage. Fish from warm waters carry a different bacterial flora than those from colder waters. Common cold water microbial flora include *Pseudomonas*, *Aeromonas*, *Flavobacterium*, *Shewanella*, *Listeria* and *Vibrio* species (Jay, 2000a). Many of these bacteria on cold-water species are gram-negative, psychrotrophic bacteria and are more likely to grow in typical storage conditions because they grow well in cold conditions (Jay, 2000b).

Determination of Seafood Freshness

Seafood freshness may be difficult to determine. Acceptability as measured by bacterial load is the most primitive method. Each species of fish may have different species of bacteria and a unique chemical makeup that will vary the acceptable bacterial load from ~10^2 to 10^4 CFU/g and unacceptable load from ~10^6 to 10^8CFU/g. Research has been performed to determine shelf life and microbial spoilage levels for specific species of fish (Koutsoumanis and Nychas, 2000). Additionally, several different chemical indices have been developed to estimate the freshness of seafood. These methods include total volatile base nitrogen [(TVBN) (Botta *et al.*, 1984)] determination, trimethylamine (TMA) determination (Hungerford, 1998), and the K index and the P index. The K index is defined by the following equation: [(inosine + hypoxanthine) / (ADP, AMP, IMP, I, Hx and ATP)] (Ehira and Uchiyama, 1986). The P index, another index used to determine fish freshness, is defined by the following equation: (TMA concentration / TVBN concentration) x 100 (Malle and Poumeyrol, 1989). Correlations that tie microbial counts to levels of degradation compounds (TVBN and TMA, etc.) and sensory perceived freshness have had limited success and are species specific. For example, TVBN and K-value were found to be inappropriate for determining freshness of carp that had been treated with irradiation (Icekson *et al.*, 1996). Emborg *et al.* (2002) discovered in a study of modified atmosphere packaged salmon that TMA concentrations indicative of unacceptablility in cod (30 mg/100 g fish) were inappropriate in salmon due to low trimethylamine oxide in salmon. A K-value of 70 to 80% was suggested as a

"good-quality" indicator for Atlantic salmon (Erikson *et al.*, 1997) with values lower than 40 to 50% indicating excellent quality. The authors also discovered that the onset of rigor began within two to four hours after death in stressed fish compared to 25 hours in unstressed fish. In contrast, Ehira and Uchiyama (1986) in a study of species of fish found in Japanese waters determined that, in general, K-values of 20% or below are appropriate for sashimi grade fish whereas K-values above 75% indicate poor quality fish.

Shelf Life of Seafood

Shelf life of seafood varies by species, method of kill and temperature conditions. Reddy *et al.* (1997) found that the sensory shelf life of aquacultured salmon fillets varied greatly depending on storage temperature. The shelf life of salmon was between 16 and 20 days at 4°C but at 8°C the shelf life dropped to between 8 and 10 days. At 16°C, shelf life was only three to four days.

Emborg *et al.* (2002) conducted a study with salmon harvested at two different seasonal periods, (September/October) and (February/March), and held at modified atmosphere (MAP). Differences in the shelf life at 2°C of the MAP (60% CO_2 / 40% N_2) salmon were discovered based on season of harvest alone. Shelf life of salmon harvested in the September/October time frame had a sensory shelf life of 14 days whereas the sensory shelf life of the February/March harvested salmon was approximately 21 days. The reason for this difference was attributed to lower levels of the spoilage bacteria on the fish harvested in the colder months compared to those harvested in the warmer months.

The shelf life of black skipjack stored in ice at 0°C was found by Manzano *et al.* (2000) to be approximately 18 days. This was determined by two methods, sensory assessment and K-value (75%), both of which were found to correlate well to predict shelf life of this species of fish.

Koutsoumanis and Nychas (2000) studied the shelf life of Mediterranean gilt-head seabream stored aerobically at temperatures of 0, 5, 10 and 15°C. Multiple methods were used to determine the shelf life including trimethylamine analysis, total volatile base nitrogen analysis, aerobic plate counts and sensory analysis. Shelf life, as determined by sensory analysis, was 212 hours (~9 days) at 0°C and 104 hours (~4 days) at 5°C. The *Pseudomonas* population at these two times was log 7.1 for both treatments. TMA concentrations were very low throughout the experiment and were determined not to be a good indicator of quality for this fish. TVBN concentrations at sensory rejection time for 0 and 5°C storage were approximately 22 mg/100 g of fish for both treatments, which is lower than rejection concentrations of TVBN for other species of fish.

Jeon *et al.* (2002) reported the TVBN concentrations of cod and herring stored at at 4°C reached rejection levels (30 mg/100 g fish) at day six and eight, respectively. Corresponding trimethylamine concentrations for cod and herring on day six and eight were 5.1 and 3.07 mg/100 g, respectively. This, again, demonstrates the variability of these shelf life indicators based on species.

Shelf life of minced fish products is often lower than that of intact fish portions due to the greater surface area of the mince. Shelf life of catfish mince was found to be only five days at 5°C and seven days at 0°C as researched by

Suvanich *et al.* (2000a). Shelf life was determined by TVBN concentrations and sensory analysis. Strong odor was detected at TVBN concentrations of 30 mg/100 g of fish. Aerobic plate counts in the same study (Suvanich *et al.,* 2000b) were found to be log 5.5 in the fresh mince and those values rose to log 8 by day three, prior to detection of sensory spoilage or TVBN indicated spoilage.

Seafood Preservation Techniques

Seafood is preserved in many ways including traditional methods such as salting, drying, and smoking (Ashie *et al.*, 1996). Reduction of water activity is the goal of all three of these methods. By reducing the water activity of the food, the microorganisms find it difficult to proliferate on the surface of the food. These three methods also result in cessation of normal autolytic enzymes in the fish but do not preserve the fresh flavor qualities of the fish. In industrialized nations the most common methods of seafood preservation are freezing and low temperature storage. Shelf life extension of fresh fish usually begins with high pressure spraying before applying a dip or chemical treatment. This method involves spraying the whole fish to remove the bacteria that inhabit the slime on the surface of the fish (Kosak and Toledo, 1981). The high-pressure wash utilizes fresh seawater but this water needs to be decontaminated first. Often chemicals such as chlorine dioxide are added to the seawater to accomplish this.

Low Temperature Storage

Low temperature storage involves storing fish, generally on ice, at low temperatures, below 4°C, but as close to 0°C as possible as the shelf life can be reduced by as much as one day for each hour the fish is kept above 0°C (Ashie *et al.*, 1996). Low temperature storage reduces the growth rate of the microbial flora but other factors can counteract this reduction such as handling, storage after catch, species of fish, the way the fish were caught, and the history of the fish. Often the fish will not be held at the proper temperature at some point from capture to the dinner plate, resulting in a rapid increase in the microbial growth. Low temperature storage also selects for those organisms adapted to low temperatures (Ashie *et al.*, 1996). Fish that live in cold waters have microbes such as *Listeria monocytogenes* and *Clostridium botulinum* that are adapted to cold environments. These two bacteria are human pathogens and can produce toxins at all temperatures, including refrigerated temperatures.

Cold storage of fish on ice may be time consuming, labor intensive as well as costly (Reppond *et al.*, 1983). A newer method of cold storage after catch on board vessels is utilization of mechanically refrigerated seawater (RSW). One study indicated that storing salmon in RSW reduced microbial growth compared to storage on ice (Bronstein *et al.*, 1985). It is assumed that storage on ice allows aerobic bacteria to more easily grow because there is more exposure to air in ice. A drawback of a RSW system is that if some of the fish do undergo spoilage, all of the fish in the tank may be affected and the contents of the entire tank may not be acceptable (Lee and Kolbe, 1982). Refrigerated seawater

technology has also been used in conjunction with CO_2. Addition of CO_2 reduces the pH and reduces bacterial growth. Drawbacks of this refrigerated seawater technology include salt uptake by the fish, which results in flesh discoloration and other sensory changes (Bullard and Collins, 1978).

Another temperature strategy to increase shelf life of fish is super chilling at temperatures just under the freezing point, generally between -3 and -4°C (Ashie *et al.*, 1996). This method slows down the natural metabolic degradation of the fish as well as seriously inhibiting microbial growth. Shelf life of sea bass was found by Chang *et al.* (1998) to vary greatly depending on storage temperature. Changes in microbial growth and nucleotide breakdown as measured by K value and total volatile base nitrogen (TVBN) were studied at temperatures of 10, 5, 0 and -3°C over storage of up to 45 days. Maximum shelf life cut-off values were defined as TVBN greater than 15 mg/100 g fish, K-value greater than 50% and aerobic plate counts exceeding 3×10^6 (CFU/g). Shelf life of partially frozen (-3°C) sea bass was found to be 37 days whereas at 0°C, the shelf life dropped to 14 days. At 5 and 10°C the shelf life decreased to three and two days, respectively. The TVBN-based shelf life in this study was longer than the microbial-based shelf life and K-value-based shelf life was the shortest of all three indicators. The microbial count was only found to be an accurate measurement of quality at or above 0°C. Superchilling may not be practical though as the low temperatures required are difficult to maintain throughout the distribution process.

Emborg *et al.* (2002) identified *Photobacterium phosphoreum* as the major spoilage organism in Atlantic salmon. The authors were able to extend the shelf life of salmon stored at modified atmosphere (60% CO_2/40% N_2) by one or two weeks by first freezing the product at -20°C or -30°C prior to storage at 2°C. The researchers postulated that the freezing killed a sufficient number of the *Photobacterium phosphoreum* to extend the shelf life of the packaged salmon.

Dips/Chemical Treatments

Much research has been done to study the feasibility of using various organic acids such as acetic, lactic, malic and tartaric, as dips. Dips containing organic acids may act as antimicrobials by causing the cell membrane of the bacteria to become permeable. Phosphates such as trisodium phosphate (TSP), sodium tripolyphosphate (STP), and sodium metaphosphate (SMP) (Marshall and Jindal, 1997) have also been studied as seafood dips to extend shelf life. Polyphosphates have been shown to enhance antimicrobial activity possibly because they act as metal ion chelators causing changes in the cell membrane of the bacteria which interferes with normal functioning (Ashie *et al.*, 1996). Phosphates may also act as pH buffers, prevent lipid oxidation and promote water binding capacity. Other chemicals such as chlorine dioxide (Kim *et al.*, 1999), sodium lactate (Williams *et al.*, 1995), sodium acetate and potassium sorbate both with and without a lactic acid bacteria cultures (Kim and Hearnsberger, 1994) have also been investigated as possible dips for seafood products.

Sodium lactate at levels of 2% applied in a vacuum tumbler was able to extend the shelf life of catfish fillets stored at 1°C from four days to seven days (Williams *et al.*, 1995). Statistically lower ($p<0.05$) plate counts were evident compared to the controls. In addition, pH and water activity were not affected by the addition of the sodium lactate. Cooking yields were higher as well in the sodium lactate treated fillets due to increased water retention. Sodium lactate solutions at levels of 1% were not as effective as the 2% treatment.

Marshall and Kim (1996) studied the effectiveness of acetic and lactic acid dips on the sensory and microbiological qualities of catfish fillets. Fillets that were treated with 3-4% acetic acid or a combination of 2% acetic and 2% lactic acid for a total of 30-60 seconds showed suppressed growth of the normal spoilage bacteria for up to four days. Acetic acid was found to be a better antimicrobial agent than lactic acid. One drawback of the use of acids was they were disliked by the sensory panel. The acetic acid resulted in flesh discoloration and an acidic odor in the fish treated with levels of 2% acetic acid or above. Exposure times to the dip were also advised to be no more than thirty seconds to avoid the objectionable odor.

Bal'a and Marshall (1998) investigated the effects on color, pH, aerobic plate counts and growth of *Listeria monocytogenes* of several different 2% organic acid (citric, malic, hydrochloric, lactic, tartaric and acetic) treatments on catfish fillets for eight days at 4°C. The fillets were dipped for ten minutes in one of the treatments or in distilled water (controls) and were then allowed to drip for five minutes at 4°C. Fillets in the *Listeria* study were inoculated for five minutes

in a one-liter bath to which 0.1 mL of an eight-strain culture of *Listeria* had been added prior to dipping in the other treatments. Results indicated the acid treatments significantly reduced the surface pH of the catfish fillets throughout the experiment with the hydrochloric acid resulting in the lowest pH values. A gradual rise in pH over time was observed in all treatments. Hunter color analysis revealed acid treatments caused a bleaching effect on the fish tissue, with citric acid causing the most effect on color change and malic acid, the least effect. All of the acid treatments also caused a yellowing of the fish flesh except for hydrochloric acid, which actually reduced the yellow tones compared to the control. Initial aerobic plate counts for all acid treatments were lower than the control with hydrochloric acid producing the lowest counts. Control samples reached log six by day eight of the study, indicating spoilage, while the acid dipped samples remained below log four. *Listeria* counts on acid-dipped fillets were only one log lower during the study compared to the controls.

Marshall and Jindal (1997) studied the antimicrobial effects of various phosphate treatments on catfish frames. Ten percent phosphate (trisodium phosphate (TSP), sodium tripolyphosphate (STPP), and sodium metaphosphate (SMP) solutions were prepared in autoclaved tap water. The solutions were refrigerated to 5°C and the catfish frames (which had been packed in ice and were used within four hours after death) were randomly chosen and dipped in one of the treatments for five minutes. After dipping the frames were removed and drained for two minutes on sterile muslin cloth. The frames were then shaken manually with peptone diluent and were plated onto petri film to assess

aerobic plate counts. After determining that TSP was the most effective treatment, a shelf life study was conducted at 5°C using TSP. Another treatment involved dipping the frames in the 10% TSP solution followed by an additional two minute rinse in tap water at 5°C. Controls in this study were frames that had been dipped in autoclaved tap water at 5°C. Aerobic plate counts were analyzed over an eight day period. Results of this study showed that the control frames were above acceptable APC (log 7) at day four. The washed treatments had shelf lives of three to four days past day four with counts of log 4 on day eight. Frames that were rinsed after phosphate dip treatment had higher counts, log 7, than the unrinsed frames on day eight.

Phosphates have also been used as dips on other meat products such as chicken skin. Capita *et al.* (2001) studied the effects of trisodium phosphate (TSP) solutions on the *Listeria* microflora of chicken skin during refrigerated (2°C) storage. Chicken skin was inoculated with *Listeria monocytogenes* and then dipped in either distilled water (control) or TSP solutions of 8, 10 or 12% for 15 minutes. Microbial analyses were conducted on days 0, 1, 3 and 5. Results of this experiment showed that initial log reductions for the TSP dips compared to the control ranged from 1.5 to 2.7 for the 8 and 12% treatments, respectively. By day five, the 12% TSP dip reduced *Listeria* counts by log 3.6 whereas the 8% TSP treatment resulted in a log 2.1 reduction compared to the water dipped control.

Chlorine dioxide, used as a drinking water sanitizer in Europe, has been studied as a possible dip to extend shelf life of fish. Kim *et al.* (1999) studied the

effects of different concentrations (20, 40, 100 and 200 ppm) chlorine dioxide dips prepared in 3.5% salt solutions on two species of whole or filleted fish, grouper and Atlantic salmon as well as shrimp and scallops. The fish were dipped in one of the various solutions for five minutes. All seafood products were stored in bags in crushed ice at 5°C for seven days except for the whole fish which were stored in crushed ice in a display case at 3°C. Sensory and microbial analysis were conducted on days zero, three and seven. Results varied for the different types of seafood with no significant difference observed among the treatments on day seven in the grouper fillet. However, there was a significant difference among treatments in the salmon fillets on day seven; the APC of the control fillet and 200 ppm chlorine treated fillet were log 7.6 and log 6.5, respectively. Significantly lower APC were also observed on day seven for chlorine dioxide treated scallops, whole grouper, and whole salmon (muscle, not skin). The most effective antibacterial treatments in this study resulted in less than desirable sensory effects. Chlorine dioxide at levels of 100 and 200 ppm resulted in some discoloration (rust color) of the scallops. Negative sensory effects, melanosis and chlorine odor were also observed in the shrimp treated with 100 and 200 ppm chlorine dioxide. These levels of chlorine dioxide also caused a rusty color to develop in the salmon fillets and resulted in a bleaching of the grouper fillets.

The effects of both storage temperature (0-2, and 5°C) and 2.5% potassium sorbate (only at 5°C) on the shelf life of whole freshwater silver perch over the course of 25 days were studied by Gelman *et al.* (2001). The potassium

sorbate dipped fish were dipped for a total of 30 minutes. Analyses conducted on days 3, 5, 10, 15, 20 and 25 included sensory, aerobic plate counts (flesh and skin), total volatile base nitrogen (TVBN), and hypoxanthine concentrations. At day 25 sensory scores remained highest in the 0 to 2°C stored fillets but potassium sorbate increased sensory scores compared to the 5°C treatment with no potassium sorbate. No significant differences in TVBN levels were observed in the 5°C treatment compared to 5°C with potassium sorbate although TVBN did remain steady in the 0 to 2°C treatment throughout the 25 days. The same observations were observed with the hypoxanthine concentrations. Addition of potassium sorbate slowed the microbial growth during the first 15 days, especially on the skin, compared to temperature conditions of 5°C with no potassium sorbate. Overall, lower temperatures were more effective in improving shelf life of the fish compared to the chemical treatment.

Chitin and Chitosan

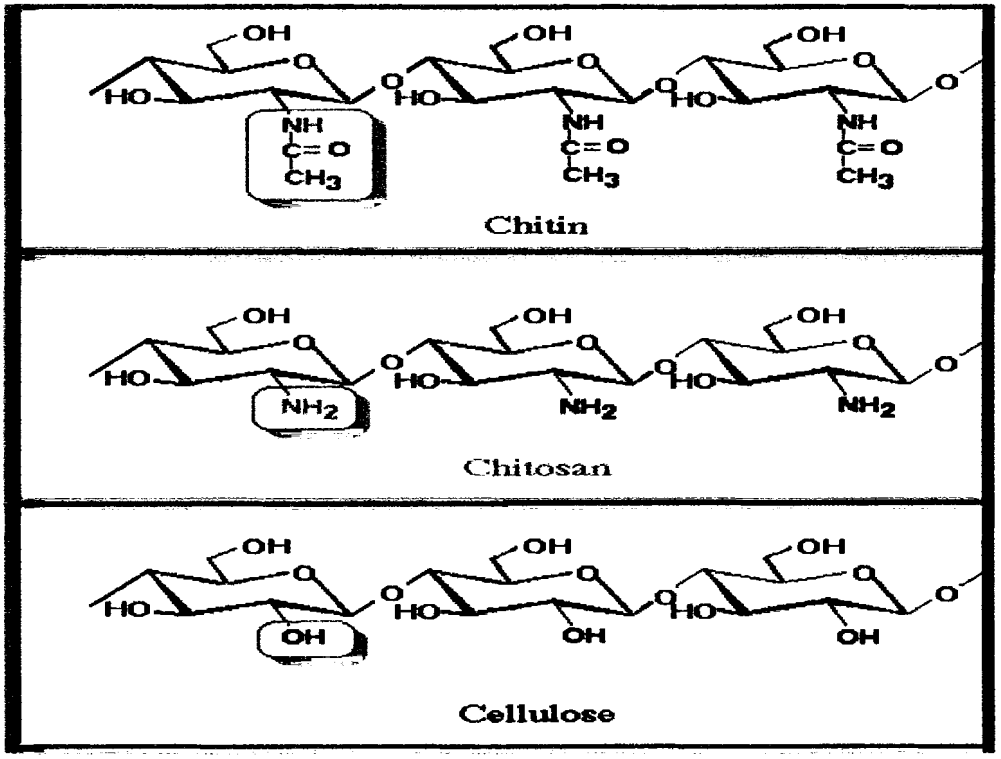
Chitin is found in a variety of places in nature. Sources include fungi, insect exoskeletons, and marine invertebrates (Hirano, 1997). Shellfish chitin from crab and shrimp comprises from 17% to 32% of the dry weight of the shell (Shahidi and Naczk, 1989). Other sources such as fungi contain more chitin per weight and are more prevalent. It is estimated that fungi and microorganisms produce more than a hundred billion tons of chitin per year.

Worldwide crab and shrimp processing waste, as of 1991, was estimated to be $1.5x10^6$ metric tons a year (Knorr, 1991). This waste is mostly dumped at

sea or in landfills, but stricter laws are limiting this practice (Shahidi and Naczk, 1989) which causes a dilemma for the industry. Utilizing the shellfish waste for chitin production provides a solution for the waste disposal problem. It is estimated that the chitin that could be produced from the worldwide shellfish processing waste is $1.0x10^9$ metric tons annually (Jeon *et al.*, 2000).

The chemical name of chitin is poly-β-(1,4)-N-acetyl-D-glucosamine (Tsugita, 1989). The chemical name of chitosan, which is derived from chitin by deacetylation, is poly-β-(1,4)-glucosamine. The structure of both of these compounds is similar to cellulose (Figure 1). Both chitin and chitosan are insoluble in water but can be dissolved in organic acids.

Figure 1. Chemical Structures of Cellulose, Chitin and Chitosan



Production of Chitosan from Chitin

Preparation of chitosan from crustacean shells involves several steps, alternating treatments of base and acid (Shahidi and Naczk, 1989; Skaugrud and Sargent, 1990; Healy *et al.*, 1994; Jeon *et al.*, 2000). The first step is to remove protein from the shells with a weak basic solution (usually sodium hydroxide or potassium hydroxide). The shells are then treated to de-mineralized with an acid, resulting in chitin. The chitin is then treated with a strong basic solution, which causes de-acetylation. The precipitate formed in this step is crude chitosan. The crude chitosan is washed with water and dissolved in dilute aqueous acid. A clear supernatant results from this step and the addition of a basic solution neutralizes the supernatant and pure chitosan precipitates out.

The traditional method of chitin/chitosan production involves the use of large amounts of acids and bases which can be cost-prohibitive and environmentally unfriendly. As enzymatic (Ilyina *et al.*, 2000; Jeon and Kim, 2000; Gildberg and Stenberg, 2000) and microbiological (Healy *et al.*, 1994) methods of chitosan production are perfected, the process could potentially become more environmentally friendly and cost-efficient.

Applications of Chitosan

Chitosan can be used in a vast array of applications. Chitosan has properties that allow it to be incorporated into films and gels. Chen *et al.* (1996) incorporated chitosan into a film with methylcellulose and potassium sorbate or sodium benzoate. The resulting film released up to 49% of the incorporated

preservatives within four hours of application with no changes in physical properties of the film.

Uses for chitosan have been found in other fields such as medicine, wastewater treatment, agriculture, cosmetics, and in food (Knorr, 1991). Chitosan has been found to be useful as a wound management aid to reduce scar tissue (Lloyd *et al.*, 1998). Applications have also been discovered in controlled release of lactic acid bacteria during butter and cottage cheese production by encapsulating the bacteria in chitosan beads (Zhou *et al.*, 1998).

Recently, chitosan has also been studied as an antimicrobial agent in foods. Chitosan films have been used to improve shelf life of strawberries by delaying fungal growth and preventing moisture loss (Ghaouth *et al.*, 1991a). In agriculture, chitosan has been demonstrated to be useful for aflatoxin control (Cuero *et al.*, 1991). Chitosan has also been used to coat cucumbers and bell peppers and was effective in reducing the amount of moisture loss and allaying fungal growth (Ghaouth *et al.,* 1991b).

Chitosan has been studied extensively by Korean researchers as an additive in many food products including sweet potato starch noodles (Baek *et al.*, 2001), whipping cream (Kim *et al.*, 2000), shortened cake (Ha *et al.*, 1999), sausage (Park *et al.*, 1999) and fish meat paste (Cho *et al.*, 1998) although only the abstracts are available in English.

Non-Meat Food Applications of Chitosan

Roller and Covill (2000) studied the antimicrobial properties of chitosan glutamate, a chitosan derivative, in mayonnaise and mayonnaise-based shrimp salad. The researchers initially tested the effects of chitosan glutamate on controlling microbial growth in laboratory media. Afterwards, the derivative was tested in mayonnaise that was inoculated with common fresh mayonnaise spoilage organisms; *Salmonella enteritidis*, *Z. bailii*, and *L. fructivorans*. The mayonnaise was prepared with acetic acid or lemon juice. Chitosan was added to the mixtures at a level of 3 g/L. Growth of organisms in the chitosan dissolved in lemon juice was only slightly inhibited, by one log cycle. When chitosan was dissolved in acetic acid, the effects were much more significant, resulting in bacterial reductions of three to four log cycles. The mayonnaise was reported to have a gelled appearance at chitosan levels of 3 g/L.

Chitosan glutamate was also tested as an anti-fungal agent in apple juice (Roller and Covill, 1999). In this study, chitosan glutamate was tested for its use in controlling bacterial growth in laboratory media prior to use in apple juice. Eight strains of molds and yeasts were tested in the apple juice trials. Levels of chitosan glutamate in the apple juice ranged from 0.1 to 5 g/L. All eight species tested were inhibited by chitosan glutamate. Viable organisms inoculated into the apple juice were reduced by three log cycles initially and an extended lag phase was observed. Some of the organisms showed normal growth after the initial lag phase but others such as *Zygosaccharomyces bailii* were completely inhibited. The level of chitosan needed to inhibit the fungal species varied widely

as well. *Z. bailii* was inhibited at levels of 0.1 g/L whereas *Saccharomyces ludwigii* required a level of 5 g/L for inhibition. These results demonstrated the variability of chitosan action on microorganisms.

Degraded chitosan and its effects on antimicrobial action have also been studied. Rhoades and Roller (2000) theorized that degrading chitosan enhances its antimicrobial action. The investigators studied the effects of degraded chitosan versus native chitosan in laboratory media, saline solution, and apple juice. The investigators degraded the chitosan with lysozyme and crude papaya latex. When mildly degraded chitosan was tested in laboratory media against various strains of bacteria and fungi, an increase in antimicrobial action was observed. Highly degraded chitosan displayed no antimicrobial action. When the degraded chitosan was tested in hummus, a chickpea dip, at levels of 5 g/L the total natural mesophilic organism counts were reduced between four and five log cycles. According to the researchers, the slight increase in activity of the degraded chitosan compared to the native chitosan was not enough to justify the extra time and effort required to degrade the chitosan.

Since traditional chitosan can only be dissolved in an acid solution work is being done to make chitosan applicable to non-acidic foods. Tsai *et al.* (2000) evaluated the use of degraded chitosans, chitoligosaccharides, as antibacterial agents in milk. Chitoligosaccharides prepared by enzymatic degradation of chitosan by cellulase were used in this experiment. The chitoligosaccharides were first tested in nutrient broth against common raw milk pathogens. Subsequent testing took place in sterilized milk that was inoculated with the

same organisms used in the nutrient broth. The final test took place in raw milk containing chitoligosaccharides.

In nutrient broth the minimal inhibitory concentrations of chitoligosaccharides required for the organisms tested ranged from 5 to 29 ppm. In contrast the native chitosan required concentrations from 50 to 100 ppm to be effective. In raw milk samples inoculated with bacterial cultures chitoligosaccharides were able to reduce counts by three log cycles. The use of chitoligosaccharides extended the shelf life of the raw milk by four days at 4°C.

Use of Chitosan in Meat Products

Chitosan has recently been studied as an additive in meat products with much more research being conducted in Korea and other Asian countries (Lin and Chao; 2001; Jo *et al.*, 2001). Earlier research was conducted incorporating chitosan into ground beef (St. Angelo and Vercellotti, 1992; Darmadji and Izumimoto, 1994).

Jo *et al.* (2001) researched the quality of a sausage product prepared with water soluble chitosan oligomers (molecular weight of 5000 kDa). The sausage was formulated with the chitosan oligomers at a level of 0.2% and then was either vacuum packaged or stored in air at 4°C for three weeks. Each week, the sausage was tested for microbial growth, lipid oxidation (TBARS), Hunter color and sensory attributes. Results of this experiment indicated that the addition of the chitosan oligomer had no effect on the microbial growth in the sausage, regardless of storage conditions. At week three of storage under aerobic storage

conditions, lipid oxidation was significantly ($p < 0.05$) reduced compared to the control sausage while no differences were observed in the vacuum packaged sausage. Under aerobic conditions the sausage with the chitosan oligomer had slightly higher L-values than the control sausage with the exception of day zero. The L-values of the vacuum packaged sausage with chitosan oligomer showed no difference compared to the control. No changes in the a-values were observed with any packaging method or sausage treatment compared to the controls. Addition of chitosan to the sausage resulted in higher b-values compared to the controls. Sensory analysis revealed no significant differences among treatments

Addition of chitosan to a low fat Chinese sausage was studied by Lin and Chao (2001). Three different molecular weight chitosans [(LMW, 150 kDa), (MMW, 600 kDa) and (HMW, 1250 kDa)] in 1% lactic acid were added to sausage at a level of 0.1%. The sausages were vacuum packaged and stored at 4°C for a period of nine weeks. Analyses conducted at week zero, three, six and nine included: pH, water holding capacity, lipid oxidation, Hunter color, sensory evaluation and aerobic plate counts. Results of this study revealed that pH of the chitosan treated sausage were lower than the controls, probably due to the lactic acid. Water holding capacity was greater in sausages formulated with MMW and LMW chitosan compared to the control. Hunter color a values were lower, compared to the control and the L and b values were higher than the control. No differences were found among treatments for any sensory attributes. The

addition of the chitosan treatments did not reduce the microbial counts of the sausage formulations throughout the storage period, compared to the control.

Ouattara *et al.* (2000) took a different approach to applying chitosan to meat products. The film-forming properties of chitosan were taken advantage of by incorporating organic acids (acetic and propionic) alone or together with cinnamaldehyde or lauric acid into a chitosan film. These films were studied in contact with various processed meat products (bologna, ham and pastrami) vacuum packaged at 4 or 10°C for 21 days. Microbial counts (of bacteria inoculated onto the meats) and the release of acid from the film matrix were determined on days 0, 7, 14, and 21. Release of the acetic acid from the chitosan films was relatively slow with between 2 and 22% of the acid remaining in the matrix after 168 hours of storage. Propionic acid was completely released from the matrix within 48 hours of storage, regardless of meat product. The growth of lactic acid bacteria were not inhibited by the chitosan film but the growth of *Enterobacteriaceae* and *Serratia liquifaciens* were either delayed or completely inhibited by the films.

St. Angelo and Vercellotti (1992) studied the effects of N-carboxymethylchitosan as a flavor protector in ground beef. The purpose of this study was to utilize N-carboxymethylchitosan to preserve the beefy flavor in warmed over meat products. Some off flavors in meat products are due to lipid oxidation. N-carboxymethylchitosan was used instead of chitosan because it is water-soluble at all pH levels, and can chelate transition metals that catalyze lipid oxidation. The N-carboxymethylchitosan was added directly to the meat at a

level of 0.5%. The hamburger meat patties were then cooked and refrigerated. The control patties were frozen, then thawed, and re-heated.

After two days of refrigerated storage a trained sensory panel evaluated the re-heated hamburger patties. It was discovered that N-carboxymethylchitosan effectively prevented the formation of warmed over flavor in the hamburger patties. No difference in the flavor of the patties with N-carboxymethylchitosan compared to the freshly prepared control was detected. A thiobarbituric acid assay (TBARS) was performed to determine the role of N-carboxymethylchitosan in preventing lipid oxidation. When N-carboxymethylchitosan was added at a level of 600 ppm lipid oxidation was inhibited by 60%.

Darmadji and Izumimoto (1994) also studied the effect of chitosan in meat preservation. In this study minced beef was mixed with powdered chitosan at levels of 0.2, 0.5 and 1%. A multitude of tests were performed on the samples including sensory, chemical, microbiological, and color attributes. After the chitosan was mixed with the minced beef, the meat was wrapped in polyethylene film (Glad Products Company; Oakland, CA) and incubated either at 30°C for 0, 12, 24, or 48 hours or at 4°C for 0, 3, 5, or 10 days. At all of the time intervals, total volatile base nitrogen was tested as well as TBARS, microbial analysis, color analysis and sensory analysis.

Microbiological inhibition was greatest at both temperatures in samples with 1% chitosan. A reduction of two log cycles was evident in the refrigerated samples at 1% chitosan levels compared to the untreated control. Inhibition of

bacteria by chitosan varied by species; *Staphylococci* were the most resistant. Gram-negative bacteria were inhibited by up to two log cycles. Chitosan was also able to inhibit the formation of total volatile basal nitrogen and was most effective at the 1% level.

Use of Chitosan in Seafood Products

The use of chitosan as a preservative in seafood products has been studied to a small degree. Simpson *et al.* (1997) studied the use of chitosan in raw shrimp, both whole and beheaded. The dip consisted of sodium acetate and Tween 80 and chitosan in levels of 0, 1 or 2%. The pH of the chitosan dip was adjusted to 5.6 to assure full solubility. The raw shrimp was dipped and stored in vacuum-sealed bags on ice at 4-7°C for a twenty-day period.

During the course of the experiment the shrimp samples were subjected to TVBN, nucleotide, and sensory analyses. The sensory analysis focused on odor, appearance, and degree of melanosis. Microbiological sampling was performed using inoculated samples. Natural spoilage bacteria in the shrimp were not studied.

The results of this study indicated that chitosan was able to inhibit microbial growth in all of the samples. However, the headless samples were not as greatly affected by chitosan which was theorized by the researchers to be due to higher initial bacterial levels in the shrimp due to contamination from the guts. The total microbial counts for both whole and headless shrimp were significantly reduced ($p<0.05$). Lag phases of all of the spoilage organisms tested were

extended as well. Some organisms such as *B. cereus* had an initial lag phase extension but then the growth of bacteria increased rapidly and surpassed the control by 25 hours of incubation. Results of the TVBN analysis of the shrimp samples indicated a significant reduction in the formation of total volatile bases ($p<0.05$) with the use of chitosan. Chitosan was able to prevent melanosis only in samples that had not been beheaded.

This study demonstrated chitosan's varied effect on different organisms. Differences in the molecular weight of the chitosan were theorized to be the cause of some of the variability from this study compared to other studies conducted using the same spoilage microorganisms. Low molecular weight chitosan seemed to be a more effective inhibitor of microbial growth for some organisms such as *E. coli* (Chang *et al.*, 1989).

Jeon *et al.* (2002) studied the preservative effects of different molecular weight chitosan coatings on Atlantic cod and herring for a 12 day period at refrigerated temperature (4°C). Three different chitosans were prepared from crab processing waste by altering the deacetylation times. Molecular weight and apparent viscosity of these chitosans were determined by using a rotational viscometer. The apparent viscosities of the three chitosans were 360, 57 and 14 centipoise (cP). The edible coatings were produced by first dissolving 1% chitosan in a 1% acetic acid solution. Glycerol was then added to the solutions at a concentration of 1 mL per gram of chitosan. The resulting dips were applied to the fish fillets for 30 seconds, then the fillets were allowed to stand for two minutes at which time they were dipped again for 30 seconds. After dipping, the

fillets were dried for two hours at 40°C in a forced air oven to form a film. All samples were then stored at 4°C for the rest of the experiment. Fillets were tested at days four, six, eight, 10 and 12 for moisture loss, lipid oxidation, total volatile base nitrogen, trimethylamine, hypoxanthine and microbial growth.

Results of this experiment indicated that the 360 and 57 cP chitosan solutions were the most effective in preventing moisture loss, reducing lipid oxidation, reducing TVBN and TMA concentrations and lowering microbial counts. The most highly degraded, 14 cP chitosan coating was not as effective as the more viscous chitosan solutions. TVBN concentrations were kept below 30 mg of N per 100 g of fish, the quality cutoff value, throughout the entire study in both the cod and the herring with the 360 and 57 cP chitosan treatments whereas the untreated cod fillet exceeded this value by day six. Bacterial counts in the untreated and 1% acetic acid dipped cod fillets exceeded the quality cutoff of 10^6 CFU/g between day six and day ten whereas counts in the chitosan treated fillets stayed below this level throughout the 12 day experiment. The chitosan treatments kept the bacteria in the stationary phase through day six whereas the counts continued to climb in the control and acetic acid dipped treatment. This study indicates that the degree of degradation of the chitosan may alter the preservative power of chitosan treatments when used as a dip.

Chen *et al.* (1998) studied the preservation of oysters with two chitosan derivatives, N-sulfobenzoyl and N-sulfonated chitosan. It was discovered that N-sulfobenzoyl was much more effective than N-sulfonated chitosan at inhibiting microbial growth. The authors theorized that sulfonating the chitosan created a

negative charge and thus repelled the cell membrane of the bacteria, which also had a negative charge.

This study did not investigate natural flora within the oyster meat. The oysters were inoculated with potential contaminants. Sulfobenzoyl chitosan at 2000 ppm was able to reduce bacterial counts between three and four log cycles. Sulfobenzoyl chitosan was also found to be much more water soluble than native chitosan. Water solubility is useful in food applications, although sensory analyses were not conducted in this study to determine consumer acceptability.

Chitosan is currently being studied as an anti-oxidative agent in cooked herring (Janak Kamil *et al.*, 2000). Over a ten day period the effects of chitosan on lipid oxidation in cooked herring were studied using peroxide value analysis, conjugated dienes, 2-thibarbituric acid-reactive substances, and headspace propanol. Levels of 150 and 200 ppm of chitosan were effective in reducing lipid oxidation products. Chitosan was demonstrated to be an effective tool for reducing lipid oxidation in this high fat fish.

Antimicrobial Action of Chitosan

There has been much speculation on the mechanisms involved in chitosan's antimicrobial properties. Antibacterial properties are thought to be a result of several factors. Chitosan's chelating properties may remove metals needed by bacterial enzymes (Muzzarelli, 1977). Other theories postulate that chitosan's cationic nature disrupts the cell membranes by reacting with the negatively charged membrane (Chen *et al.*, 1998).

Wang (1992) examined the antibacterial effects of chitosan in vitro against a variety of bacteria typical in food. The organisms tested were *Staphylococcus aureus, Escherichia coli, Yersinia entercolitica, Listeria monocytogenes* and *Salmonella typhimurium*. Chitosan, at levels of 0, 0.5, 1.0, 1.5, 2.0 and 2.5% was added to nutrient broth that had been acidified with acetic acid to a pH of either 5.5 or 6.5. The broths were inoculated with bacteria after the mixtures had been autoclaved and cooled. The inoculated flasks were incubated at 30°C for eight days and were sampled and spread plated on each day of the experiment. Results of the experiment indicated that chitosan was not effective in inhibiting microbial growth in the pH 6.5 broths. However, the growth of most of the organisms was significantly reduced at pH 5.5. Chitosan was most effective against *S. aureus, S. typhimurium, E. coli* and *Y. entercolitica* but was not as effective against *L. monocytogenes*.

Helander *et al.* (2001) studied the effects of chitosan on the cell membranes of gram-negative bacteria including *Escherichia coli, Pseudomonas aeruginosa* and *Salmonella typhimurium*. The effects of chitosan on the outer membrane of the bacteria were analyzed by studying the uptake of a hydrophobic probe, 1-*N*-phenylnapthylamine (NPN), both in the presence and absence of $MgCl_2$ (which protects gram-negative bacteria from outer membrane damage), and by electron microscopy. The chitosan concentrations used were 100 and 250 ppm at two different pH levels, 5.3 and 7.2. Results of the study indicated that 250 ppm chitosan at a pH of 5.3 caused uptake of NPN by *E. coli, P. aeruginosa* and *S. typhimurium*. The uptake was reduced in the presence of

$MgCl_2$, in the case of *E. coli* and *S. typhimurium,* and was totally inhibited in *P. aeruginosa*. Electron microscopy revealed that 250 ppm of chitosan at a pH of 5.3 caused visible changes in the outer membrane of both *E. coli* and *S. typhimurium* as evidenced by thickening and formation of vesicular structures on the outer membrane, thereby affecting the barrier properties of the bacterial cell.

Chitosan derivatives have been demonstrated to have greater antimicrobial activity than native chitosan (Sudarshan *et al.*, 1992). When studying various gram-positive and gram-negative bacteria in media, it was discovered that chitosan glutamate and chitosan lactate were able to reduce bacterial log counts from one to five cycles in an hour for both types of bacteria. (Sudarshan *et al.*, 1992) also reported evidence that leakage of intracellular material was one of the mechanisms of chitosan action at low concentrations. At higher concentrations, antibacterial activity was attributed to chitosan coating the surface of the bacteria, thereby impeding mass transfer across the cell membrane in both directions.

Jeon and Kim (2000) studied the antimicrobial effects against *E. coli* of a chitooligosaccharide (COS) mixture produced by chitinase in a bioreactor. A 1% chitosan solution (89% degree of deacetylation) prepared in lactic acid was adjusted to pH 5.5. After producing the COS mixture, the antibacterial effects of the mixture against *E. coli* were analyzed by adding 0.5 mL of the 1.0% mixture in 0.05 M acetate buffer (pH 6.0) to 49 mL of trypticasease soy broth solution containing 0.5 mL of cultured *E. coli* suspension. This mixture was incubated at 37°C with shaking, and inhibition was measured spectrophotometrically at 640

nm. The addition of native chitosan totally inhibited growth of *E. coli* compared to the control and the addition of COS also inhibited growth but not to the same degree. A study of just the COS in different percentages (0.01, 0.05, 0.1 and 0.5) revealed an increasing inhibition of growth linked to increasing COS concentration.

The antibacterial effects of three different molecular weight chitosans were further studied by Jeon *et al.* (2001). The three different chitoligosaccharides defined as high molecular weight chitoligosaccharides (HMWCOS), medium molecular weight chitoligosaccharides (MMWCOS) and low molecular weight chitoligosaccharides (LMWCOS) were produced by enzymatically degrading 1.0% chitosan (89% degree of deacetylation) solutions with chitosanase (694U/ g protein) in a membrane reactor with three membranes of 10, 5 and 1 kDa respectively. Four gram-negative (*E. coli, E. coli 0157-H7, Salmonella typhimurium* and *Pseudomonas aeruginosa*) and five gram-positive (*Streptococcus mutans, Micrococcus luteus, Staphylococcus aureus, Staphylococcus epidermidis* and *Bacillus subtilis*) bacteria were tested. Both minimum inhibitory concentrations and bactericidal activity of each COS fraction were analyzed. A bactericidal study was conducted in which a bacterial culture was added to acetate buffer at pH 6.0 and incubated at 37°C for one hour at which time serial dilutions were made and plated onto trypticase soy agar. Minimum inhibitory concentrations were determined in trypticase soy broth (no pH stated). Results of the study showed that bactericidal activity was most effective with the HMWCOS and undegraded chitosan. The most effective

treatment against *P. aeruginosa* was the undegraded chitosan, resulting in a 68% kill. The LMWCOS treatment resulted in only a 22% kill of *P. aeruginosa*. All treatments performed better with the other gram-negative bacteria but were more effective against the gram-postive bacteria. Bactericidal activity against all bacteria decreased with decreasing molecular weight. Minimum inhibitory concentrations (MIC) were lowest for the undegraded chitosan treatments for all bacteria. *Pseudomonas aeruginosa* required the highest concentrations of every treatment to inhibit growth. The most susceptible gram-positive organisms were *Streptococcus mutans, Micrococcus luteus* and *Staphylococcus aureus* with MIC for all treatments ranging from 0.01 to 0.1%. The results of this experiment indicate that the higher molecular weight chitosans had more antibacterial activity and that gram-positive organisms were more susceptible to the effects of chitosan.

Liu *et al.* (2000) also studied the antibacterial effects of chitoligosaccharides on *E.coli*. The chitosan was placed in glass vials and was degraded by using γ irradiation at doses of up to 100 kGy under vacuum. The chitosans were dissolved in 2M acetic acid and were added to the nutrient broth. The *E. coli* culture was inoculated into the media at different pH levels and concentrations and was incubated at 37°C for 24 hours. Every two hours the growth was tracked with a spectrophotometer at 610 nm. The authors also attached a fluorescent marker label (fluorescein isothiocyanate) to the chitosan oligomers and the movement of the marker was tracked with a confocal laser scanning microscope. Results of the experiment indicated that the water soluble

chitosan oligomers had a good antimicrobial effect against *E.coli*. Above pH 6.3 the antimicrobial activity decreased and no antimicrobial activity was observed above pH 7.0. The reason for the inhibition was proposed to be the uptake of the chitosan oligomers into the bacterial cell as evidenced by the labeled marker. The authors proposed that the uptake of the chitosan caused the inhibition of transcription from DNA.

Enzymatic Degradation of Chitosan

Enzymatic degradation of chitosan has been studied as a way to confer solubility to chitosan at neutral pH. Many different enzymes and methods have been successfully utilized (Ohtakara *et al.*, 1988; Muzzarelli *et al.*, 1994; Nordtveit *et al.*, 1994; Muzzarelli *et al.*, 1995; Kim *et al.*, 1997; Ilyina *et al.*, 2000; Shin-ya *et al.*, 2001). Pantaleone *et al.* (1992) studied the effects of some commercially available enzymes on chitosan solutions prepared in either 5% acetic acid or in acetate buffer at pH levels of 3.3, 3.6 4.0, and 5.5. Low and high viscosity chitosans, both with a declared 85% deacetylation level, were studied at concentrations of 1.0 or 2.5% w/v for the low viscosity and 0.5 or 1.0% for the high viscosity chitosan prepared in the acetic acid solution or the acetate buffer. The enzymes tested included glycanases, amylases, proteases, tannases and lipases, 38 in total. The enzymes were tested at a 1% level by weight of the chitosan. Enzymes were added to the chitosan solutions at ambient temperature and the reduction in viscosity was measured after a 24 hour period using a Brookfield DV II viscometer. Viscosity reductions varied widely by enzyme class

depending on the source of the enzyme. In the glycanase class viscosity reduction ranged from 68-99%. In the protease class viscosity reductions of 0 to 98% occurred, with papain achieving the greatest and most rapid viscosity reductions. Viscosity reductions of 35 to 100% occurred in the lipase enzyme class.

A more extensive study was conducted by Yalpani and Pantaleone (1994) who tested a number of factors including pH, temperature, original molecular weight of the substrate, chitosan concentration (0.5, 1.0 and 2.0%) and degree of chitosan acetylation. Many different enzymes were studied including cellulase, lysozyme, lipase, hemicellulase, papain, chitinase, and a human saliva preparation. The conversion of chitosan to degraded chitosan was measured viscometrically and by a reducing sugar assay. Many commercial enzymes were able to use chitosan as a substrate. In many cases the viscosity reduction was much greater with some of the commericial enzymes rather than with chitinase. Specific activity of the enzymes was highest with lower concentrations of chitosan. The authors theorized that there was no common lytic agent shared by the enzymes because there were different ideal pH, temperature, molecular weight, degree of deacetylation and substrate concentrations for each enzyme.

Zhang and Neau (2001) studied the degradation of chitosan solutions (0.1, 0.3, 0.5 and 0.7%) with five different degrees of deacetylation (77.8, 76.0, 77.0, 85.6 and 92.4%) by β-glucosidase (3811 U/mg) from an almond emulsion. Hydrolysis was conducted in a 0.1M acetate buffer at pH 5.0.The enzyme was added to the solution at a level of 0.02% w/v. The changes in viscosity were

measured over a five hour period using an Ubbelholde capillary viscometer. Results of the experiment indicated that the initial degradation rate was dependent on molecular weight of the chitosan and the degree of deacetylation. Chitosans with a lower molecular weight and lower degree of deacetylation were more susceptible to hydrolysis by the β-glucosidase. The most rapid depolymerization occurred in the first two hours. The researchers theorized that the enzyme complex may have included a chitinase which may have been responsible for the hydrolytic activity.

Zhang *et al.* (1999) successfully degraded chitosan solutions with a mixture of enzymes that included cellulase (0.8 U/mg), alpha amylase (500 U/mg) and proteinase (40 U/mg). The conditions of hydrolysis were 0.5 g of chitosan (molecular weight 1,500,000 and a degree of deacetylation of 76%) dissolved in 10 mL of 2% acetic acid, pH adjusted to 5.6. Five mg of the enzyme mixture (dissolved in 0.05 mol/L acetate buffer) was added to the chitosan solution and the enzyme was allowed to act on the substrate for 40 minutes at 40°C. The reaction was stopped by boiling the mixture for 10 minutes. Water solubility was confirmed when a portion of the mixture was removed and mixed with concentrated sodium hydroxide, with no subsequent precipitate formation. Characterization of the chitosan oligomers formed was conducted using a hollow membrane filtration membrane to fraction off portions, followed by mass spectrophotometer analysis. By continuous removal of degraded products of a specific molecular weight range, a small scale continuous method of chitosan degradation was developed.

Safety and Regulatory Status of Chitosan

Food ingredients must be generally recognized as safe (GRAS) by the FDA in the United States before they can be used in food products. Chitosan has not been proclaimed GRAS officially by the FDA but one company has tried to get approval for their chitosan. Primex Ingredients ASA, a Norwegian shrimp-derived chitosan manufacturer, proclaimed to the FDA self affirmed GRAS status for their product on March 15, 2001 (Food and Drug Association, 2001). After a year, Primex withdrew this proclamation without receiving a response from the FDA. However, the FDA has approved chitosan for medical uses such as bandages and drug encapsulation. Although chitosan has not achieved GRAS status in the United States, it is widely used in foods in Italy, Finland, Korea and Japan.

Preliminary research studies indicate that chitosan ingestion has some beneficial effects on health including an improved HDL-cholesterol/total cholesterol ratio and lower total serum cholesterol (Maezaki *et al.*, 1993). Kim *et al.* (2001) studied the oral toxicity of chitosan oligosaccharides in rats. The authors reported that the sub acute toxicity of chitosan oligosaccharides was low even at the maximum dosage used in the study, 2000 mg/kg by weight of the rats. A double blind human study by Pittler *et al.* (1999) studied the effects of chitosan supplementation on 34 overweight individuals. After four weeks, no significant weight loss was observed and no differences in serum levels of vitamin A, D, E and beta carotene were observed between the placebo group and those who received chitosan. However, the group receiving chitosan

supplementation did have higher vitamin K levels than the placebo group. No serious side effects of chitosan supplementation were noted. A review article by Ylitalo *et al.* (2002) reported that no significant symptoms have been observed in any human studies, some lasting up to 12 weeks, other than mild constipation or diarrhea in a small percentage of the participants. Conversely, there are some concerns regarding chitosan ingestion including proliferation of bacterial pathogens in the digestive system due to loss of beneficial flora (Tanaka *et al.*, 1997), chelation of calcium and other minerals and possibly other metabolic concerns (Deuchi *et al.*, 1995). Growth suppression has been reported in tilapia (Shiau and Yu, 1999) fed chitosan-supplemented diets containing between two and ten percent chitosan. Shellfish allergenicity may also be a concern since chitosan is derived from crustacean shells.

MATERIALS AND METHODS: ANTIMICROBIAL EFFICACY OF CHITOSAN DIPS AND POWDERED CHITOSAN ON ATLANTIC SALMON PRODUCTS

Objectives

The objectives of this study were to determine the antimicrobial efficacy of a solubilized chitosan dip on Atlantic salmon fillets and powdered chitosan on ground salmon trim. Differences in aerobic plate counts, total volatile base nitrogen, trimethylamine levels and pH between salmon products treated with high and low molecular weight chitosans were also studied.

Experimental Design

The experiment was designed to analyze the effects of different chitosan treatments on pH, total volatile base nitrogen (TVBN), aerobic plate counts, and levels of trimethylamine in Atlantic salmon during a two week storage period at refrigerated temperatures. The experiment was also designed to test effects of low and high molecular weight chitosan, chitosan percentages (0.5, 1.0 and 2.0), and solubilized versus non-solubilized chitosan on fish quality. The chitosan dip portion of the study consisted of four fillet treatments prepared in triplicate, and the powdered chitosan portion of the experiment consisted of seven ground trim treatments, also prepared in triplicate batches (Tables 1 and 2). Analyses were conducted on days one, three, six, 10 and 13 of refrigerated storage.

Salmon Trim Study

Preparation of Chitosan

High and low molecular weight crab chitosan both with a declared deacetylation percentage of 75-85% were purchased from Aldrich (Milwaukee, WI). In order to achieve a greater surface area and greater distribution in the salmon trim the chitosan flakes were ground in a Wiley mill (Arthur Thomas Co.; Philadelphia, PA) with a size 20 sieve attachment two days prior to use. The powdered chitosan was then weighed into small sample bags (VWR; West Chester, PA) prior to mixing with the salmon trim.

Application of Chitosan

Fifty pounds of salmon, consisting of thirty pounds of trim (skinless portions of salmon remaining after commercial filleting) and twenty pounds of fillet were purchased from a commercial Atlantic salmon farm (Heritage Salmon; Eastport, ME). The salmon trim was transported in styrofoam totes with ice to Holmes Hall at the University of Maine the same day it was processed. The salmon was then ground once through a commercial food grinder (Hobart Manufacturing Corporation (Model 84141); Troy, OH). The ground salmon trim was stored in metal bowls covered with Cling Wrap® (Glad Products Company; Oakland, CA) in the refrigerator at 4°C until further treatment. Each batch of salmon was mixed with the previously prepared powdered chitosan in an electric mixer (Kitchenaid; St. Joseph, MI) for one minute on speed four with the paddle blade attachment. Both the mixer blade and bowl were washed between each

treatment. Each batch (625 g) was divided between five separate sample bags (~125 g each), one for each day of analysis.

Table 1. Treatment Codes for Salmon Trim Study

Code	Treatment
T	Salmon Trim with no Chitosan Added
.5TL	0.5% Low Molecular Weight Chitosan in Salmon Trim
1TL	1.0% Low Molecular Weight Chitosan in Salmon Trim
2TL	2.0% Low Molecular Weight Chitosan in Salmon Trim
.5TH	0.5%High Molecular Weight Chitosan in Salmon Trim
1TH	1.0% High Molecular Weight Chitosan in Salmon Trim
2TH	2.0% High Molecular Weight Chitosan in Salmon Trim

Chitosan Dip Preparation and Use on Salmon Fillet

The remaining 20 pounds of salmon was in the form of whole fillets. Upon arrival at the University of Maine, the fillets were cut into approximately 125 g portions. The chitosan dips were prepared by mixing 10 g of either high or low molecular weight chitosan (Aldrich Chemical Company Inc.; Milwaukee, WI) with 1 L of a 1.0% acetic acid (EM Science; Gibbstown, NJ) solution. The dips were mixed in a household Kitchenaid (Model KSM90) mixer (Kitchenaid; St. Joseph, MI) at a setting of four for five minutes with the paddle attachment. The

remaining dip consisted of the 1.0% acetic acid (EM Science; Gibbstown, NJ) solution. All dips were poured into shallow plastic containers. Random fillet portions were placed in one of the three dips for 30 seconds, then removed from the dip and allowed to drip while held vertically for 15 seconds. Then they were placed on styrofoam trays and over wrapped twice with plastic wrap and immediately placed in the refrigerator (General Electric Model CTX14CYTDRWH; Louisville, KY) at 4°C. Control fillets were placed on styrofoam trays without dipping, then over wrapped and refrigerated.

Table 2. Treatment Codes for Salmon Fillet Study

Code	Treatment
F	Control/No Dip
FA	1% Acetic Acid Dip
FL	1% Low Molecular Weight Chitosan in 1% Acetic Acid
FH	1% High Molecular Weight Chitosan in 1% Acetic Acid

Chemical Analyses

Moisture

Five gram samples of both raw trim and fillet were put in pre-weighed scintillation vials (Wheaton Scientific, Millville, NJ). The vials containing the samples were dried in a vacuum oven (National Appliance Co.; Portland, OR) for 24 hours at a temperature of 70°C. The vials were cooled in a desiccator and then re-weighed. The percent moisture was calculated according to the following

calculation: [((vial wt. + sample wt.) – (vial + dry sample wt))/sample wt.] x 100. Both samples were analyzed in triplicate.

Ash

The dried samples from the moisture analysis were removed from the vials and were ground with a mortar and pestle and at least one gram of sample was weighed into pre-weighed scintillation vials. The vials were placed in a muffle oven (Thermodyne F-A1730, Dubuque, IA) at a temperature of 550°C for 6 hours. The vials were then reweighed and the percent ash was determined according to the following equation: [((vial wt. + ash wt.) – (vial wt.))/(sample wt.)] x 100. Samples were analyzed in triplicate.

Minerals

Each ashed sample was dissolved in 1 mL each of concentrated nitric acid (EM Science; Gibbstown, NJ) and hydrochloric acid (J.T. Baker; Phillipsburg, NJ) in the scintillation vial. Ten mL of distilled water was then added to each vial and the samples were then agitated with a vortexer (Vortex-Genie Model K-550-G; Bohemia, NY). Each sample was transferred to a 100 mL quantitative flask and was brought to volume with distilled water. Samples were analyzed utilizing an Inductively Coupled Argon Emission Spectrophotometer (ICP) by the Analytical Lab in Deering Hall at the University of Maine. The samples were tested for aluminum, boron, calcium, copper, iron, potassium, magnesium, manganese, sodium, phosphorous and zinc. Mineral concentrations were determined

according to the following calculation: (mineral ppm x dilution factor)/ wt. of wet sample. Samples were analyzed in triplicate.

Total Volatile Base Nitrogen

Fillet and trim samples were removed from the refrigerator on each day of analyses and a 25 g sample was taken for TVBN analysis according to the method of Botta *et al.* (1984). Fifty mL of 7.5% trichloroacetic acid (Sigma; St. Louis, MO) were added to 25 g of each sample and the mixture was homogenized for 30 seconds in a microblender (Waring; New Hartford, CT). The homogenized contents of the blender were transferred to 200 mL centrifuge tubes (VWR; Boston, MA) and were placed in a centrifuge (Sorvall RC-5B; Newtown, CT) for 20 minutes at 4000rpm (2611 x g). The resulting supernatant was collected in a Falcon screw top test tube by passing it through a funnel lined with Whatman #1 fluted filter paper (Fisher Scientific; Pittsburgh, PA). The extracted samples were refrigerated at approximately 5°C until they could be analyzed. Fifteen mL of each sample were placed in the glass addition funnel of a micro Kjeldahl rapid distillation unit (Labconco Corp.; St. Louis, MO) set on seven. Four mL of a 10% sodium hydroxide solution were added to the funnel and the resulting distillate was collected in 50 mL Erlenmeyer flasks containing 15 mL of 4% boric acid (Fisher Scientific; Fair Lawn, NJ) solution plus eight drops of indicator solution (two parts 0.2% alcohol methyl red : one part 0.2% alcohol methylene blue). The flasks containing the distilled sample were then titrated with 0.25N hydrochloric acid until the color changed back to the color

prior to distillation. The number of mg of nitrogen per 100 g of fish was calculated with the following equation: [(mL HCl titrated x Normality of HCl x 14.007 x (67.5 mL / 15 mL)] x (100 g / 25 g).

Trimethylamine Analysis

Extracts of each sample were taken as described in the TVBN method and the analysis was conducted as described by Hungerford (1998). Twenty five grams of each sample were placed in microblender (Waring; New Hartford, CT). Fifty mL of a 7.5% trichloroacetic acid solution (Sigma; St. Louis, MO) were added to the blender and the sample was blended for 30 seconds until completely homogenized. The homogenized mixture was then poured into centrifuge tubes (VWR; Boston, MA) and centrifuged at 4000 rpm (2611 x g) for 20 minutes. The supernatant was collected in Falcon tubes by passing it through a funnel lined with fluted filter paper. The collected supernatant was kept refrigerated at approximately 5°C until analysis. Between one and three mL of filtrate were pipetted into large glass test tubes for each analysis, depending on the day of collection. A stock solution of TMA was made consisting of 0.682 g of trimethylamine hydrochloride, one mL of concentrated hydrochloric acid brought to 100 mL with distilled water. A working solution was made with the standard solution by taking one mL of stock solution and mixing it with one mL of hydrochloric acid, diluted to 100 mL with distilled water. A standard curve was made with the working solution by pipetting four volumes (0.5, 1, 2, and 3 mL) of standard solution into test tubes. All samples were brought up to four mL with

distilled water. One mL of 20% formaldehyde solution (Mallinckrodt Baker Inc.; Paris, KY), 10 mL of dried toluene (Burdick & Jackson Inc.; Muskegon, MI) and three mL of a one g/mL potassium carbonate solution (Fisher Scientific; Fairlawn, NJ) were added to each test tube and each test tube was capped with a rubber stopper and shaken vigorously by hand forty times. Seven to nine mL of the toluene layer was then pippetted into small test tubes containing approximately 0.1 g of anhydrous sodium sulfate (Fisher Scientific; Fair Lawn, NJ). The test tubes were capped and then mixed briefly with a vortexer (Vortex-Genie Model K-550-G; Bohemia, NY). Five mL of the toluene layer in each small test tube was transferred to another test tube and five mL of a 0.02% picric acid in toluene working solution (EM Science; Gibbstown, NJ) was added. The test tubes were then mixed on a vortexer and the absorbance was read at 410nm against a blank (4mL of distilled water). Milligrams of TMA per 100 g of sample were calculated according to the following equation: (Absorbance of sample/ Absorbance of Standard closest to sample absorbance) x (mg TMA-N/mL standard solution) x mL standard solution used x 67.5 x (100 g/25 g).

pH

Fifteen grams of each sample were weighed into a Falcon test tube to which 15 mL of distilled water were added. Each sample was then homogenized for 30 seconds with a polytron (Kinematica; Switzerland) set at five. The pH of the samples was measured utilizing an Orion Model 320 PerpHecTLogR meter (Beverly, MA) calibrated with pH 4 and 7 buffer (Orion; Beverly, MA) range,

washing the probe thoroughly with distilled water between each sample. Samples were analyzed in triplicate.

Microbial Analysis

Total plate counts were conducted with plate count agar (Difco; Detroit, MI) using standard AOAC method (Maturin and Peeler, 1998). Aseptically, twenty-five gram samples were removed from each fillet or trim treatment on each day of analyses and were transferred to Whirl-Pak stomacher bags (Nasco; Fort Atkinson, WI). Two hundred and twenty five mL of a 0.1% bactopeptone solution (Difco; Detroit, MI) were added and the samples were mixed for two minutes using a Model 400 stomacher-lab blender (Tekmar Co.; Cincinnati, OH). Serial dilutions were prepared with 0.1% bactopeptone and one mL aliquots were asepectically transferred from the test tubes to sterile Petri dishes (Fisher Scientific Co. LLC; Agawam, MA). Sterilized plate count agar cooled to approximately 45 °C was poured into the plates and the plates were cooled and then stored upside down for 48 hours at room temperature, approximately 23°C. The bacterial colonies were hand counted with the aid of a Quebec colony counter (American Optical Company; Buffalo, NY). Results were expressed as colony forming units (CFU) per gram of sample. All samples were analyzed in duplicate.

Statistical Analysis

Differences between treatments were evaluated using Systat 10 (SSPS Inc.; 2001) to calculate one way analysis of variance (ANOVA) with the confidence level set at $p \leq 0.05$. Tukey's post hoc test (Neter *et al.*, 1996) was used to analyze differences among treatment means. Multiway ANOVA was used to study the effects of chitosan type (high molecular weight, low molecular weight, no chitosan) and day of storage (1, 3, 6, 10 and 13) on aerobic plate counts (APC), total volatile base nitrogen (TVBN), and pH of the salmon fillets. Multiway ANOVA was also used to study the effects of chitosan percent (0.5, 1.0 and 2.0), chitosan type (high molecular weight and low molecular weight), and day of storage (1, 3, 6, 10, and 13) on APC, TVBN and pH of salmon trim.

MATERIALS AND METHODS: ENZYMATIC DEGRADATION OF CHITOSAN SOLUTIONS

Objectives

The objectives of this study were to study the feasibility of degrading chitosan to water soluble oligomers using the commercially available enzymes, alpha amylase and bromelain, and to determine which pH and reaction length were most effective for enzymatic degradation of chitosan. Once the optimal pH, reaction length, and type of enzyme were established a more extensive study of the enzymatic breakdown of chitosan was conducted using alpha amylase at pH 4. Enzymatic breakdown of chitosan was followed by measuring changes in chitosan solution viscosity after 10, 30, and 60 minutes of degradation.

Experimental Design

The experiment was designed to first test the efficacy of the two enzymes at degrading low molecular weight chitosan at three different pH levels (4, 5.5, and 7) at ambient room temperature. Various enzyme concentrations were also tested: 1, 5, and 10% by weight of the chitosan. Observations and measurements, temperature, pH and viscosity readings were taken at time 0, 8 hours and 24 hours of degradation. Time periods were 10 minutes, 30 minutes and 60 minutes in the case of the second portion of the study with alpha amylase.

Enzymes

Alpha amylase derived from *Bacillus subtilis* (lot number 7153F) was purchased from ICN (Costa Mesa, CA). The declared activity of this enzyme was 307,000 units/gram defined as "one unit will dextrinize one mg of starch per min at pH 6.6 at 30 °C." Bromelain derived from pineapple (lot number 86283) was also purchased from ICN. The declared activity was 1169 units/gram defined as "one unit will hydrolyze one mg of amino nitrogen from gelatin in 20 minutes at pH 4.5 at 45 °C."

Procedures

One percent acetic acid solutions were prepared and 50 mL of these solutions were added to 100 mL beakers. The pH of the solutions was adjusted with 20% NaOH solution to one log under the target pH (3.0 for 4.0, 4.5 for 5.5, and 6.0 for 7.0). One half of a gram of low molecular weight chitosan (with a declared viscosity of between 20 and 200 centipoise at a level of 1% in a 1% acetic acid solution) that had been ground in a Wiley mill, as described earlier, was added to the beakers. The powdered enzyme was added to the solutions and the solutions were covered with plastic wrap and put on a lab rotator (Lab Line Instruments; Melrose Park, IL) set at seven. Temperature and pH were measured and observations were made at all of the time periods mentioned before. An attempt to quantify the degree of water solubility afforded by enzymatic activity was also attempted but was found to not be feasible. After

determining that alpha amylase was the most promising enzyme and that a pH of 4.0 yielded the best results, another experiment was performed to follow the enzymatic degradation during the first hour by taking viscosity measurements at 0, 10, 30 and 60 minutes.

Protein Determination of Enzyme Solutions

A 10 mg/mL enzyme solution was made with both alpha amylase and bromelain (ICN Biomedicals Inc.; Costa Mesa, CA) by adding 50 mg of enzyme to 5 mL of water and mixing. Twenty, 100 and 200 microliters of each solution were pipetted into test tubes in duplicate and evaluated for protein content using the method of Lowry *et al.* (1951). The volume in the tubes was brought up to five mL with distilled water. Five mL of solution D which consisted of 100 mL of solution A [(2% Na_2CO_3 (Malinckrodt Baker; Paris, KY) in 0.4% NaOH)] plus one mL of solution B [(1% cupric sulfate (Fisher Scientific; Fair Lawn, NJ) in distilled water)] plus one mL of solution C [(2.7% sodium postassium tartrate (Malinckrodt Baker; Paris, KY) in distilled water)] were added to each test tube. After 10 minutes, 0.5 mL of solution E [(1N Folin and Ciocalteu's phenol reagent (Sigma Chemical Company; St. Louis, MO)] was added to each test tube that was then vortexed. The absorbance was read at 700 nm after at least 25 but no more than 50 minutes had passed. A standard curve was established using bovine serum albumin (BSA). The amount of protein in the enzyme samples was calculated by extrapolating the micrograms of protein per milliliter (x) from the

absorbance data from the standard curve (y=0.0176x + 0.0188) where y = the absorbance of the sample.

Specific Gravity

The specific gravity of the undegraded and enzymatically degraded (for 10 minutes, 60 minutes, 8 hours and 24 hours) low molecular weight chitosan solutions, at pH levels of 4.0 and 5.5, was measured according to AOAC method 26.1.08 (AOAC, 1998) by preparing 200 mL of each 1% chitosan solution. The solutions were poured into the clear glass graduated cylinder and the 165 mm hydrometer (VWR Brand; Boston, MA) was inserted into the liquid. The specific gravity was read at the meniscus of the liquid on the hydrometer. No measurements were taken for the non-solubilized chitosan at pH seven.

Viscosity

Viscosity of the enzymatically degraded chitosan solutions was determined at each time period (initial, 10 minutes, 30 minutes, 60 minutes, 8 hours and 24 hours) by utilizing four different sizes (#50, 75, 150 and 200) of a Cannon-Fenske capillary type viscometer (Cannon; State College, PA) according to ASTM method D445 (ASTM, 2000). Measurements were taken by inverting the viscometer and inserting the receiving end into the chitosan solution. A bulb type syringe was used on the other end of the viscometer to draw the solution into the unit to a specified line. The unit was then inverted and attached to a holding unit, suspending the bottom of the viscometer in a beaker

of distilled water at ambient temperature. The time required for the meniscus of the solution to flow from the top line to the bottom line was measured in seconds with a digital stopwatch. The calculation for the viscosity was as follows: (number of seconds) x (viscometer constant) x (specific gravity of the solution). Viscosity units were expressed as Centipoise (cP).

Intrinsic Viscosity

Intrinsic viscosity of degraded (10 min, 30 min, 60 min, 8 hrs, and 24 hrs) and undegraded chitosan were calculated viscometrically according to the method of Zhang and Neau (2001). The efflux time of the solvent (1.0% acetic acid solution) was measured for each size of viscometer used. The efflux time of each chitosan solution was measured with the appropriate size viscometer. The initial concentration of each chitosan solution was 1.0 g/dL. Dilutions of 0.125, 0.25, 0.50, and 0.75 g/dL were prepared for each degraded chitosan solution (0 min., 30 min., 60 min., 8 hr. and 24 hr. degradation time) and the undegraded chitosan solution. The efflux time of each dilution was measured and the specific viscosity was determined for each dilution by the following equation [(efflux time of the solution) – (efflux time of the solvent)]/(efflux time of the solvent). The reduced viscosity for each dilution was then determined by the following equation (specific viscosity)/(concentration of solution in g/dL). The intrinsic viscosity was determined for each solution by plotting the concentration (in g/dL) on the x-axis against the corresponding value for reduced viscosity on the y-axis. The

equation for the line was determined and extrapolated back to a zero concentration, which was the intrinsic viscosity [*n*].

Quantification of Insoluble Chitosan

The pH of the solutions were brought to seven by drop wise addition of 20% NaOH while stirring with an automatic stirrer. Adjusting the pH to seven precipitated the insoluble chitosan. The whole solution was then transferred to centrifuge tubes and centrifuged for 20 minutes at 6000 rpm (5875 x g). The supernatant was then poured off and the insoluble chitosan pellet was transferred to pre-weighed foil dishes (VWR Brand; Boston, MA) that were then dried in a Fisher Isotemp, model 350, drying oven (Fisher Scientific, Fair Lawn, NJ) for 24 hours at 95°C. The foil dishes were then cooled and re-weighed to determine the amount of chitosan that was insoluble.

Temperature

The temperature of all enzymatically degraded chitosan solutions was measured with a mercury in glass thermometer (VWR Brand; Boston, MA) at each of the previously mentioned time periods.

MATERIALS AND METHODS: IN VITRO ANTIMICROBIAL EFFICACY OF CHITOSAN AGAINST *LISTERIA INNOCUA* AND *PSEUDOMONAS AERUGINOSA*

Objectives

The objectives of this study were to study the antimicrobial effects of enzymatically (alpha amylase) degraded (for 10 min, 60 min and 24 hours) and undegraded low molecular weight chitosan against two bacteria, *Listeria innocua* and *Pseudomonas aeruginosa*, common in fish products. The *Pseudomonas* study was conducted using both nutrient broth and trypticase soy broth to study the effects of the two different broths on the antimicrobial efficacy of the chitosan.

Experimental Design

Factors studied included species of bacteria, chitosan addition (0.5, 1.0 and 2.0% of chitosan solution added; v/v of the broth), and type of chitosan (undegraded, 10 minute degraded, 60 minute degraded and 24 hour degraded). In the *Pseudomonas* study, the effects of two different broths, nutrient broth versus trypticase soy broth, were studied. Each of the 14 treatments was prepared in triplicate with duplicate microbial analyses. Treatment codes are listed in Table 3.

Preparation of Pour Plates

Trypticase Soy Agar (Becton Dickinson and Company; Cockeysville, MD) was prepared according the directions on the container and was then autoclaved and allowed to cool to approximately 45°C. The agar was then poured into

Table 3. In Vitro Treatment Codes

Code	Treatment
CON1	Unadjusted pH (7.3) Nutrient Broth or Trypticase Soy Broth
CON2	pH Adjusted (5.5) Nutrient Broth or Trypticase Soy Broth
CH05	0.5% LMW Chitosan* in Nutrient Broth or Trypticase Soy Broth (pH 5.5)
CH1	1.0% LMW Chitosan in Nutrient Broth or Trypticase Soy Broth (pH 5.5)
CH2	2.0% LMW Chitosan in Nutrient Broth or Trypticase Soy Broth (pH 5.5)
COX05	0.5% 10 min Degraded LMW Chitosan in Nutrient Broth or Trypticase Soy Broth (pH 5.5)
COX1	1.0% 10 min Degraded LMW Chitosan in Nutrient Broth or Trypticase Soy Broth (pH 5.5)
COX2	2.0% 10 min Degraded LMW Chitosan in Nutrient Broth or Trypticase Soy Broth (pH 5.5)
COY05	0.5% 60 min Degraded Chitosan in Nutrient Broth or Trypticase Soy Broth (pH 5.5)
COY1	1.0% 60 min Degraded Chitosan in Nutrient Broth or Trypticase Soy Broth (pH 5.5)
COY2	2.0% 60 min Degraded Chitosan in Nutrient Broth or Trypticase Soy Broth (pH 5.5)
COZ05	0.5% 24 hr Degraded Chitosan in Nutrient Broth or Trypticase Soy Broth (pH 5.5)
COZ1	1.0% 24 hr Degraded Chitosan in Nutrient Broth or Trypticase Soy Broth (pH 5.5)
COZ2	2.0% 24 hr Degraded Chitosan in Nutrient Broth or Trypticase Soy Broth (pH 5.5)

*Volume of 1% chitosan solution added to flasks by volume of broth.

disposable plastic petri dishes which were immediately covered after being poured. After cooling, the plates were inverted and reinserted into the original plastic bags. The plates were stored at room temperature and were used within a week of production (plates for the first trial of the *Listeria* study were stored in the refrigerator prior to use but a condensation problem made this practice unacceptable).

Propagation of Bacterial Cultures

The *Listeria innocua* culture was a teaching culture supplied by Dr. Al Bushway (University of Maine). The *Pseudomonas aeruginosa* was supplied by ATCC (American Type Culture Collection; Manassas, VA). The *Pseudomonas* was a freeze dried culture (10145-U) and was propagated according the directions given by the supplier. The bacteria were transferred into sterilized nutrient broth (Difco; Detroit, MI) and were incubated at 37°C for 24 hours. The culture was plated onto trypticase soy agar plates and was isolated from the plate and re-inoculated into trypticase soy broth (Becton Dickinson and Company; Cockeysville, MD). Plates with isolated colonies were stored in the refrigerator for later propagation for each subsequent trial. Bacterial cultures were transferred from broth to new broth exactly 17 hours before inoculation into the flasks containing chitosan or the two control broths, see Table 4, and were incubated at 37° C. Serial dilutions were made of the bacterial culture used to inoculate the plates just prior to flask inoculations. The serial dilutions were plated, incubated, and read in the same manner as with the flasks. Results were

recorded in colony forming units (CFU) per mL. Microbial analyses were completed in duplicate.

Preparation of Chitosan Solutions

Four 1% low molecular weight chitosan solutions were prepared in 1% acetic acid by adding the chitosan to the acetic acid solutions while stirring with a stir bar. While still stirring, 10% alpha amylase (by weight of chitosan) was added to the beakers. The enzymatic reaction was stopped in each beaker at the appropriate time (10 minutes, 60 minutes and 24 hours) by autoclaving the beaker, covered with a watch glass, for 10 minutes. The solutions were stored at room temperature until used. The undegraded chitosan solution was made the day the 24 hour solution was completed.

Table 4. Broth Recipes for In Vitro Studies

Treatment	Volume of TSB* or NB (mL)	Volume of 1% Chitosan or Chitooligosaccharide Solution (mL)
Control 1 (pH 7.3)	40.0	0.0
Control 2 (pH 5.5)	40.0	0.0
0.5% Chitosan	39.8	0.2
1.0% Chitosan	39.6	0.4
2.0% Chitosan	39.2	0.8

*TSB = Trypticase Soy Broth
NB = Nutrient Broth

Inoculation and Spread Plating

One tenth of one mL of bacterial culture in TSB or NB was removed aseptically from the culture tube with a sterilized plastic tip on an automatic pipet-man (VWR Brand; Boston, MA) and inoculated into each 50 mL Erlenmeyer flask (14 in total) containing the sterilized cool broth and added chitosan solution (Table 4). One tenth of one mL of the bacterial culture, *Pseudomonas aeruginosa*, was used for the TSB experiment in order to achieve the same inoculation level (approximately 10^7 CFU/mL) as the *Listeria* experiment. After inoculation the flasks were capped with #2 rubber stoppers and placed in the refrigerator in a metal tray taped to the top of a lab rotator. The rotator was set on seven to gently agitate the broth while incubating. The rotator was placed on the bottom shelf of the refrigerator and the seal of the refrigerator was reinforced with packing tape where the cord emerged from the refrigerator. The temperature of the refrigerator was adjusted so it remained between four and six degrees centigrade for the entire experiment. Each day of analysis (one, two, three and four) each flask was randomly removed from the refrigerator and serial dilutions were made in sterilized bactopeptone. One tenth of one mL aliquots of the dilutions were aseptically transferred to TSA plates and were spread with ethyl alcohol sterilized glass rods using an inoculating turntable (Fisher Scientific; Fair Lawn, NJ). The plates were incubated at 37°C for 24 hours before being counted with the aid of a Quebec colony counter (American Optical Company; Buffalo, NY). Contents of each flask were plated and enumerated in duplicate.

Viscosity

The viscosity of the chitosan solutions was measured both before and after autoclaving as described previously. The purpose was to estimate the molecular weight and degree of degradation of the chitosan solutions and to test the effect of autoclaving on molecular degradation.

Statistical Analysis

Differences between treatments were evaluated using Systat 10 (SSPS Inc.; 2001) to calculate one way analysis of variance (ANOVA) with the confidence level set at $p \leq 0.05$. Tukey's post hoc test (Neter *et. al.*, 1996) was used to analyze differences among treatment means. Multiway ANOVA was conducted to test the effects of chitosan addition (0.5, 1.0 and 2.0%) and chitosan treatment (undegraded, 10 minute degraded, 60 minute degraded and 24 hour degraded) on both raw and log transformed data. Multiway analysis was also used to analyze differences among treatments and time of refrigerated incubation on both raw and log transformed data.

RESULTS:
ANTIMICROBIAL EFFICACY OF CHITOSAN DIPS AND POWDERED CHITOSAN ON ATLANTIC SALMON PRODUCTS

Observations

Salmon Trim

Visible flecks of chitosan could be seen in the trim, especially in the treatments containing 2% powdered chitosan. By day six, visible differences in color could be seen between the chitosan treated trim and the untreated trim. Untreated samples were brown and those containing chitosan continued to appear as they had on the first day of storage.

Salmon Fillet

The high molecular weight (HMW) chitosan dip was very viscous and formed a thick coating on the salmon fillets. The low molecular weight chitosan (LMW) dip was thinner and formed a thinner coating on the surface of the fillets. As the study progressed the coating on the fillets degraded to some extent, forming a pool of slightly yellow fluid on the foam trays.

Moisture, Ash and Mineral Composition

Average moisture content of the salmon fillet and the salmon trim were 67.9 ± 0.56 and 70.2 ± 4.59%, respectively. Ash contents were 4.2 ± 0.79 and 5.0 ± 0.41% for the fillet and ground trim, respectively. Mineral concentrations in both trim and fillet are recorded in Table 5.

Table 5. Mineral Concentrations (mg/kg) of Salmon Trim and Fillet *

Element	Trim	Fillet
Aluminum	11.8 ± 2.7	13.6 ± 2.3
Boron	114.1 ± 8.3	132.7 ± 16.0
Calcium	127.8 ± 20.2	138.3 ± 8.3
Copper	2.119 ± 1.8	0.9 ± 0.4
Iron	9.783 ± 1.0	17.8 ± 3.4
Potassium	8117.6 ± 1125.1	9616.6 ± 997.8
Magnesium	642.8 ± 77.2	707.9 ± 59.5
Sodium	1261.2 ± 90.5	1410.3 ± 141.5
Phosphorous	5074.6 ± 558.1	5760.3 ± 451.5
Zinc	10.2 ± 3.4	8.5 ± 1.0

*Each value is the average of three analyses ± standard deviation on a wet weight basis.

Microbial Analyses

Salmon Trim

Aerobic plate counts started out at very low levels for all seven treatments, ranging from 3.80×10^3 to 6.95×10^3 CFU/g (Table 6). Large increases in APC counts occurred between day three and day six, rising from 10^4 to 10^6 CFU/g. No significant differences ($p>0.05$) were observed among the different treatments after day one. In some cases slightly higher APCs were observed in the chitosan treated trim. On day one, the 0.5% HMW treated trim had significantly higher ($p<0.05$) aerobic plate counts compared to 1.0% LMW treated trim. No differences were observed between low and high percentages of chitosan, based on multiway ANOVA.

Salmon Fillet

No significant differences were observed among treatments on the first day of analysis although values were very low, 10^3 CFU/g (Table 7). Fillets

Table 6. Aerobic Plate Counts (CFU/g) of Atlantic Salmon Trim With and Without Chitosan During Refrigerated (4°C) Storage *

Treatment Code**	Day 1	Day 3	Day 6	Day 10	Day 13
T	$5.43x10^3 \pm 3.25x10^2$ ab	$8.25x10^3 \pm 5.48x10^3$	$1.31x10^7 \pm 1.14x10^7$	$1.02x10^8 \pm 7.49x10^7$	$2.03x10^8 \pm 1.47x10^8$
.5TL	$5.74x10^3 \pm 4.42x10^2$ ab	$9.50x10^3 \pm 4.27x10^3$	$1.46x10^6 \pm 1.11x10^6$	$5.10x10^7 \pm 2.13x10^7$	$4.19x10^8 \pm 2.72x10^8$
.5TH	$6.95x10^3 \pm 1.13x10^3$ b	$3.53x10^4 \pm 1.44x10^4$	$1.19x10^6 \pm 7.06x10^5$	$1.26x10^8 \pm 5.80x10^7$	$2.52x10^8 \pm 7.01x10^7$
1TL	$3.80x10^3 \pm 7.1x10^1$ a	$8.52x10^4 \pm 1.00x10^5$	$6.28x10^6 \pm 4.72x10^6$	$8.46x10^7 \pm 6.26x10^7$	$6.84x10^8 \pm 7.90x10^8$
1TH	$4.38x10^3 \pm 7.15x10^2$ ab	$2.63x10^4 \pm 2.20x10^4$	$7.17x10^6 \pm 6.20x10^6$	$9.93x10^8 \pm 1.25x10^9$	$4.35x10^8 \pm 5.41x10^7$
2TL	$4.15x10^3$ ab***	$2.18x10^4 \pm 1.53x10^4$	$7.38x10^6 \pm 8.43x10^6$	$6.99x10^8 \pm 7.54x10^8$	$4.14x10^8 \pm 1.74x10^8$
2TH	$4.18x10^3 \pm 1.30x10^3$ ab	$3.15x10^4 \pm 3.86x10^4$	$6.07x10^6 \pm 5.49x10^6$	$6.38x10^8 \pm 3.84x10^8$	$6.03x10^8 \pm 5.53x10^7$
p-value	0.033	0.396	0.394	0.304	0.573

*Each value is an average of three replications, each analyzed in duplicate ± standard deviation. Different letters within each column indicate a significant difference ($p < 0.05$) among treatments based on one-way analysis of raw data followed by Tukey's post hoc test.

**T = Trim control; .5TL = trim with 0.5% LMW chitosan; .5TH = trim with 0.5% HMW chitosan; 1TL = trim with 1.0% LMW chitosan; 1TH = trim with 1.0% HMW chitosan; 2TL = trim with 2.0% LMW chitosan; 2TH = trim with 2.0% HMW chitosan.

***Single sample analyzed.

Table 7. Aerobic Plate Counts (CFU/g) of Atlantic Salmon Fillet With and Without Chitosan During (4°C) Refrigerated Storage *

Treatment Code**	Day 1	Day 3	Day 6	Day 10	Day 13
F	$1.95x10^3 \pm 1.49x10^3$	$7.56x10^4 \pm 4.89x10^3$ b	$1.97x10^8 \pm 1.56x10^7$ a	$1.21x10^9 \pm 8.56x10^8$	$9.49x10^9 \pm 1.37x10^{10}$
Fa	$1.92x10^3 \pm 1.24x10^3$	$7.35x10^3 \pm 1.00x10^4$ a	$8.47x10^4 \pm 9.89x10^4$ a	$1.15x10^7 \pm 1.38x10^7$	$1.11x10^9 \pm 7.29x10^8$
FL	$8.75x10^2 \pm 3.18x10^2$	$1.23x10^3 \pm 1.15x10^3$ a	$1.09x10^6 \pm 1.08x10^6$ a	$9.52x10^7 \pm 6.01x10^7$	$3.14x10^9 \pm 3.66x10^9$
FH	$1.37x10^3 \pm 5.01x10^2$	$1.25x10^3 \pm 1.36x10^3$ ab	$4.53x10^3 \pm 6.06x10^3$ a	$3.53x10^6 \pm 4.83x10^6$	$2.38x10^8 \pm 3.50x10^8$
p-value	0.644	0.029	0.038	0.074	0.429

*Each value is an average of three replications, each analyzed in duplicate ± standard deviation. Different letters within each column indicate a significant difference ($p < 0.05$) among treatments based on one-way analysis of variance of raw data followed by Tukey's post hoc test.

** F = Control fillet; Fa = Fillet dipped in 1.0% acetic acid; FL = Fillet dipped in 1.0% LMW chitosan in 1.0% acetic acid; FH = Fillet dipped in 1.0% HMW chitosan in 1.0% acetic acid.

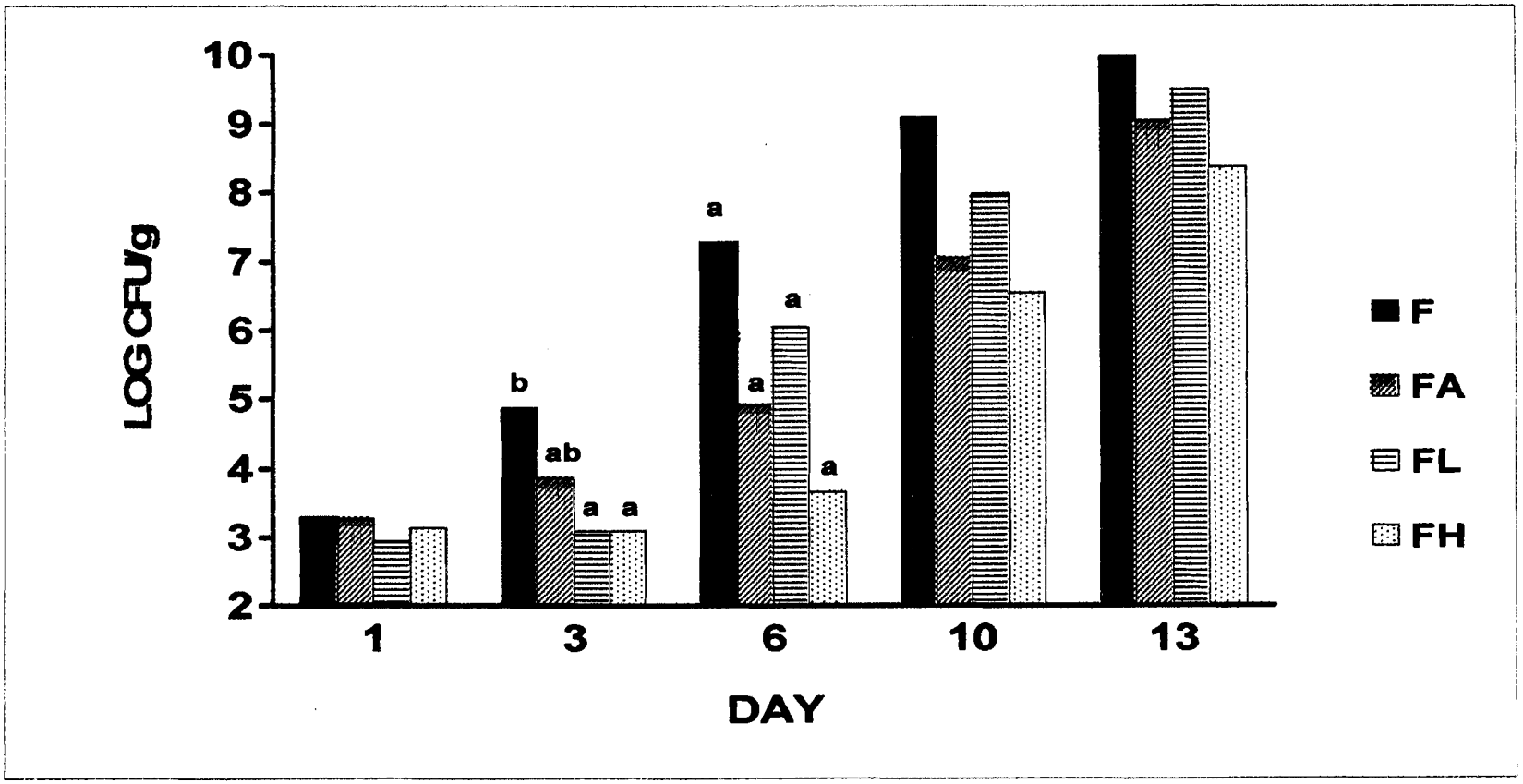
dipped in HMW chitosan dips had the lowest microbial counts compared to the control throughout the study although only significantly ($p<0.05$) so on days three and six based on one way ANOVA. Dipping fillets in 1.0% acetic acid alone resulted in a reduction in microbial counts compared to the control fillets on day three through day 10 but only significantly ($p=0.03$) so on day three; however the reductions were not as pronounced as those for the HMW chitosan treatment. Fillets treated with LMW chitosan dips had lower microbial counts between days one and 13 compared to the control fillets but only significantly ($p=0.03$) so on day three based on one way ANOVA. None of the treatments showed effective antimicrobial activity after 10 days of refrigerated storage (Figure 2)

Total Volatile Base Nitrogen

Salmon Fillet

There were no significant differences in TVBN concentrations in fillets on any day during the study (Figure 3 and Table 8). TVBN values in all fillet treatments were relatively low throughout the study in general, ranging from 14.4 to 23.4 mg N/100 g fish, and a similar trend to the APC data can be seen (Figures 2 and 3). TVBN values were lowest in the fillets treated with HMW chitosan dips and were highest in the untreated fillets. Linear regression analysis of both the fillet APC and TVBN data revealed a significant ($p=0.0004$) positive association between the two factors (Figure 4). The R^2 value of this relationship was relatively low ($R^2=0.5053$) probably due to relatively large increases in aerobic plate counts resulting in small increases in TVBN concentrations.

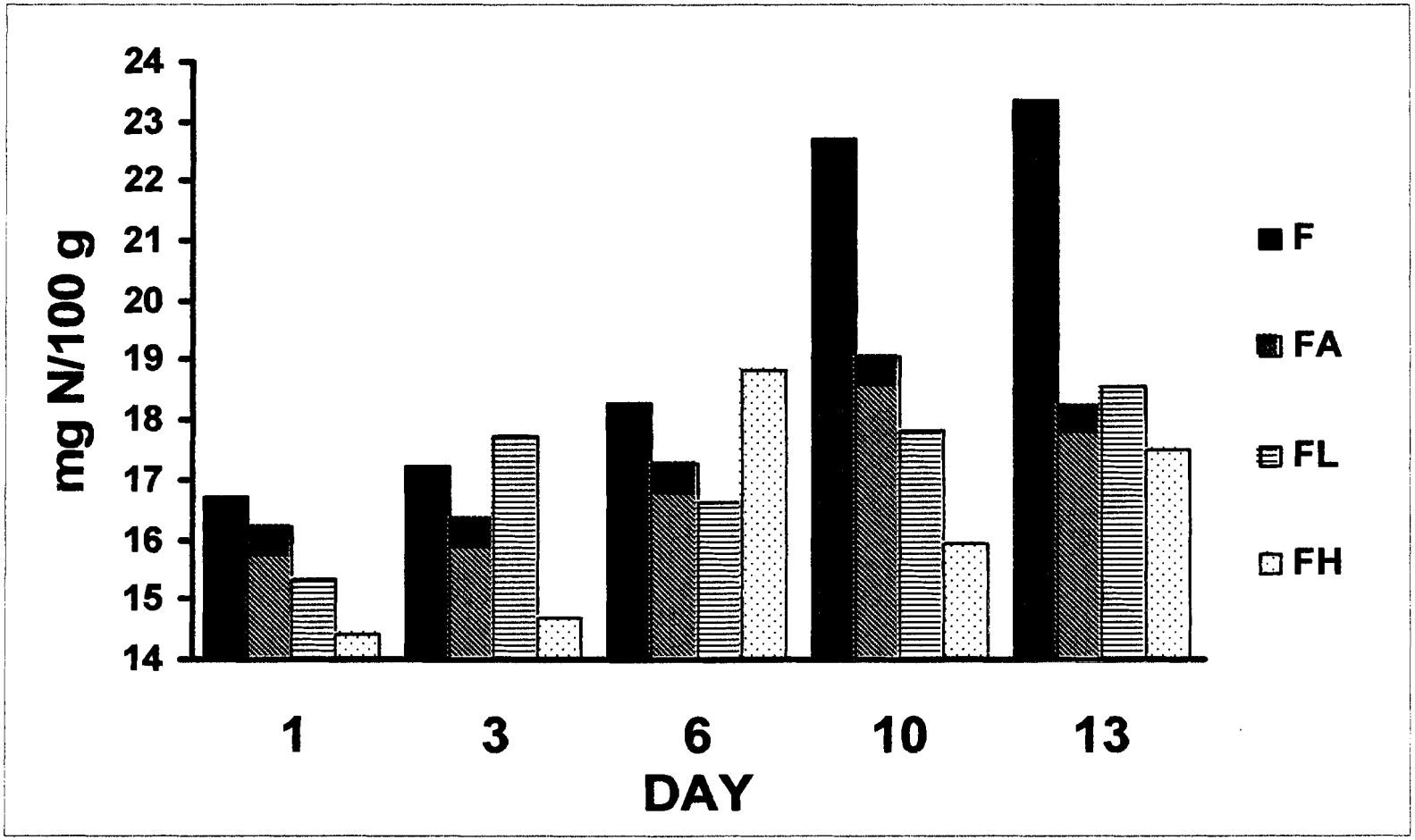
Figure 2. Aerobic Plate Counts (LOG CFU/g) of Chitosan Treated Salmon Fillet During Refrigerated (4°C) Storage



* Each value is an average of three replications, each analyzed in duplicate. Different letters over columns indicate a significant difference ($p < 0.05$) among treatments based on one-way analysis of variance of raw data followed by Tukey's post hoc test.

** F = Control fillet; Fa = Fillet dipped in 1.0% acetic acid; FL = Fillet dipped in 1.0% LMW chitosan in 1.0% acetic acid; FH = Fillet dipped in 1.0% HMW chitosan in 1.0% acetic acid.

Figure 3. TVBN (mg N/100g) Concentrations of Chitosan Treated Salmon Fillet During Refrigerated (4°C) Storage*



* Each value is an average of three replications, each analyzed singly.

** F = Control fillet; Fa = Fillet dipped in 1.0% acetic acid; FL = Fillet dipped in 1.0% LMW chitosan in 1.0% acetic acid; FH = Fillet dipped in 1.0% HMW chitosan in 1.0% acetic acid.

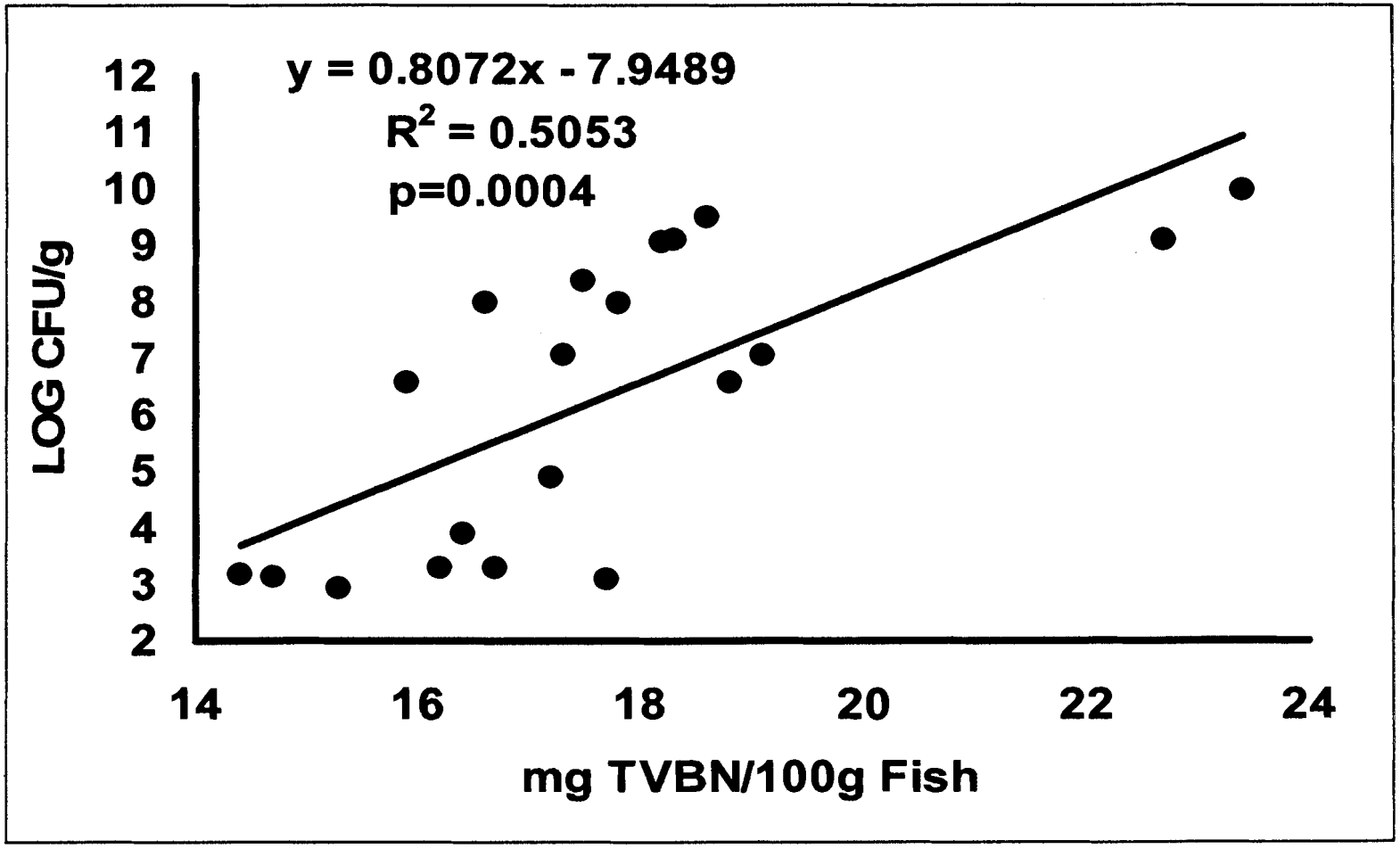
Table 8. TVBN (mg N/100g) Concentrations in Chitosan Treated Salmon Fillet During Refrigerated (4°C) Storage*

Treatment Code**	DAY 1	DAY 3	DAY 6	DAY 10	DAY 13
F	16.7 ± 2.5	17.2 ± 4.0	18.3 ± 0.3	22.7 ± 4.0	23.4 ± 5.1
Fa	16.2 ± 1.1	16.4 ± 2.8	17.3 ± 0.5	19.1 ± 2.8	18.2 ± 1.5
FL	15.3 ± 1.8	17.7 ± 1.3	16.6 ± 0.5	17.8 ± 1.3	18.6 ± 1.3
FH	14.4 ± 0.5	14.7 ± 1.1	18.8 ± 3.7	15.9 ± 1.1	17.5 ± 1.5
p-value	0.396	0.080	0.513	0.066	0.124

* Each value is the average of three replications ± standard deviation, analyzed singly.

** F = Fillet control; Fa = Fillet dipped in 1% acetic acid; FL = Fillet dipped in 1% LMW chitosan in 1% acetic acid; FH = Fillet dipped in 1% HMW chitosan in 1% acetic acid.

Figure 4. Linear Regression of TVBN and APC (LOG CFU/g) of Chitosan Treated Fillet



Salmon Trim

There were no significant differences among any of the trim treatments on any day throughout the study based on one way ANOVA (Table 9). TVBN values were relatively low throughout the study, ranging from 15.4 to 23.0 mg N/100 g of fish. The trim values were similar to those observed in the fillet study. A slow increase in values occurred with increasing storage time.

pH

Salmon Fillet

pH values initially rose from day 1 to day 3 but then fell again till a rise between days 10 and 13 (Table 10). Significant differences in the pH of the different treatments only were present on days 1 ($p=0.019$) and 10 ($p=0.007$). In both cases, the pH of the high molecular weight chitosan dipped fillets were lower than the pH of the untreated fillets. All of the treated fillets had lower pH values than the untreated control including the 1.0% acetic acid dipped fillets.

Salmon Trim

There were significant differences ($p<0.05$) among all of the treatments on every day of the study (Table 11). Addition of powdered chitosan to the salmon trim resulted in higher pH values compared to the control. The higher the percentage of chitosan, the higher the resulting pH value.

Table 9. TVBN (mg N/100g) Concentrations in Chitosan Treated Salmon Trim During Refrigerated (4°C) Storage*

Treatment Code**	DAY 1	DAY 3	DAY 6	DAY 10	DAY 13
T	16.6 ± 0.3	16.1 ± 0.7	18.8 ± 2.4	23.0 ± 3.9	22.0 ± 2.0
.5TL	16.4 ± 0.6	16.3 ± 0.8	16.8 ± 0.4	19.5 ± 1.2	21.8 ± 0.9
.5TH	16.7 ± 0.1	17.3 ± 0.3	16.8 ± 0.7	21.9 ± 2.8	21.7 ± 1.3
1TL	16.3 ± 0.1	17.6 ± 1.2	16.2 ± 0.5	19.4 ± 2.8	20.5 ± 1.3
1TH	16.3 ± 0.2	17.2 ± 1.8	16.6 ± 0.6	21.6 ± 1.8	20.9 ± 1.1
2TL	15.4 ± 1.2	17.1 ± 0.9	16.5 ± 0.7	20.5 ± 1.2	17.9 ± 6.7
2TH	15.7 ± 0.5	18.0 ± 0.1	16.3 ± 0.2	18.5 ± 0.5	22.4 ± 1.6
p-value	0.292	0.286	0.101	0.263	0.560

* Each value is the average of three replications ± standard deviation, analyzed singly.

** T = Trim control; .5TL = Trim with 0.5% LMW chitosan; .5TH = Trim with 0.5% HMW chitosan; 1TL = Trim with 1% LMW chitosan; 1TH = Trim with 1% HMW chitosan; 2TL = Trim with 2% LMW chitosan; 2TH = Trim with 2% HMW chitosan.

Table 10. pH of Chitosan Treated Salmon Fillet During Refrigerated (4°C) Storage*

Treatment Code **	DAY 1	DAY 3	DAY 6	DAY 10	DAY 13
F	6.16 ± 0.03b	6.23 ± 0.05	6.12 ± 0.02	6.12 ± 0.02b	6.22 ± 0.07
Fa	6.10 ± 0.01ab	6.23 ± 0.02	6.08 ± 0.03	6.03 ± 0.01a	6.08 ± 0.11
FL	6.09 ± 0.02ab	6.25 ± 0.05	6.12 ± 0.05	6.03 ± 0.04a	6.10 ± 0.07
FH	6.06 ± 0.05a	6.20 ± 0.05	6.07 ± 0.05	6.01 ± 0.04a	6.07 ± 0.06
p-value	0.019	0.543	0.300	0.007	0.141

* Each value is the average of three replications ± standard deviation, analyzed singly. Means in the same column not sharing a superscript are significantly ($p < 0.05$) different based on one-way ANOVA followed by Tukey's post hoc test.

** F = Fillet control; Fa = Fillet dipped in 1% acetic acid; FL = Fillet dipped in 1% LMW chitosan in 1% acetic acid; FH = Fillet dipped in 1% HMW chitosan in 1% acetic acid.

Table 11. pH of Chitosan Treated Salmon Trim During Refrigerated (4°C) Storage*

Treatment Code **	DAY 1	DAY 3	DAY 6	DAY 10	DAY 13
T	6.16 ± 0.01a	6.30 ± 0.03a	6.14 ± 0.02a	6.08 ± 0.01a	6.11 ± 0.02a
.5TL	6.34 ± 0.02c	6.49 ± 0.04c	6.40 ± 0.04c	6.29 ± 0.04c	6.29 ± 0.01c
.5TH	6.36 ± 0.02c	6.51 ± 0.06c	6.42 ± 0.03c	6.31 ± 0.04c	6.30 ± 0.01c
1TL	6.29 ± 0.02b	6.43 ± 0.04b	6.32 ± 0.04b	6.23 ± 0.03b	6.23 ± 0.02b
1TH	6.45 ± 0.02c	6.68 ± 0.01d	6.56 ± 0.02d	6.48 ± 0.02d	6.45 ± 0.03d
2TL	6.54 ± 0.01d	6.76 ± 0.01de	6.64 ± 0.02e	6.59 ± 0.03e	6.54 ± 0.03e
2TH	6.52 ± 0.02d	6.80 ± 0.01e	6.59 ± 0.02de	6.56 ± 0.01e	6.49 ± 0.03d
p-value	0.000	0.000	0.000	0.000	0.000

* Each value is the average of three replications ± standard deviation, analyzed singly. Means in the same column not sharing a superscript are significantly ($p < 0.05$) different based on one-way ANOVA followed by Tukey's post hoc test.

** T = Trim control; .5TL = Trim with 0.5% LMW chitosan; .5TH = Trim with 0.5% HMW chitosan; 1TL = Trim with 1% LMW chitosan; 1TH = Trim with 1% HMW chitosan; 2TL = Trim with 2% LMW chitosan; 2TH = Trim with 2% HMW chitosan.

Trimethylamine

Salmon Fillet

TMA-N concentrations were undetectable until day 10 (Table 12). On day 10 the highest level of TMA-N, 1.21mg/100g of fish, was observed in the fillet control. On day 13, the acetic acid dipped fillet was significantly ($p=0.03$) higher in TMA-N than either chitosan dip treatment but not the control. Chitosan treated fillets were lower in TMA-N than the control on both day 10 and 13.

Salmon Trim

TMA-N concentrations in salmon trim were also not detected until day 10 of storage (Table 13). There were no significant differences among the different chitosan treatments on day 10 or 13. On day 10 the highest concentrations occurred in the 1.0% LMW chitosan treatment and the lowest concentration was seen in the 2.0% HMW chitosan treatment. The highest value, 3.30mg TMA-N/100g fish, on day 13 was the control trim treatment. High values were also observed in trim treated with 2.0% HMW chitosan and the 0.5% HMW chitosan. No visible trends regarding chitosan type or percent could be observed.

Table 12. Trimethylamine Concentrations (mg TMA-N/100g) of Chitosan Treated Salmon Fillet During Refrigerated (4°C) Storage*

Treatment Code**	Day 1	Day 3	Day 6	Day 10	Day 13
F	n.d.***	****	n.d.	1.21 ± 0.93	0.64 ± 0.21 ab
Fa	n.d.	****	n.d.	0.39 ± 0.15	0.88 ± 0.15 a
FL	n.d.	****	n.d.	0.49 ± 0.03	0.28 ± 0.22 b
FH	n.d.	****	n.d.	0.44 ± 0.12	0.29 ± 0.26 b
p-value				0.190	0.030

* Each value is the average of three replications ± standard deviation, analyzed in duplicate. Means in the same column not sharing a superscript are significantly ($p < 0.05$) different based on one-way ANOVA followed by Tukey's post hoc test.

** F = Fillet control; Fa = Fillet dipped in 1% acetic acid; FL = Fillet dipped in 1% LMW chitosan in 1% acetic acid; FH = Fillet dipped in 1% HMW chitosan in 1% acetic acid.

*** n.d. indicates undetectable levels of TMA-N

**** Analyses not conducted.

Table 13. Trimethylamine Concentrations (mg TMA-N/100g) of Chitosan Treated Salmon Trim During Refrigerated (4°C) Storage*

Treatment Code **	Day 1	Day 3	Day 6	Day 10	Day 13
T	n.d.***	****	n.d.	0.65 ± 0.93	3.30 ± 0.29
0.5TL	n.d.	****	n.d.	1.43 ± 1.82	2.14 ± 0.26
0.5TH	n.d.	****	n.d.	1.37 ± 0.98	3.09 ± 0.49
1TL	n.d.	****	n.d.	2.05 ± 2.29	1.28 ± 0.18
1TH	n.d.	****	n.d.	0.91 ± 0.26	2.48 ± 1.31
2TL	n.d.	****	n.d.	1.16 ± 0.64	2.38 ± 1.26
2TH	n.d.	****	n.d.	0.54 ± 0.24	3.07 ± 1.02
p-value				0.750	0.110

* Each value is the average of three replications ± standard deviation, analyzed in duplicate.

** T = Trim control; .5TL = Trim with 0.5% LMW chitosan; .5TH = Trim with 0.5% HMW chitosan; 1TL = Trim with 1% LMW chitosan; 1TH = Trim with 1% HMW chitosan; 2TL = Trim with 2% LMW chitosan; 2TH = Trim with 2% HMW chitosan.

*** n.d. indicates undetectable levels of TMA-N

**** Analyses not conducted.

RESULTS:
ENZYMATIC DEGRADATION OF CHITOSAN SOLUTIONS

Enzyme Effectiveness

The protein contents of the alpha amylase and bromelain were found to be 30.0% and 47.9%, respectively. Both alpha amylase and bromelain were initially tested for breakdown of 1.0% chitosan solutions at pH 4.0 at a level of one percent by weight of chitosan. White, cloudy precipitate formed in the beaker after 24 hours of enzyme activity after the pH was adjusted to 7.0 with 10% sodium hydroxide, indicating the chitosan solution was not made water soluble. The enzyme level was then increased to 50% and it was discovered that the alpha amylase produced a minute amount of precipitate after the pH was adjusted to seven. However, bromelain appeared to produce the same amount of white precipitate that it had at the 1.0% enzyme level. The amount of precipitate formation in the solution containing alpha amylase indicated that this enzyme had effectively degraded the chitosan to afford water solubility. Based on these results it was decided that the next phase of the quantification study would continue only with alpha amylase.

Observations

Quantification studies conducted with a 10% level of alpha amylase at pH levels of 4.0, 5.0 and 7.0 for time periods of 8 hours and 24 hours revealed that the most visible indications (little precipitation when pH adjusted to 7.0) of water solubility occurred in the pH 4.0 treatment (Table 14). The least amount of

Table 14. Observations of 1% Chitosan Solutions Degraded with 10% Alpha Amylase

Treatment	Initial Appearance	8 Hour Appearance after pH adjustment to 7.0	24 Hour Appearance after pH adjustment to 7.0
Control (no enzyme) pH 4.0	Thick, opaque	Thick, gelatinous, white, clumpy precipitate	Thick, gelatinous, white, clumpy precipitate
pH 4.0	Thick, opaque	Cloudy liquid, white, smooth precipitate	Slightly cloudy, small amount of fine precipitate
pH 5.5	Thinner, some undissolved flakes, opaque	Slightly cloudy liquid and undissolved flakes, ropy	Slightly cloudy liquid and undissolved flakes, ropy
pH 7.0	Thin, watery, all undissolved chitosan flakes	Thin, watery, all undissolved chitosan flakes	Thin, watery, all undissolved chitosan flakes

precipitate was formed after 24 hours of enzymatic degradation at pH 4.0 but a slight amount of precipitate formed in the pH 5.5 treatment, probably due to the lack of fully solubilized chitosan to begin with. A large amount of thick, clumpy precipitate formed in the control treatment and no precipitate formed at all in the pH 7.0 treatment when the pH was adjusted to 7.0.

Gravimetric Measurements

The results from the eight-hour degradation study indicate that none of the treatments became completely water-soluble. All of the treatments displayed either 100% or higher degree of insolubility when the amount of dried precipitate (formed after pH adjustment to 7.0) was compared to the initial weight of chitosan and enzyme used in each treatment (Table 15). This indicates that this method used to quantify chitosan insolubility was inaccurate. The results from the 24 hour study indicated that the pH 4.0 enzymatic treatment resulted in the most soluble chitosan. When the pH was brought back up to 7.0, only 11.4% of the chitosan remained insoluble (Table16). Enzymatic degradation at pH 5.5 was somewhat effective at producing water soluble chitosan. After 24 hours, 75% of the chitosan remained insoluble based on the gravimetric measurements. Alpha amylase appeared to have little enzymatic activity on chitosan that was not solubilized, as was the case with the pH 7.0 treatment (Table 16).

Table 15. Weight Measurements of 1% LMW Chitosan Solutions Degraded with 10% Alpha Amylase for 8 Hours

Treatment	Initial Weight of Chitosan + Enzyme (g)	Weight of Insoluble Chitosan Precipitate+ Enzyme (g)	% Insolubility*
Control (no enzyme)	0.501 ± 0.001	0.707 ± 0.088	141.0
pH 4.0	0.552 ± 0.003	0.600 ± 0.004	108.7
pH 5.5	0.552 ± 0.003	0.555 ± 0.033	100.5
pH 7.0	0.552 ± 0.001	0.676 ± 0.035	122.5

*Calculated by dividing (dried weight of the insoluble chitosan precipitate + enzyme) by (initial weight of chitosan + enzyme) x 100.

Table 16. Weight Measurements of 1% LMW Chitosan Solutions Degraded with 10% Alpha Amylase for 24 Hours

Treatment	Initial Weight of Chitosan + Enzyme (g)	Weight of Insoluble Chitosan Precipitate + Enzyme (g)	% Insolubility*
Control (no enzyme)	0.501 ± 0.002	0.590 ± 0.082	117.8
pH 4.0	0.554 ± 0.001	0.064 ± 0.017	11.4
pH 5.5	0.551 ± 0.002	0.412 ± 0.120	74.6
pH 7.0	0.555 ± 0.002	0.475 ± 0.015	85.6

*Calculated by dividing (dried weight of the insoluble chitosan precipitate + enzyme) by (initial weight of chitosan + enzyme) x 100.

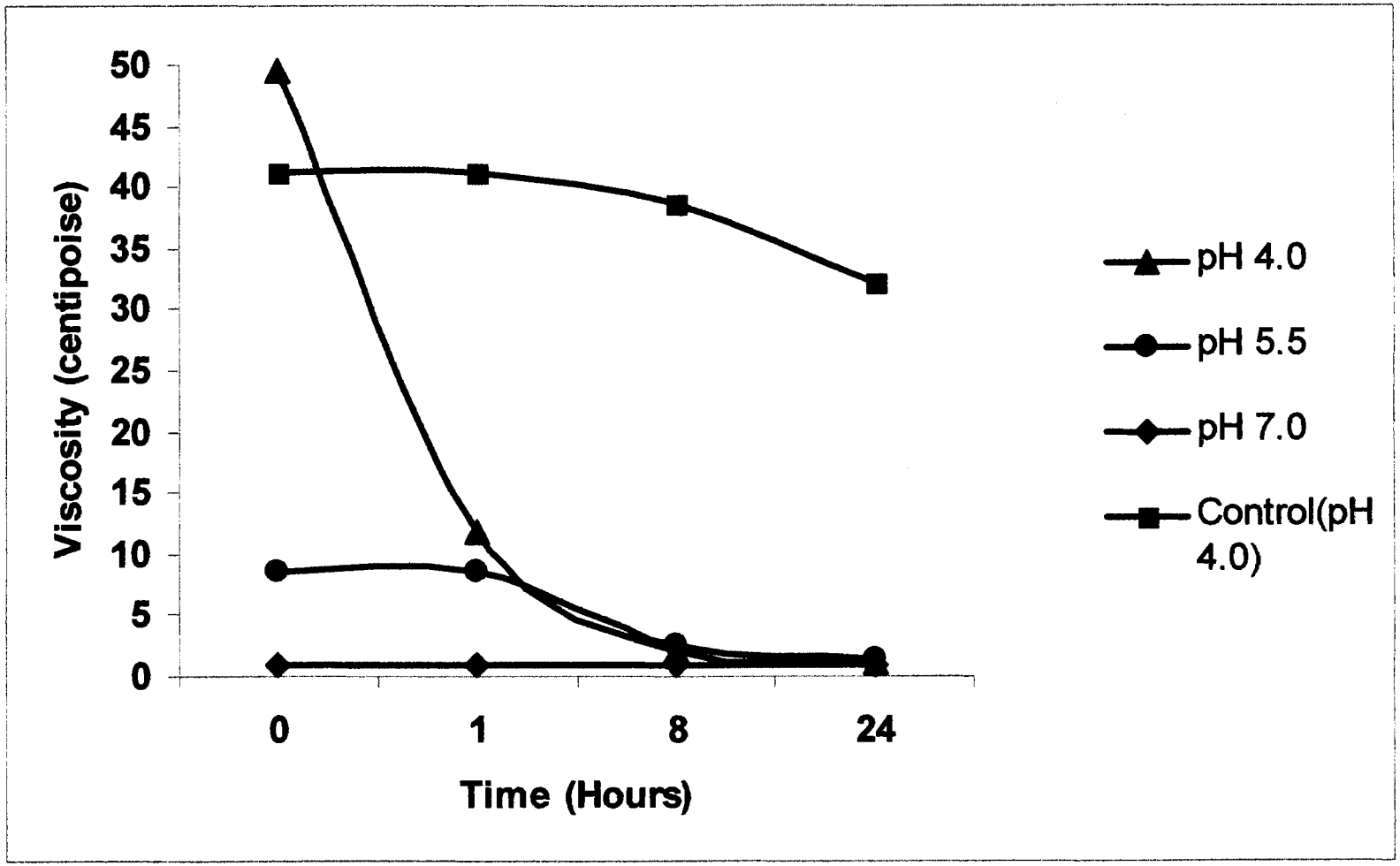
Viscosity

Results of the viscosity measurements indicate that the greatest rate of enzymatic degradation occurred within the first hour of introduction of the alpha amylase to the chitosan solution. Figure 5 illustrates that alpha amylase was most effective in the solution with a pH of 4.0 as indicated by the speed and degree of viscosity reduction. Minimal changes in viscosity were visible in all of the other treatments. A slight reduction in viscosity, from 49.6 cP to 32.2 cP was evident in the control treatment, possibly a result of the acetic acid solution causing a slow hydrolysis of some of the polymer's (chitosan) bonds.

Enzyme Concentration

A comparison of the viscosity data of the 5% alpha amylase (by weight of chitosan) versus 10% alpha amylase indicates a slower rate of degradation by the lower enzyme concentration (Figures 6 and 7). Rate of viscosity reduction during the first ten minutes for the 10% and 5% alpha amylase treatment was 1.23 centipoise per minute and 0.97 centipoise per minute, respectively (Figures 6 and 7). From ten minutes to 30 minutes the rate decreased for the 10% and 5% enzyme treatment to 0.85 centipoise per minute to 0.68 centipoise per minute, respectively. The rate of degradation for both treatments was nearly the same from 30 minutes to 60 minutes, 0.21 centipoise per minute for the 10% enzyme treatment and 0.20 centipoise per minute for the 5% treatment. In the first hour, the difference in the degradation rate resulted in a 76% viscosity reduction for the 10% alpha amylase versus a 61% viscosity reduction for the 5%

Figure 5. Effect of pH and Time on 1% LMW Chitosan Solutions Degraded with Alpha Amylase*



*All solutions were prepared in 1% acetic acid and enzyme concentrations were 10% by weight of the chitosan.

Figure 6. Viscosity Reduction of 1% Chitosan Solution by 10% (w/w of Chitosan) Alpha Amylase

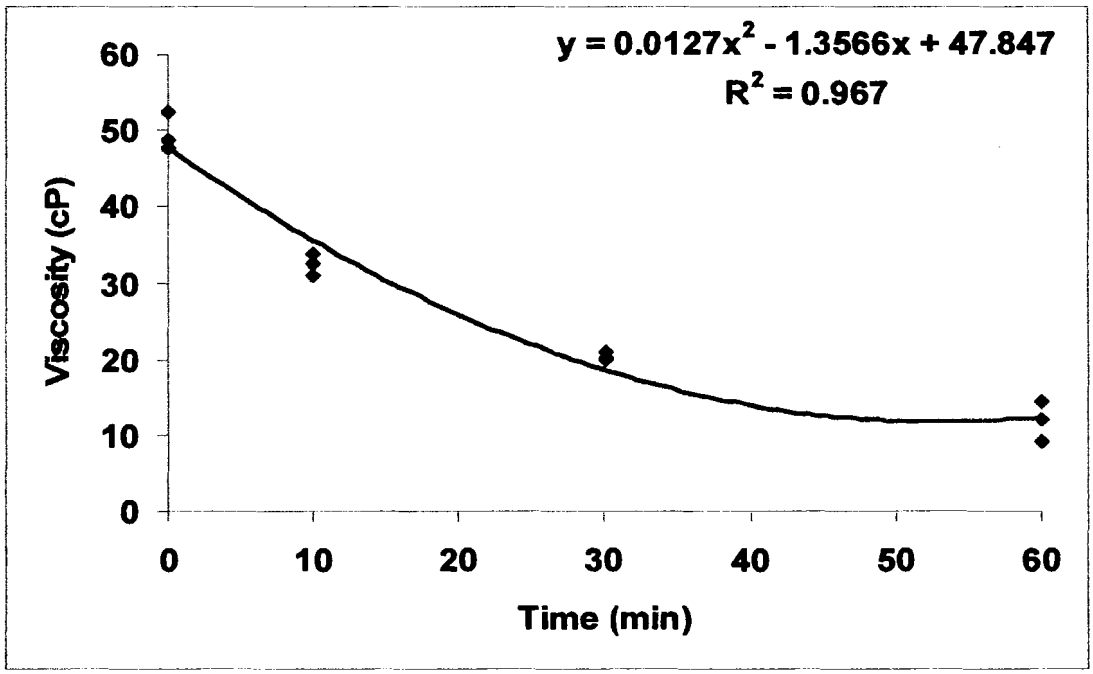
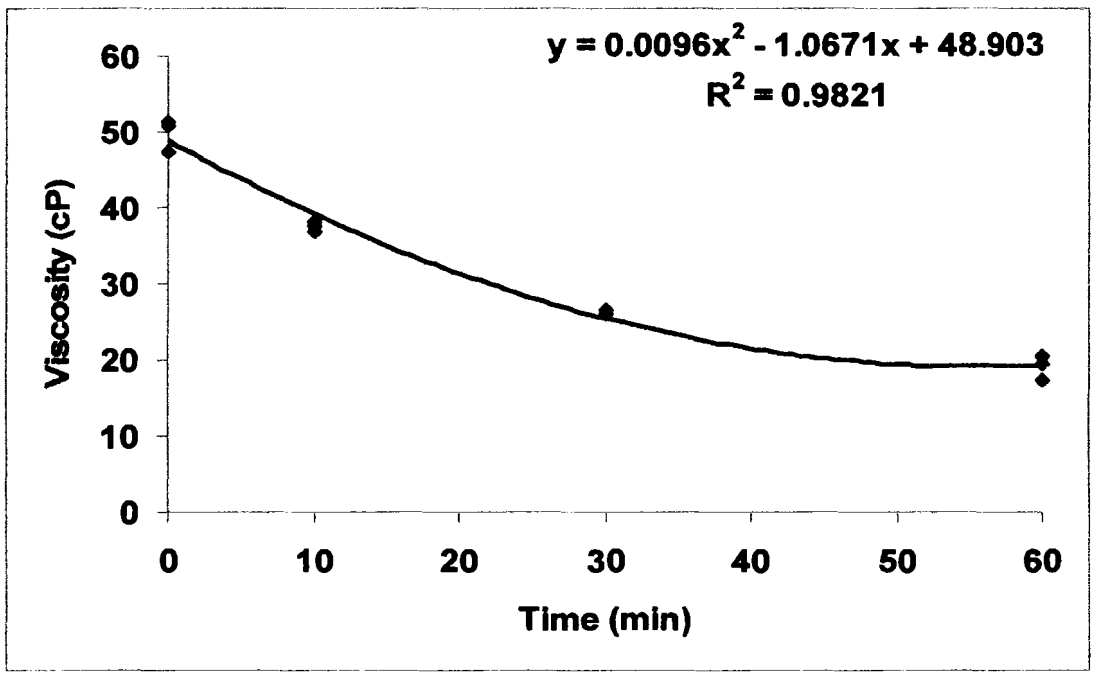


Figure 7. Viscosity Reduction of 1% Chitosan Solution by 5% (w/w of Chitosan) Alpha Amylase



alpha amylase treatment. The viscosity of the 5% enzyme treatment was 2.3 centipoise after 24 hours which was the same viscosity as the 10% enzyme treatment after eight hours, indicating the degree of degradation achieved by the 5% treatment was not as high as that of the 10% treatment (Figures 8 and 9).

Intrinsic Viscosity

The undegraded chitosan had an intrinsic (reduced) viscosity of 25.67 (Figure 10). The degraded chitosan solutions;10 min degraded, 30 min degraded, 60 min degraded, 8 hour degraded and 24 hour degraded had intrinsic viscosities of 12.83, 10.34, 8.75, 3.08 and 0.79 mL/g respectively (Figure 10)

Figure 8. Viscosity Reduction of 1% LMW Chitosan Solutions at pH 4.0 by 10% (w/w of Chitosan) Alpha Amylase

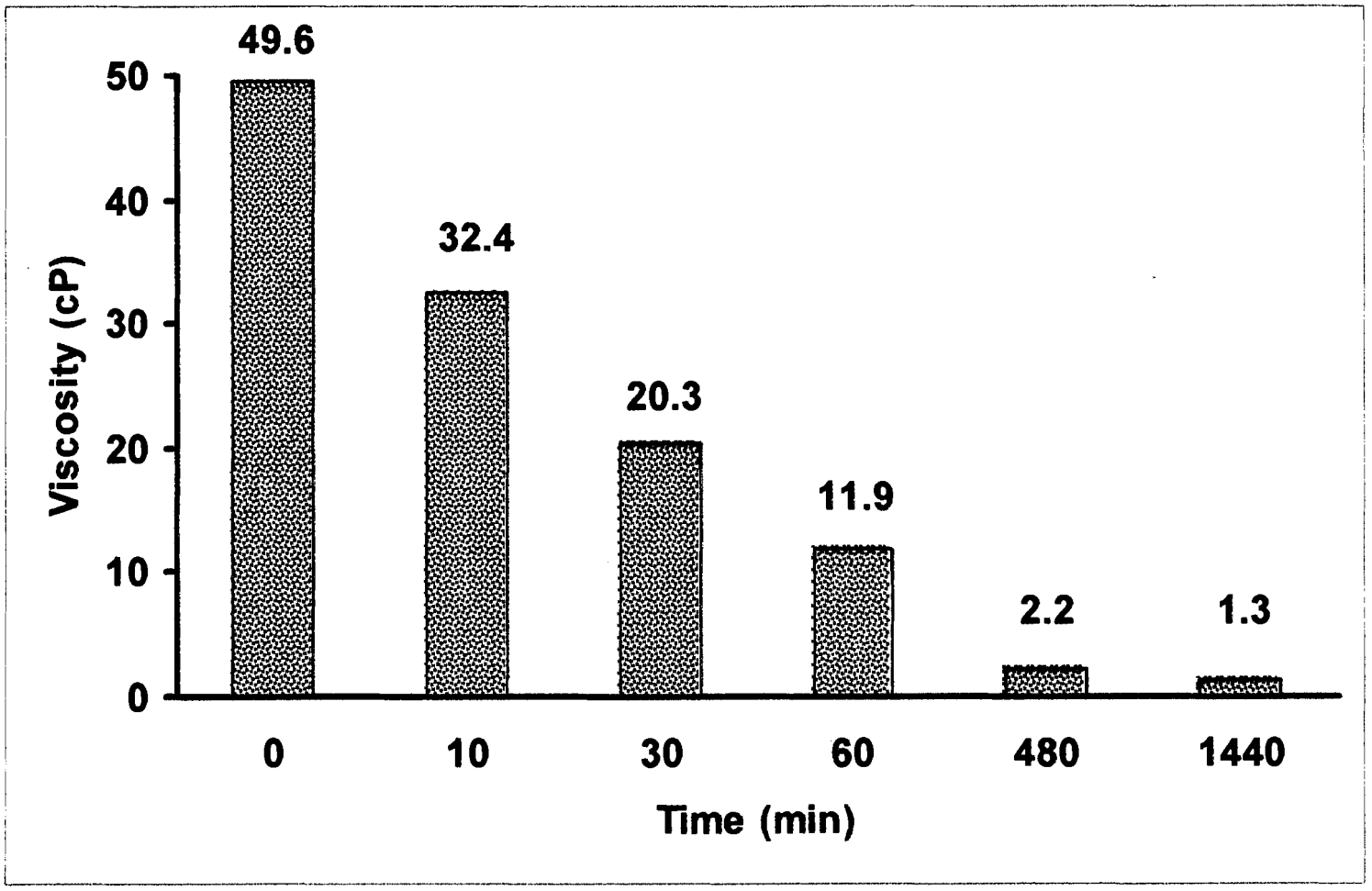


Figure 9. Viscosity Reduction of 1% LMW Chitosan Solutions at pH 4.0 by 5% (w/w of Chitosan) Alpha Amylase

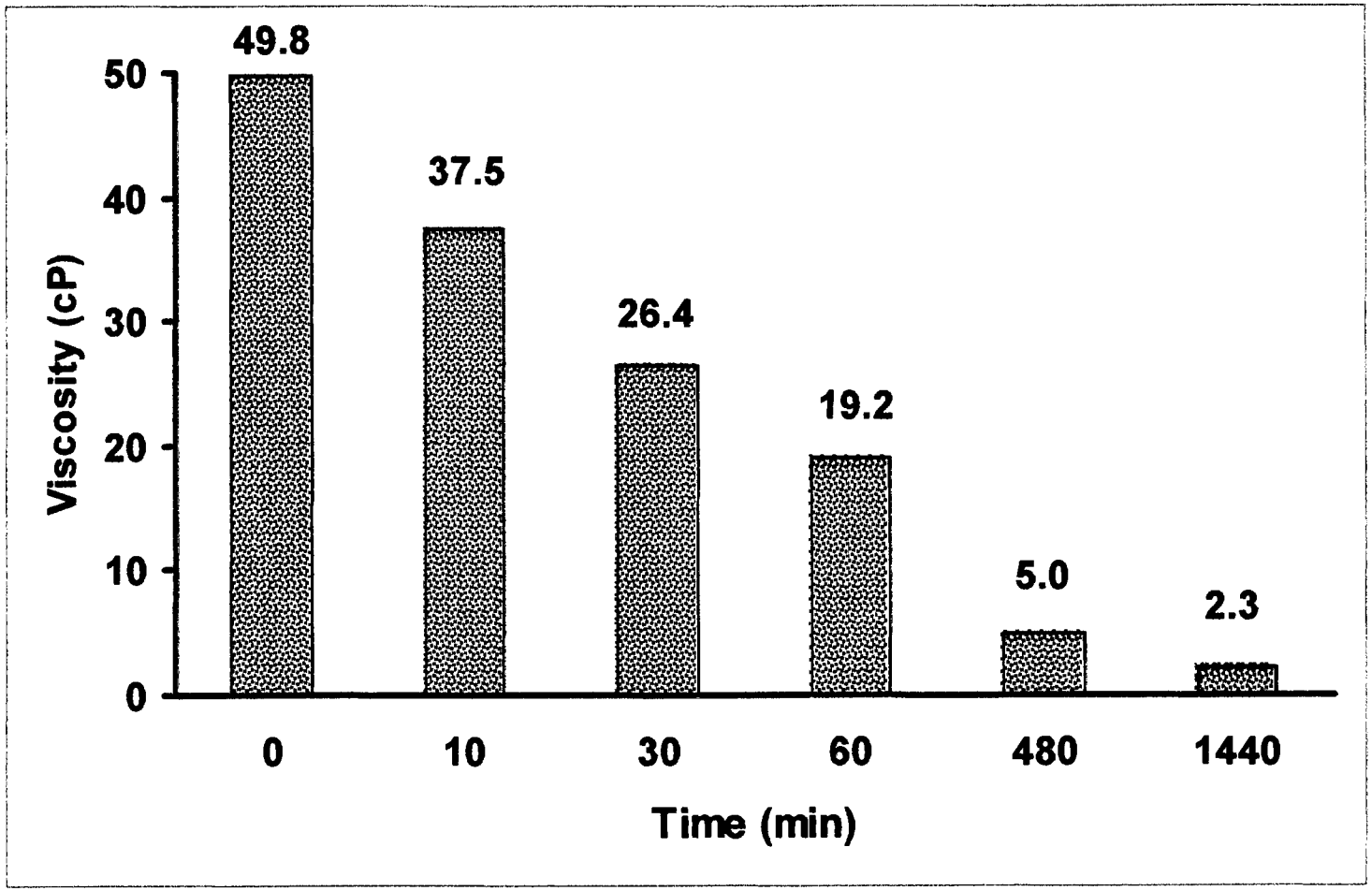
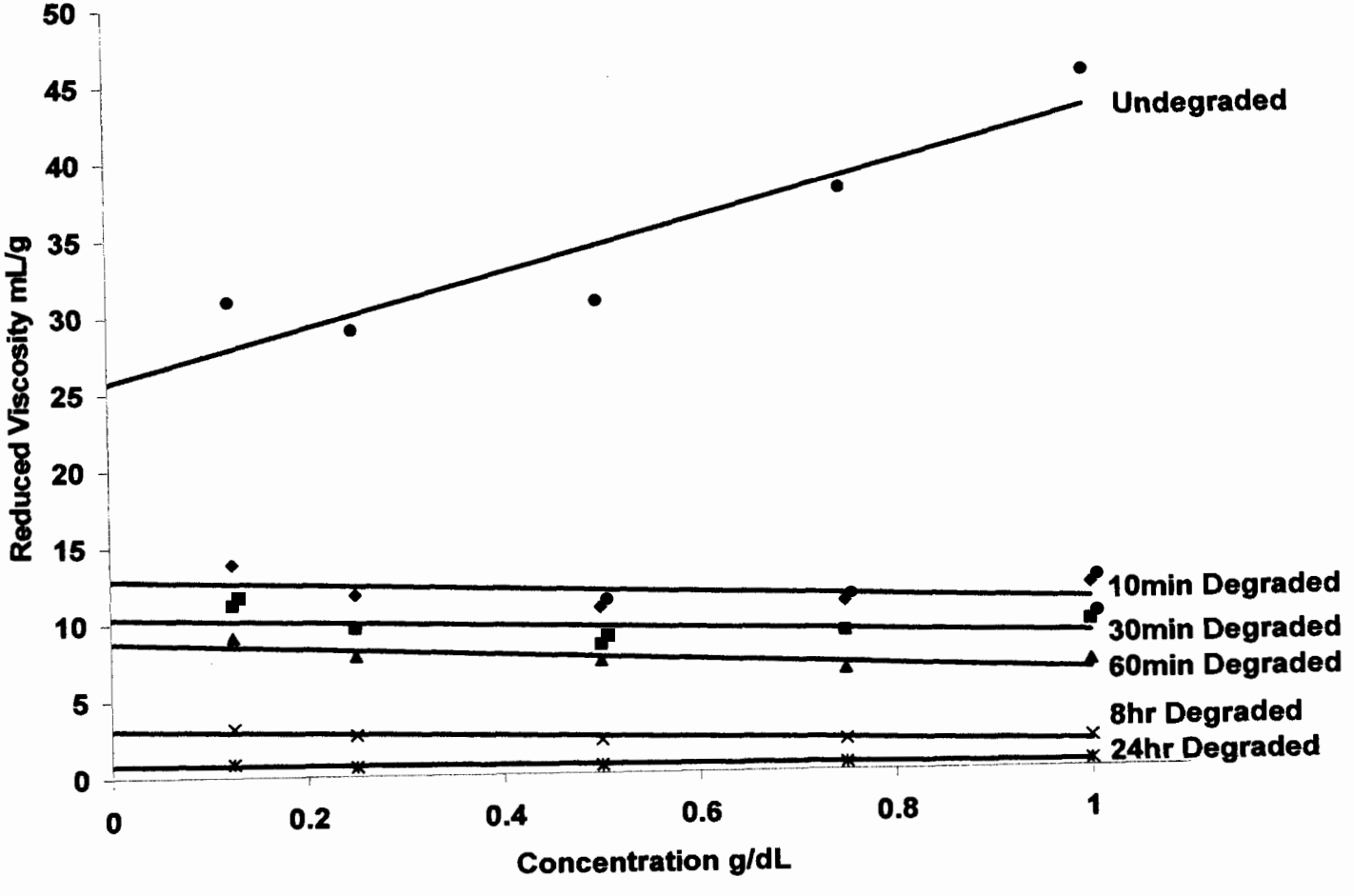


Figure 10. Intrinsic Viscosity of Degraded and Undegraded 1% Chitosan Solutions



RESULTS:
IN VITRO ANTIMICROBIAL EFFICACY OF CHITOSAN AGAINST *LISTERIA INNOCUA* AND *PSEUDOMONAS AERUGINOSA*

Listeria in Trypticase Soy Broth

The inoculation level into each treatment flask was approximately $2.49x10^8$ CFU/mL. The chitosan treated flasks were up to one log lower than both controls on day one (Table 17). Bacterial counts of the controls gradually rose to $8.15x10^8$ and $3.54x10^8$ CFU/mL for control 1 and control 2, respectively, by day four while bacterial counts in chitosan treated flasks remained fairly constant all four days and by day four all had counts at least one log lower than the controls (Table 17). On day three *Listeria* counts in the acidified control were significantly higher ($p<0.05$) than all of the chitosan treatments with the exception of the 24 hour degraded chitosan treatments, regardless of percentage of chitosan. When averaged over four days, there was approximately a one log higher *Listeria* count in the 0.5% chitosan treatment versus the 2.0% treatment (Figure 11). There was a trend toward lower *Listeria* counts with increasing degradation time except in the case of the 24 hour degraded chitosan, which had higher *Listeria* counts than all other chitosan treatments. There were no significant differences among treatments observed in terms of degradation time or chitosan percent based on multiway ANOVA. However, there was a significant effect of days of incubation and the interaction between days of incubation and treatment.

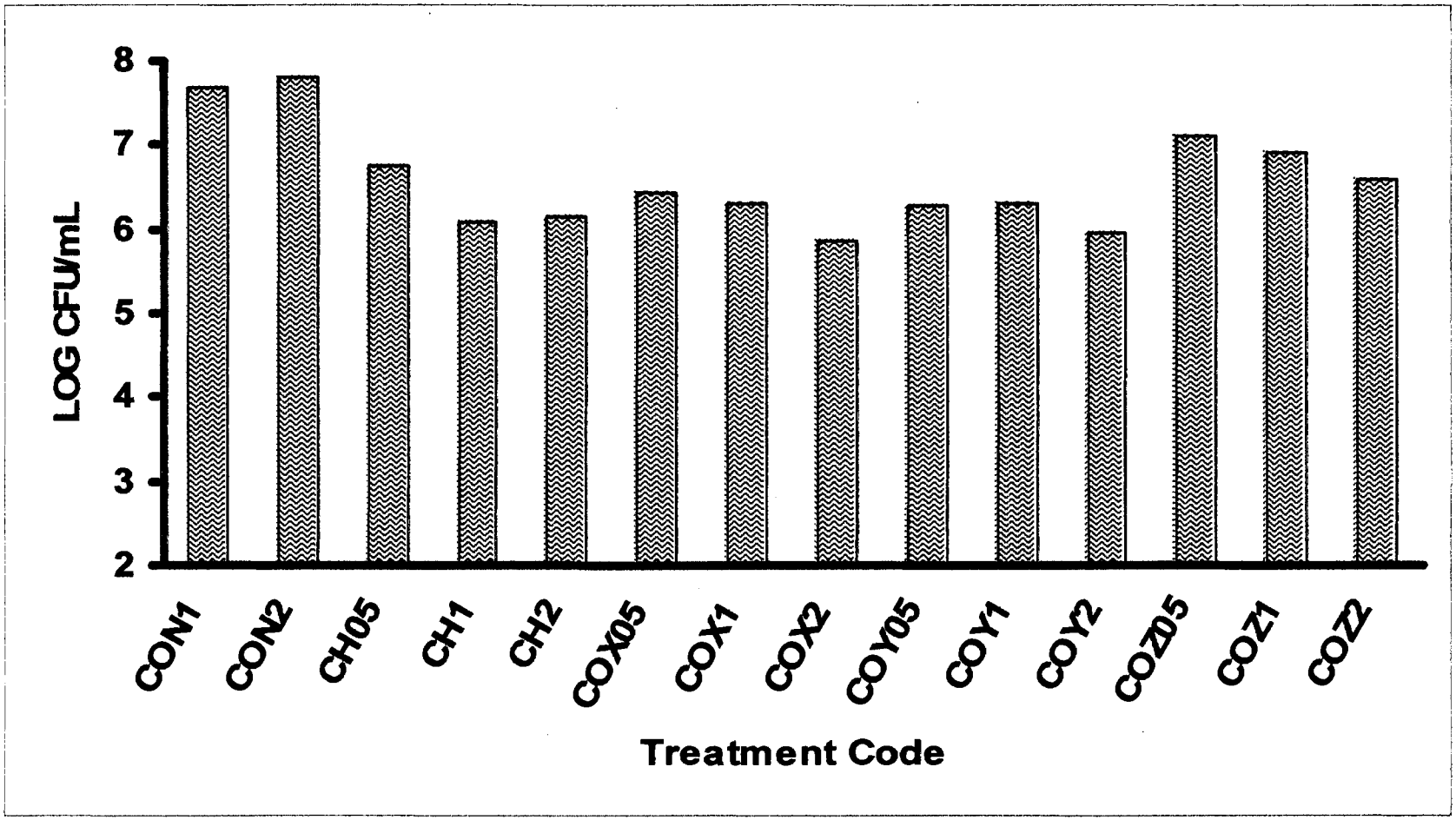
Table 17. *Listeria* Counts of Control and Chitosan Treated Trypticase Soy Broth During Refrigerated (4°C) Storage*

Code**	DAY 1	DAY 2	DAY 3	DAY 4
CON1	$2.45\times10^{7} \pm 2.75\times10^{7}$	$7.81\times10^{7} \pm 1.04\times10^{8}$	$4.79\times10^{7} \pm 4.98\times10^{7}$ ab	$8.15\times10^{8} \pm 9.92\times10^{8}$
CON2	$5.41\times10^{7} \pm 3.22\times10^{7}$	$5.50\times10^{7} \pm 7.79\times10^{7}$	$1.40\times10^{8} \pm 1.31\times10^{8}$ a	$3.54\times10^{8} \pm 3.63\times10^{8}$
CH05	$3.05\times10^{7} \pm 4.90\times10^{7}$	$1.99\times10^{7} \pm 4.31\times10^{6}$	$5.98\times10^{6} \pm 6.92\times10^{6}$ b	$1.02\times10^{7} \pm 1.51\times10^{7}$
CH1	$7.97\times10^{6} \pm 8.78\times10^{6}$	$3.01\times10^{6} \pm 3.07\times10^{6}$	$1.59\times10^{7} \pm 2.65\times10^{7}$ b	$6.02\times10^{6} \pm 9.94\times10^{6}$
CH2	$1.21\times10^{7} \pm 8.25\times10^{6}$	$2.81\times10^{6} \pm 4.37\times10^{6}$	$2.52\times10^{6} \pm 4.06\times10^{6}$ b	$1.07\times10^{7} \pm 1.80\times10^{7}$
COX05	$9.82\times10^{6} \pm 2.14\times10^{6}$	$4.96\times10^{6} \pm 7.41\times10^{6}$	$9.82\times10^{6} \pm 1.31\times10^{7}$ b	$5.75\times10^{6} \pm 9.14\times10^{6}$
COX1	$4.79\times10^{7} \pm 6.41\times10^{7}$	$3.65\times10^{6} \pm 5.27\times10^{6}$	$6.18\times10^{6} \pm 9.56\times10^{6}$ b	$6.22\times10^{6} \pm 1.02\times10^{7}$
COX2	$3.14\times10^{6} \pm 4.00\times10^{6}$	$1.94\times10^{6} \pm 2.01\times10^{6}$	$3.77\times10^{6} \pm 6.01\times10^{6}$ b	$4.80\times10^{6} \pm 8.05\times10^{6}$
COY05	$2.37\times10^{6} \pm 2.00\times10^{6}$	$3.86\times10^{6} \pm 5.12\times10^{6}$	$6.94\times10^{6} \pm 1.14\times10^{7}$ b	$2.54\times10^{7} \pm 2.26\times10^{7}$
COY1	$1.01\times10^{7} \pm 9.71\times10^{6}$	$4.01\times10^{6} \pm 3.91\times10^{6}$	$4.27\times10^{6} \pm 6.83\times10^{6}$ b	$1.48\times10^{7} \pm 1.87\times10^{7}$
COY2	$5.02\times10^{6} \pm 4.01\times10^{6}$	$7.79\times10^{5} \pm 1.04\times10^{6}$	$1.66\times10^{6} \pm 1.63\times10^{6}$ b	$1.77\times10^{7} \pm 3.01\times10^{7}$
COZ05	$5.08\times10^{7} \pm 5.73\times10^{7}$	$3.63\times10^{7} \pm 3.73\times10^{7}$	$3.84\times10^{7} \pm 4.93\times10^{7}$ ab	$3.65\times10^{6} \pm 1.85\times10^{6}$
COZ1	$1.69\times10^{7} \pm 1.77\times10^{7}$	$2.07\times10^{7} \pm 3.82\times10^{6}$	$2.13\times10^{7} \pm 3.18\times10^{7}$ ab	$9.23\times10^{6} \pm 1.21\times10^{7}$
COZ2	$1.09\times10^{7} \pm 1.09\times10^{7}$	$1.26\times10^{7} \pm 9.48\times10^{6}$	$1.84\times10^{7} \pm 2.48\times10^{7}$ ab	$4.80\times10^{6} \pm 7.20\times10^{6}$
p-value	0.412	0.334	0.045	0.069

*Each value is an average of three replications, each analyzed in duplicate ± standard deviation. Different letters within each column indicate a significant difference ($p < 0.05$) among treatments based on one-way analysis of raw data followed by Tukey's post hoc test.

**CON1 = non-acidified control; CON2 = acidified control; CH05 = 0.5%v/v 1%LMW chitosan; CH1 = 1.0%v/v 1%LMW chitosan; CH2 = 2.0%v/v 1%LMW chitosan; COX05 = 0.5%v/v 1%10 min degraded chitosan; COX1 = 1.0%v/v 10 min degraded chitosan; COX2 = 2.0%v/v 10 min degraded chitosan; COY05 = 0.5%v/v 1% 60 min degraded chitosan; COY1 = 1.0%v/v 1% 60 min degraded chitosan; COY2 = 2.0%v/v 1% 60 min degraded chitosan; COZ05 = 0.5%v/v 0.5% 24 hour degraded chitosan; COZ1 = 1.0%v/v 1.0% 24 hour degraded chitosan; COZ2 = 2.0% 24 hour degraded chitosan

Figure 11. Four Day Mean *Listeria* Counts in Trypticase Soy Broth*



*Each value is the average of four days in triplicate.
CON1 = non-acidified control; CON2 = acidified control; CH05 = 0.5%v/v 1%LMW chitosan; CH1 = 1.0%v/v 1%LMW chitosan; CH2 = 2.0%v/v 1%LMW chitosan; COX05 = 0.5%v/v 1%10 min degraded chitosan; COX1 = 1.0%v/v 10 min degraded chitosan; COX2 = 2.0%v/v 10 min degraded chitosan; COY05 = 0.5%v/v 1% 60 min degraded chitosan; COY1 = 1.0%v/v 1% 60 min degraded chitosan; COY2 = 2.0%v/v 1% 60 min degraded chitosan; COZ05 = 0.5%v/v 0.5% 24 hour degraded chitosan; COZ1 = 1.0%v/v 1.0% 24 hour degraded chitosan; COZ2 = 2.0% 24 hour degraded chitosan

Pseudomonas in Trypticase Soy Broth

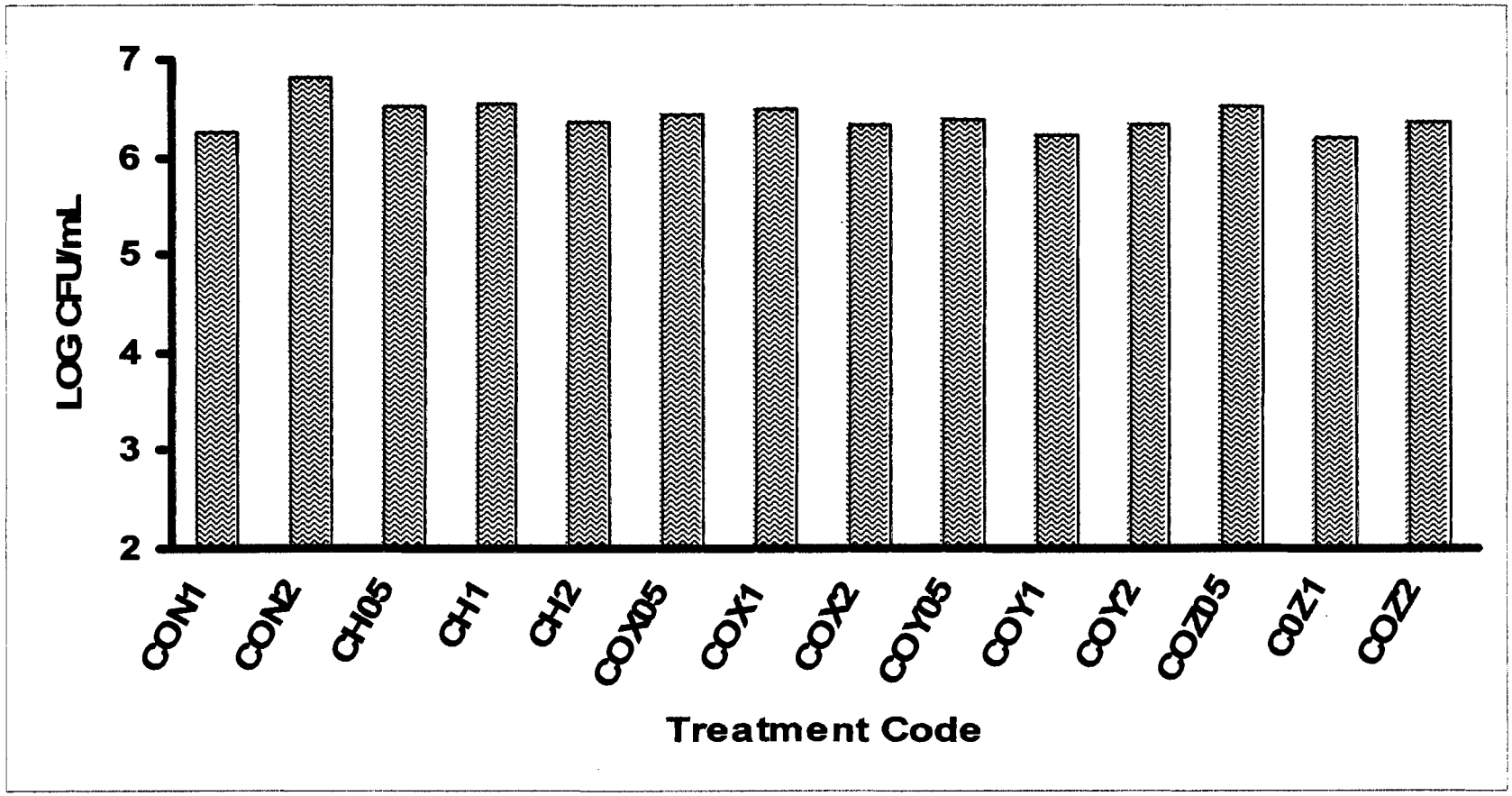
Initial inoculation levels of *Pseudomonas* were approximately $2.50x10^7$ CFU/mL, which was less than the inoculation level of the *Listeria*. *Pseudomonas* did not grow as well as *Listeria*, therefore a higher inoculation amount, one mL compared to 0.1 mL, was required to achieve the desired level. Initial counts on day one indicated an approximately one log reduction for all treatments, including the controls, compared to the inoculum level (Table 18). CON2, the acidified control, had approximately a half log higher *Pseudomonas* count compared to CON1 throughout the experiment. All chitosan treatments had a slightly lower *Pseudomonas* count throughout the experiment compared to the CON2 but not CON1 which is apparent when evaluating the mean counts for all four days of the experiment (Figure 12). One way ANOVA revealed no significant differences among any treatment on any day of analysis (Table 18). There was a higher amount of precipitate when chitosan was added to this broth compared to nutrient broth. No significant differences were observed regarding chitosan percent, day or degradation time based on multiway ANOVA.

Table 18. *Pseudomonas* Counts of Control and Chitosan Treated Trypticase Soy Broth During Refrigerated (4°C) Storage*

Treatment Code**	DAY 1	DAY 2	DAY 3	DAY 4
CON1	$3.75x10^6 \pm 2.59x10^6$	$1.79x10^6 \pm 1.48x10^6$	$1.78x10^6 \pm 1.50x10^6$	$1.75x10^6 \pm 9.73x10^6$
CON2	$1.26x10^7 \pm 7.61x10^6$	$7.10x10^6 \pm 2.72x10^6$	$7.04x10^6 \pm 4.00x10^6$	$4.63x10^6 \pm 2.38x10^6$
CH05	$5.96x10^6 \pm 3.73x10^6$	$4.43x10^6 \pm 3.53x10^6$	$5.09x10^6 \pm 4.32x10^6$	$4.03x10^6 \pm 3.63x10^6$
CH1	$6.60x10^6 \pm 5.01x10^6$	$6.50x10^6 \pm 6.70x10^6$	$5.13x10^6 \pm 5.04x10^6$	$7.37x10^6 \pm 8.11x10^6$
CH2	$5.28x10^6 \pm 6.41x10^6$	$4.03x10^6 \pm 4.91x10^6$	$8.90x10^6 \pm 8.70x10^6$	$3.13x10^6 \pm 3.87x10^6$
COX05	$9.77x10^6 \pm 1.07x10^7$	$3.33x10^6 \pm 2.55x10^6$	$3.89x10^6 \pm 3.91x10^6$	$3.71x10^6 \pm 3.38x10^6$
COX1	$7.92x10^6 \pm 6.71x10^6$	$5.10x10^6 \pm 4.32x10^6$	$5.90x10^6 \pm 6.82x10^6$	$3.28x10^6 \pm 2.99x10^6$
COX2	$4.91x10^6 \pm 3.79x10^6$	$3.61x10^6 \pm 3.05x10^6$	$5.07x10^6 \pm 4.20x10^6$	$3.33x10^6 \pm 2.85x10^6$
COY05	$4.00x10^6 \pm 2.73x10^6$	$3.12x10^6 \pm 2.10x10^6$	$3.41x10^6 \pm 2.18x10^6$	$2.27x10^6 \pm 2.13x10^6$
COY1	$6.47x10^6 \pm 6.53x10^6$	$4.60x10^6 \pm 5.84x10^6$	$3.45x10^6 \pm 4.22x10^6$	$3.54x10^6 \pm 4.71x10^6$
COY2	$4.94x10^6 \pm 4.74x10^6$	$3.12x10^6 \pm 3.32x10^6$	$4.63x10^6 \pm 6.06x10^6$	$3.03x10^6 \pm 3.69x10^6$
COZ05	$5.82x10^6 \pm 4.14x10^6$	$5.29x10^6 \pm 3.29x10^6$	$4.53x10^6 \pm 3.86x10^6$	$4.41x10^6 \pm 2.74x10^6$
COZ1	$3.71x10^6 \pm 4.53x10^6$	$2.34x10^6 \pm 1.96x10^6$	$2.60x10^6 \pm 2.64x10^6$	$2.17x10^6 \pm 1.54x10^6$
COZ2	$4.36x10^6 \pm 3.21x10^6$	$4.53x10^6 \pm 3.89x10^6$	$3.68x10^6 \pm 3.13x10^6$	$2.65x10^6 \pm 2.45x10^6$
p-value	0.84	0.93	0.93	0.94

*Each value is an average of three replications, each analyzed in duplicate ± standard deviation. Different letters within each column indicate a significant difference ($p < 0.05$) among treatments based on one-way analysis of raw data followed by Tukey's post hoc test.
*CON1 = non-acidified control; CON2 = acidified control; CH05 = 0.5%v/v 1%LMW chitosan; CH1 = 1.0%v/v 1%LMW chitosan; CH2 = 2.0%v/v 1%LMW chitosan; COX05 = 0.5%v/v 1%10 min degraded chitosan; COX1 = 1.0%v/v 10 min degraded chitosan; COX2 = 2.0%v/v 10 min degraded chitosan; COY05 = 0.5%v/v 1% 60 min degraded chitosan; COY1 = 1.0%v/v 1% 60 min degraded chitosan; COY2 = 2.0%v/v 1% 60 min degraded chitosan; COZ05 = 0.5%v/v 0.5% 24 hour degraded chitosan; COZ1 = 1.0%v/v 1.0% 24 hour degraded chitosan; COZ2 = 2.0% 24 hour degraded chitosan

Figure 12. Four Day Mean *Pseudomonas* Counts in Trypticase Soy Broth*



*Each value is the average of four days in triplicate.
CON1 = non-acidified control; CON2 = acidified control; CH05 = 0.5%v/v 1%LMW chitosan; CH1 = 1.0%v/v 1%LMW chitosan; CH2 = 2.0%v/v 1%LMW chitosan; COX05 = 0.5%v/v 1%10 min degraded chitosan; COX1 = 1.0%v/v 10 min degraded chitosan; COX2 = 2.0%v/v 10 min degraded chitosan; COY05 = 0.5%v/v 1% 60 min degraded chitosan; COY1 = 1.0%v/v 1% 60 min degraded chitosan; COY2 = 2.0%v/v 1% 60 min degraded chitosan; COZ05 = 0.5%v/v 0.5% 24 hour degraded chitosan; COZ1 = 1.0%v/v 1.0% 24 hour degraded chitosan; COZ2 = 2.0% 24 hour degraded chitosan

***Pseudomonas* in Nutrient Broth**

An initial trial in nutrient broth at a lower inoculation level ($3.14x10^5$ CFU/mL) resulted in nearly total inhibition of *Pseudomonas* growth and uncountable plates at all dilutions by day three for all chitosan treated flasks. *Pseudomonas* counts in the controls started at log 5 and remained there all four days of the experiment (Table 19). After completing another experiment in triplicate in trypticase soy broth at a higher inoculation level ($2.50x10^7$ CFU/mL), another experiment in nutrient broth was performed (in triplicate) at the higher inoculation level ($1.98x10^7$ CFU/mL). The four day mean CON2 (acidified nutrient broth) *Pseudomonas* counts were slightly higher than the four day mean CON1 throughout the experiment (Figure 13). One way ANOVA revealed the controls had significantly higher (p=0.00) *Pseudomonas* counts than all of the chitosan treatments from day one through day four of the experiment (Table 19). Multiway ANOVA revealed that there were significant effects of day (p=0.00), degradation time (p=0.00), and chitosan percent (p=0.00) on *Pseudomonas* counts. The effects of chitosan percent on *Pseudomonas* counts can be clearly observed in Figure 13. As chitosan percent increased from 0.5 to 1.0 to 2.0%, there was a stepwise decrease in *Pseudomonas* counts. Two percent chitosan treatments were approximately one log lower than 0.5% chitosan treatments. *Pseudomonas* counts gradually fell in all chitosan treatments on each day of the experiment. There was a slight decrease in *Pseudomonas* counts for the 10 minute degraded chitosan treatment compared to undegraded chitosan. The log reductions of the 24 hour degraded chitosan treatment were slightly less than the

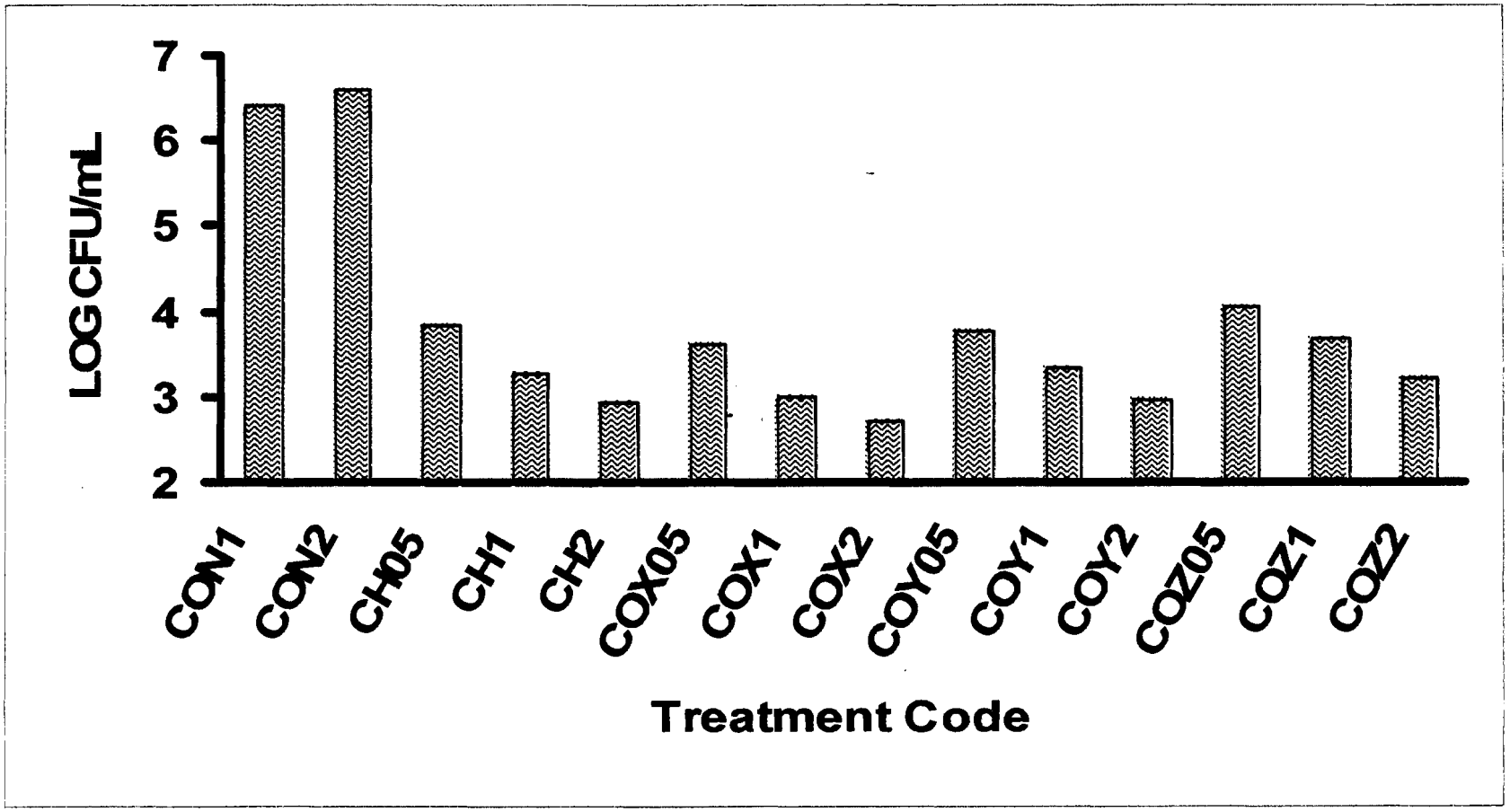
Table 19. *Pseudomonas* Counts of Control and Chitosan Treated Nutrient Broth During Refrigerated (4°C) Storage*

Treatment Code**	DAY 1	DAY 2	DAY 3	DAY 4
CON1	$2.05x10^6 \pm 1.83x10^6$ a	$3.43x10^6 \pm 2.97x10^6$ a	$2.97x10^6 \pm 2.20x10^6$ a	$2.05x10^6 \pm 1.83x10^6$ a
CON2	$1.61x10^6 \pm 2.66x10^5$ a	$4.74x10^6 \pm 1.85x10^6$ a	$3.69x10^6 \pm 9.00x10^5$ a	$1.61x10^6 \pm 2.66x10^5$ a
CH05	$5.07x10^3 \pm 6.44x10^3$ b	$1.05x10^4 \pm 9.89x10^3$ b	$7.80x10^3 \pm 8.49x10^3$ b	$5.07x10^3 \pm 6.44x10^3$ b
CH1	$8.85x10^2 \pm 7.15x10^2$ bc	$4.07x10^3 \pm 2.45x10^3$ b	$1.39x10^3 \pm 2.06x10^2$ b	$8.85x10^2 \pm 7.15x10^2$ b
CH2	$7.17x10^2 \pm 9.18x10^2$ bc	$3.63x10^3 \pm 2.75x10^3$ b	$8.18x10^2 \pm 7.30x10^2$ b	$7.17x10^2 \pm 9.18x10^2$ b
COX05	$1.90x10^3 \pm 1.27x10^3$ bc	$7.88x10^3 \pm 6.26x10^3$ b	$4.13x10^3 \pm 1.59x10^3$ b	$1.90x10^3 \pm 1.27x10^3$ b
COX1	$2.98x10^2 \pm 1.48x10^2$ c	$3.55x10^3 \pm 3.51x10^3$ b	$1.35x10^3 \pm 3.85x10^2$ b	$2.98x10^2 \pm 1.48x10^2$ b
COX2	$6.92x10^2 \pm 1.12x10^3$ bc	$1.84x10^3 \pm 1.46x10^3$ b	$1.12x10^3 \pm 1.75x10^3$ b	$6.92x10^2 \pm 1.13x10^3$ b
COY05	$3.09x10^3 \pm 3.72x10^2$ bc	$1.20x10^4 \pm 6.42x10^3$ b	$3.65x10^3 \pm 7.00x10^2$ b	$3.09x10^3 \pm 3.72x10^2$ b
COY1	$2.28x10^3 \pm 3.57x10^3$ bc	$4.23x10^3 \pm 4.15x10^3$ b	$3.51x10^3 \pm 3.02x10^3$ b	$2.28x10^3 \pm 3.57x10^3$ b
COY2	$2.68x10^3 \pm 3.70x10^3$ bc	$3.34x10^3 \pm 3.12x10^3$ b	$1.79x10^3 \pm 2.79x10^3$ b	$2.68x10^3 \pm 3.70x10^3$ b
COZ05	$6.15x10^3 \pm 5.42x10^3$ b	$3.11x10^4 \pm 2.16x10^4$ b	$7.95x10^3 \pm 5.23x10^3$ b	$6.15x10^3 \pm 5.42x10^2$ b
COZ1	$2.23x10^3 \pm 1.89x10^3$ bc	$1.71x10^4 \pm 1.56x10^4$ b	$2.41x10^3 \pm 1.06x10^3$ b	$2.23x10^3 \pm 1.89x10^3$ b
COZ2	$6.32x10^2 \pm 9.45x10^2$ bc	$8.58x10^3 \pm 5.51x10^3$ b	$2.33x10^3 \pm 1.52x10^3$ b	$6.32x10^2 \pm 9.45x10^2$ b
p-value	0.00	0.00	0.00	0.00

*Each value is an average of three replications, each analyzed in duplicate ± standard deviation. Different letters within each column indicate a significant difference ($p < 0.05$) among treatments based on one-way analysis of raw data followedby Tukey's post hoc test.

** CON1 = non-acidified control; CON2 = acidified control; CH05 = 0.5%v/v 1%LMW chitosan; CH1 = 1.0%v/v 1%LMW chitosan; CH2 = 2.0%v/v 1%LMW chitosan; COX05 = 0.5%v/v 1%10 min degraded chitosan; COX1 = 1.0%v/v 10 min degraded chitosan; COX2 = 2.0%v/v 10 min degraded chitosan; COY05 = 0.5%v/v 1% 60 min degraded chitosan; COY1 = 1.0%v/v 1% 60 min degraded chitosan; COY2 = 2.0%v/v 1% 60 min degraded chitosan; COZ05 = 0.5%v/v 0.5% 24 hour degraded chitosan; COZ1 = 1.0%v/v 1.0% 24 hour degraded chitosan; COZ2 = 2.0% 24 hour degraded chitosan

Figure 13. Four Day Mean *Pseudomonas* Counts in Nutrient Broth*



*Each value is the average of four days in triplicate.
CON1 = non-acidified control; CON2 = acidified control; CH05 = 0.5%v/v 1%LMW chitosan; CH1 = 1.0%v/v 1%LMW chitosan; CH2 = 2.0%v/v 1%LMW chitosan; COX05 = 0.5%v/v 1%10 min degraded chitosan; COX1 = 1.0%v/v 10 min degraded chitosan; COX2 = 2.0%v/v 10 min degraded chitosan; COY05 = 0.5%v/v 1% 60 min degraded chitosan; COY1 = 1.0%v/v 1% 60 min degraded chitosan; COY2 = 2.0%v/v 1% 60 min degraded chitosan; COZ05 = 0.5%v/v 0.5% 24 hour degraded chitosan; COZ1 = 1.0%v/v 1.0% 24 hour degraded chitosan; COZ2 = 2.0% 24 hour degraded chitosan

other chitosan treatments. When averaging all four days *Pseudomonas* counts of all the chitosan treatments were between 2.37 and 3.71 logs lower than the CON1 (Log 6.41) (Table 20). The addition of chitosan did not produce as much precipitate in nutrient broth as it did in trypticase soy broth.

Table 20. Average Log Reductions in *Pseudomonas* Counts Between CON1 and Chitosan Treatments in Nutrient Broth*

Treatment Code**	LOG Reduction from CON1
CH05	2.57 ± 0.50 cd
CH1	3.16 ± 0.39 abc
CH2	3.48 ± 0.68 ab
COX05	2.78 ± 0.40 bcd
COX1	3.43 ± 0.44 ab
COX2	3.71 ± 0.78 a
COY05	2.63 ± 0.33 cd
COY1	3.09 ± 0.56 abcd
COY2	3.45 ± 0.85 ab
COZ05	2.37 ± 0.51 d
COZ1	2.73 ± 0.50 bcd
COZ2	3.19 ± 0.80 abc
p-value	0.000

*Each value is the average of four days in triplicate ± standard deviation. Means in the same column not sharing a letter are significantly ($p < 0.05$) different based on one-way ANOVA followed by Tukey's post hoc test.

**CH05 = 0.5%v/v 1%LMW chitosan; CH1 = 1.0%v/v 1%LMW chitosan; CH2 = 2.0%v/v 1%LMW chitosan; COX05 = 0.5%v/v 1%10 min degraded chitosan; COX1 = 1.0%v/v 10 min degraded chitosan; COX2 = 2.0%v/v 10 min degraded chitosan; COY05 = 0.5%v/v 1% 60 min degraded chitosan; COY1 = 1.0%v/v 1% 60 min degraded chitosan; COY2 = 2.0%v/v 1% 60 min degraded chitosan; COZ05 = 0.5%v/v 0.5% 24 hour degraded chitosan; COZ1 = 1.0%v/v 1.0% 24 hour degraded chitosan; COZ2 = 2.0% 24 hour degraded chitosan

DISCUSSION:
ANTIMICROBIAL EFFICACY OF CHITOSAN DIPS AND POWDERED CHITOSAN ON ATLANTIC SALMON PRODUCTS

Moisture, Ash and Mineral Analysis

The moisture content of both the salmon fillet and trim were similar, both approximately 70% and the ash content was close to 5%. Similar moisture contents were reported by Suvanich *et al.* (1998) and Gomez-Guillen *et al.* (2000) for aquacultured salmon. Suvanich *et al.* (1998) reported Atlantic salmon ash content as 1.6%. The slightly higher ash content of the salmon used in our study may be attributed to either seasonal variations or possibly, not all of the bones were removed. The trim samples had similar proximate composition compared to the fillets because the salmon supplier had no actual trimmings available. The trim supplied to us was actually skinless pieces of salmon fillet.

pH

Salmon Trim

The pH of the salmon trim (control) was 6.16 on day one of the experiment and all of the chitosan treated trim samples had higher pH values, which increased with chitosan percentage. A possible explanation for the higher pH values in the chitosan treated trim may be due to residual base leftover from the processing of the chitosan. This leftover base may have been released into the water used to homogenize the sample, raising the pH. The chitosan did not dissolve into the trim over time, as it was visible during the entire storage period. This was because the pH of the salmon trim was over the point at which native

chitosan is soluble, 6.0. In a slightly more acidic food substance, the chitosan may have been able to solubilize within the food matrix. The pH of all of the samples, regardless of chitosan treatment, rose from day one to day three and then gradually fell to the end of the study on day 13. One would expect the pH to drop quickly as the glycogen in the muscle is converted to lactic acid and then rise again as the microbial flora produces basic compounds. Reddy *et al.*, (1997) reported that the surface pH of salmon fillets was 6.42 on day zero and then fell to 6.32 after one day. By day three, the pH again rose to 6.53 and continued to rise slightly till day 20, at which time the pH was 6.57. This is a fairly well established pattern in fish fillets. On the day the treatments were applied to the salmon in our study, pH was not measured so the pH may have started slightly higher than at day one because rigor may have been over by the first day of analysis. The rise in pH on day three was present in all samples, so calibration of the pH meter may not have been done correctly on day three. Even so, this does not explain the slow decrease or lack of change in pH over the following days. It is also possible that not enough basic substances were produced to raise the pH of the salmon trim by the end of the experiment.

Salmon Fillet

Initial pH level of the salmon fillet control (6.16) was the same as the trim control. Just as with the salmon trim, the pH values rose to day three and then fell again on day six. The fillet control did rise to 6.22 by day 13 but all of the dipped fillets only rose slightly. The pattern of pH change was more typical of

what should be expected, except for day three. The pH of all of the dipped fillets was lower than the control throughout the experiment, which may be due to inhibition of bacteria or absorption of the acetic acid into the flesh of the fish. Bal'a and Marshall (1998) reported similar reductions in the pH of fish flesh dipped in various acid treatments. The pH of the chitosan dips was approximately 4.3. In the case of the high molecular weight chitosan dip, the pH values were the lowest throughout the experiment probably due to the high viscosity of the dip, which caused the dip to cling to the fillet better than the other treatments did. The low molecular weight dip was not as viscous and did not cling as well and perhaps did not linger on the fillets as long as did the high molecular weight dip, resulting in higher pH values.

Aerobic Plate Counts

Salmon Trim

Aerobic plate counts were higher in salmon trim treatments with chitosan. No differences were observed between chitosan type; high molecular weight versus low molecular weight. Treatments with higher percentages of chitosan appeared to have higher aerobic plate counts than the lower percentage treatments. This seems to indicate that the introduction of the chitosan may have introduced bacteria into the salmon trim. Since the chitosan was ground in a Wiley mill prior to use, it is possible that the grinding introduced bacteria into the chitosan in addition to natural flora that was already present. Autoclaving the chitosan prior to use would have sterilized the product and eliminated this

contamination factor. The expected reduction in microbial counts may also not have occurred because the pH of the salmon mince was 6.16, which is above the pH at which chitosan is soluble. Although the pH of the salmon was below the pKa of chitosan, 6.3 (Helander *et al.*, 2001), throughout the experiment, it was not low enough to solubilize the chitosan. This experiment confirmed that the antimicrobial qualities of chitosan are dependent on the chitosan being solubilized in the food matrix. Lin and Chao (2001) and Jo *et al.* (2001) observed no inhibition of bacteria, at similar storage temperatures to our study, in sausage prepared with water soluble chitosan oligomers, which are lower in molecular weight than the chitosan used in this study. However, Darmadji and Izumimoto (1994) reported a two log reduction of bacterial counts in ground beef at storage temperatures of 4°C and a chitosan percentage of 1% although the type and source of chitosan were not mentioned. They may have used a water-soluble chitosan derivative because they also conducted an in-vitro antimicrobial study in yeast extract peptone broth at a pH of 6.8; native chitosan would have been insoluble in this broth. Based on a rejection limit of log six, all of the trim treatments, with the exception of 0.5% low molecular weight chitosan treatment, reached this level by day six which is consistent with the shelf life of catfish mince (5 days at 5°C) as reported by Suvanich *et al.* (2000a).

Salmon Fillet

Initial plate counts of the salmon fillet were relatively low (log 2 to log 3) due to the short time period (12 hours) between processing and application of the

treatments. The untreated control reached a level of log seven and the low molecular weight dip treatment reached log six by day six, which was in excess of the acceptability level of log six. In contrast, the acetic acid and high molecular weight chitosan (HMW) treated fillets did not surpass the acceptability level until day 10, indicating approximately a four day extension of shelf life by both treatments. Jeon *et al.* (2002) reported that a 1% acetic acid dip kept microbial counts below log six up to day eight in cod fillets and up to day six on herring fillets stored at 4°C. Shelf life of sea bass was reported to be approximately three days at 5°C as reported by Chang *et al.* (1998). In contrast, the sensory shelf life of aquacultured salmon as reported by Reddy *et al.* (1997) was reported to be between 16 and 20 days at 4°C, at which time the microbial counts were log 8.84. In our study, treated salmon fillets did not reach log eight until day 13, which is consistent with the results of Reddy *et al.* (1997). The application of the HMW dip to the salmon fillets suppressed microbial growth through day six while the other dips appear to have delayed growth only until day three.

No synergistic effect was observed between the acetic acid and the chitosan. Sagoo *et al.*(2002) reported a synergistic effect between sodium benzoate and chitosan against spoilage yeasts in a saline solution. The authors theorized the polycationic condition of the chitosan below its pKa caused a reaction with the anionic portions of the bacterial cell membrane, allowing the sodium benzoate to act upon the cells. Alakomi *et al.*, 2000 reported that lactic acid was able to weaken the outer membrane of gram-negative bacteria. Acetic

acid was used in this experiment so it is possible that the acetic acid may have allowed the solubilized chitosan in the dip to react with the bacterial cells although only the high molecular weight dip exhibited lower microbial counts than the acetic acid dipped control. The thicker coating of the HMW dip may have been responsible for suppressing microbial growth by either preventing access to oxygen or by keeping the antimicrobial components of the dip (acetic acid and chitosan) in contact with the surface of the fillet.

Total Volatile Base Nitrogen

Salmon Trim

Initial concentrations of TVBN on day one were approximately 16 mg N/100g for all treatments but then rose to higher levels ~20 mg N/100 g by day 13. The rise was not constant because for some treatments there was a drop in concentrations between day three and six. Overall, the increases in concentration throughout the experiment were not that large compared to the initial concentrations. None of the treatments reached the proposed shelf-life cut-off of 30 mg N/100 g. Jeon *et al.* (2002) reported initial TVBN concentrations of approximately 7 to 9 mg N/100 g for both cod and herring fillets stored at 4°C. By the end of 12 days of storage the concentrations had risen to 53 and 49 mg N/100 g for cod and herring, respectively. The corresponding aerobic plate counts for cod and herring on day 12 were log seven and log eight, respectively. Since salmon is a different species of fish, it may not degrade in the same way or at the same rate as cod or herring. The aerobic plate counts of the salmon trim

were relatively high by the end of the study, but the high number of bacteria did not result in corresponding higher levels of TVBN, despite starting out at higher concentrations than those found by Jeon *et al.* (2002) at the beginning of their study.

Salmon Fillet

Initial TVBN levels were similar to those of the salmon trim, between 14.5 and 16.8 mg N/100 g. By day 13, the concentration of TVBN in the control treatment had risen to approximately 23 mg N/100 g. All of the treatments that had been dipped remained between 17 and 18 mg N/100 g. All of the treatments appear to have inhibited the formation of TVBN. Similar findings were reported by Jeon *et al.* (2002) when chitosan dips were applied to cod and herring fillets. After 12 days of storage, high molecular weight chitosan dips had TVBN concentrations of 22 and 24 mg N/ 100g of fish for cod and herring, respectively. Smaller reductions were observed in the lower molecular weight dips. The difference between their experiment and ours was that glycerol was added to the dips and after dipping, the fillets were dried in a drying oven at 40°C until a film formed on the fillets. Despite the differences in application, the evidence for inhibition of TVBN formation by application of the chitosan dip is similar to our findings. The reduction in TVBN reported by Jeon *et al.* (2002) corresponded to an inhibition of microbial growth, up to three logs lower by day 12 while our TVBN values on day 13 corresponded with a one to two log reduction in aerobic plate counts.

Trimethylamine

Salmon Trim

Trimethylamine analysis was made more difficult due to the presence of chitosan in the salmon trim. The initial extraction was conducted with 7.5% trichloroacetic acid, which solubilized the chitosan flakes in the trim, causing the extraction fluid to be more viscous than normal. The chitosan was carried over into the TMA procedure which caused emulsification with the toluene layer when the test tubes were shaken, making removal of the toluene layer much more difficult. Addition of salt to the test tubes broke down the emulsion to some degree, but centrifugation was necessary to disperse the emulsion in the 2% chitosan treatments. TMA analysis was not conducted on day three because the analyses day one and six resulted in undetectable concentrations of trimethylamine. TMA was detectable on days 10 and 13 but the highest concentration detected was only 3.30 mg TMA/ 100 g. The suggested TMA concentration indicative of spoilage for fatty fish is 5 mg TMA/ 100 g (Jeon *et al.*, 2002) and for other fish, 30 mg TMA/ 100 g of fish (Reddy *et al.*, 1997). The salmon trim never reached these concentrations. Reddy *et al.* (1997) reported the initial concentration of TMA in aquacultured salmon to be 0.18 mg TMA/100g. When stored at 4°C TMA concentrations in their study slowly rose to 1.59 mg TMA/ 100 g on day 10 and did not rise drastically till day 16 at which time the concentration of TMA was 16.37 mg TMA/ 100 g. These results are consistent with our study. Perhaps if the study had been extended for three more days, higher concentrations would have been detected. The fish we obtained was very

fresh so it took a long time to produce TMA because it is produced by the spoilage bacteria. Since initial bacterial counts were very low (log two to three), it took longer to produce TMA. Reddy *et al.* (1997) reported initial aerobic plate counts of log 4.5, but the counts on day 10 were log 8.9 which corresponded with a TMA concentration of 1.59 mg / 100 g. This was also the aerobic plate count at day 16 which corresponded with a TMA concentration of 16.37 mg/ 100 g of fish, indicating it may take some time to build up the TMA concentration and that the bacterial count does not necessarily correspond with TMA concentration. Additionally, salmon does not have as much of the precursor of TMA, trimethylamine oxide (TMAO), as some other marine fish (Reddy *et al.*, 1997) hence, TVBN concentrations may have been a better indicator of degradation in our salmon samples.

Salmon Fillet

TMA concentrations were even lower in the salmon fillets. Even the untreated control fillet only had a maximum TMA concentration of 1.21 mg/100 g, and that occurred on day 10. Except for the acetic acid dipped fillets, concentrations of TMA fell between day 10 and day 13. The TMA concentrations in dipped fillets remained under 1 mg/100 g. Lower TMA concentrations were found in intact fillet portions probably because the bacteria present were mainly on the surface of the fillet, therefore only reacting with the TMAO present near the surface of the fish.

DISCUSSION:
ENZYMATIC DEGRADATION OF CHITOSAN SOLUTIONS

Enzyme Effectiveness

Our results indicated that the bromelain tested in this study was not effective in degrading chitosan solutions as indicated by the formation of precipitate at neutral pH after 24 hours of degradation even at 50% enzyme levels. Although the protein content of the bromelain mixture was higher than that of the alpha amylase mixture used, the alpha amylase activity was higher than that of bromelain. Yalpani and Pantaleone (1994) successfully degraded chitosan solutions in acetic acid with bromelain at relatively low enzyme concentrations, 0.0025 to 0.02% by weight of chitosan. The source of the enzyme was given by the authors but no mention of the enzyme activity was made. The enzyme mixture used by Yalpani and Pantaleone (1994) may have been much more active than the enzyme used in this study. The bromelain used in this study may have slightly degraded the chitosan but not to full water solubility. Viscosity changes made by the bromelain were not tested since the goal was to produce a completely water soluble chitosan. Alpha amylase, in contrast, was very effective in degrading the chitosan solution to water soluble oligomers within 24 hours at enzyme levels of 5 and 10% by weight of chitosan. Zhang *et al.* (1999) also used alpha amylase with lower activity in an enzyme complex with cellulase and proteinase to degrade chitosan. The alpha amylase used by the authors had similar activity, 500 U/mg, compared to this study, 307 U/mg. Alpha amylase was also studied indirectly by Yalpani and Pantaleone

(1994) in the form of human salivary excretions, which also contained other enzymes such as lysozyme. The authors reported an 80% reduction of chitosan solution viscosity after seven days of exposure to saliva. Alpha amylase is an endo-enzyme that works on the alpha 1-4 linkages in starch molecules. Perhaps the mode of action of alpha amylase on chitosan is the 1-4 linkages that exist in this polysaccharide, although these linkages are beta rather than alpha. The degree of deacetylation has also been proposed as a factor in enzyme effectiveness in degrading chitosan solutions. Nordtveit *et al.* (1994) and Shin-ya *et al.* (2001) both reported that highly deacetylated chitosan inhibited the effectiveness of pectinase and lysozyme, respectively, in degrading chitosan. The degree of deacetylation of the chitosan in this study was 80%. Alpha amylase and perhaps, bromelain may have been more effective reacting with the chitosan if the degree of deacetylation had been lower.

pH

The optimal pH conditions for the degradation of chitosan by alpha amylase was ~4.3, the pH obtained when the 1% chitosan solution was prepared in 1% acetic acid. Optimal conditions for function of alpha amylase on starch are pH 6.6 at 30 °C. Yalpani and Panteleone (1994) studied the effects of pH on numerous enzymes in several enzyme classes on the degradation of chitosan. They reported that most of the enzymes they tested performed best at pH levels between 3.0 and 4.5, which is consistent with the results in this study. In this study, the lower pH conditions were necessary to degrade the chitosan with the

alpha amylase as indicated by the undissolved chitosan present in the pH 5.5 and 7.0 treatments. It seems that the chitosan must be fully solubilized in order for the enzyme to be able to react with the chitosan substrate. Despite chitosan's reported solubility limit, below pH 6.0, the chitosan in the pH 5.5 treatment never fully solubilized and formed a ropy mass. Perhaps if chitosan had been fully dissolved in 1% acetic acid and then adjusted to pH 5.5, the alpha amylase may have degraded the chitosan more effectively.

Gravimetric Measurements

The attempt to quantify the degree of water solubility of the chitosan degraded by alpha amylase was, for the most part, unsuccessful. Quantification was only possible for the chitosan solutions that had fully degraded to the point where the chitosan was water soluble, after 24 hours of degradation. No method to measure solubility was found in the literature. In other studies, solubility was usually measured by adding sodium hydroxide to chitosan solutions and observing whether precipitate formed at neutral pH (Pantaleone *et al.*, 1992; Zhang *et al.*, 1999). The attempt to quantify the chitosan solutions that were not completely water-soluble most likely failed due to formation of a thick gel when the pH was adjusted to 7.0. This thick gel probably did not dry completely in the drying oven due to case hardening. The higher weights obtained after pH adjustment and drying were probably a result of water trapped in the gel matrix that could not be driven off.

Viscosity

In most of the literature enzyme degradation of chitosan was studied viscometrically with a capillary-type (Nordtveit *et al.*, 1994; Ilyina *et al.*, 2000; Zhang and Neau, 2001), Brookfield (Pantaleone *et al.*, 1992) or a Haake Rotovisco RV-20 (Muzzarelli *et al.*, 1994) viscometer. The capillary type viscometer was used in this study because it was the most commonly used method and relatively easy to use. The viscosity of the low molecular weight chitosan used in this study was between 45 and 50 centipoise when prepared as a 1% solution in 1% acetic acid. Muzzarelli *et al.* (1994) reported the initial viscosity of their 1% chitosan solution prepared in lactic acid as ~500 centipoise. Yalpani and Pantaleone (1994) reported their chitosan had a viscosity of 1260 centipoise, but they used a 2% chitosan solution prepared in 3N acetic acid. Despite the difference in concentration, this chitosan probably had a much higher starting viscosity than the chitosan used in this study.

The solvent used in the preparation can also greatly influence the viscosity of chitosan in solution. Skaugrud and Sargent (1990) reported viscosities of a 1% chitosan solution ranging from 260 centipoise in acetic and propionic acid to as low as 12 centipoise in oxalic acid. Solvent choice may also influence the degradation of a chitosan solution. Muzzarelli *et al.* (1994) reported a 40% reduction in viscosity of a 1% chitosan solution prepared in 1% acetic acid after one hour at 50°C and only 10% reductions in viscosity when malic, lactic and citric acid were used as the solvent. We observed degradation due to acid hydrolysis of our chitosan solution as well, but the reduction was 35% and this

occurred in 24 hours. The combination of heat and acetic acid probably acted synergistically to degrade the chitosan. Because this study was conducted at ambient room temperatures, usually between 20 and 25°C, this effect was slowed greatly. The synergistic effect of heat and acid was also observed when the chitosan solutions were autoclaved to deactivate the enzyme. A 55% reduction in viscosity was observed for 10 minute degraded chitosan (10% enzyme level). A 28% viscosity reduction was observed in the 60 minute degraded chitosan.

Large viscosity reductions were observed in this study with addition of alpha amylase. More rapid reductions were observed with the 10% enzyme level compared to the 5% level. The most rapid reduction in viscosity occurred in the first hour of degradation at both enzyme concentrations. Similar findings were reported by Pantaleone *et al.* (1992) for a variety of enzymes (chitinase and cellulase), rapid viscosity reductions in the first two hours were followed by a more gradual reduction up to 24 hours. Muzzarelli *et al.* (1994) also reported that the most rapid degradation of chitosan by papain occurred in the first hour. Perhaps the rapid intial reductions may be due to the nature of the enzyme, alpha amylase. Since this is an endo-enzyme the very long chains of chitosan in solutions were broken in the middle, resulting in a rapid viscosity reduction. As the number of sites the enzyme could hydrolyze decreased, the rate of degradation slowed. Pantaleone *et al.* (1992) reported a more gradual viscosity reduction pattern for less specific classes of enzymes, lipase and papain, that have different modes of action for hydrolysis. In this study, after 24 hours of

degradation, the viscosity of the chitosan solution was getting close to that of the solvent, indicating that a nearly complete hydrolysis had occurred.

Discrepancies exist in the literature regarding the effects of chitosan (substrate) concentration on the enzymatic degradation rate. Yalpani and Pantaleone (1994) reported higher enzyme activity in 0.5% chitosan solutions compared to 2% solutions for a range of enzymes (cellulase, lipase, and papain), which was theorized to have possibly been a result of inhibition by either substrate or hydrolysis products. In contrast, Muzzarelli *et al.* (1994) reported more rapid viscosity reductions during the first hour, in higher concentration chitosan solutions (12 cP/min at concentrations of 7g/L versus 110 cP/min at concentrations of 19g/L) degraded with papain. This was probably because the higher concentration chitosan solution had a higher viscosity to begin with and the enzymes had more sites to act upon because of the higher concentration of substrate. The rate of viscosity reduction in this study during the first hour for the 5 and 10% enzyme treatments was 0.49 cP/min and 0.59 cP/min, respectively. The relatively small decreases in viscosity observed in this study compared to Muzzarelli *et al.* (1994) may be attributed to the use of a low molecular weight chitosan. The reported molecular weight of Muzzarelli *et al.* (1994) chitosan was 698,340 kDa whereas the chitosan used in this study had an unspecified molecular weight. Nonetheless, it was evident that higher concentrations of alpha amylase in this study resulted in more rapid degradation of the chitosan solution, but only during the first hour.

DISCUSSION: IN VITRO ANTIMICROBIAL EFFICACY OF CHITOSAN AGAINST *LISTERIA INNOCUA* AND *PSEUDOMONAS AERUGINOSA*

Listeria in Trypticase Soy Broth

All chitosan treatments resulted in approximately a one log reduction in *Listeria* counts in TSB. The acidification of the TSB with concentrated hydrochloric acid appears to have had no effect in reducing the bacterial counts, as evidenced by the acidified broth control, which had similar counts to the non-acidified control throughout the experiment. Ita and Hutkins (1991) reported that hydrochloric acid exhibited the least antimicrobial activity toward *Listeria monocytogenes* in trypticase soy broth. The authors also reported that *Listeria monocytogenes* survived in acidified broths down as low as pH 3.5. In this study, the broth pH was only reduced to 5.5 in order to fully solubilize the chitosan in solution.

Listeria monocytogenes was reported (Wang, 1992) to be inhibited by chitosan in NB at temperatures of 30°C. Wang also reported the best inhibitory conditions occurred at a pH of 5.5 rather than 6.5, which was probably due to the insolubility of chitosan at pH values above 6.0. The lower degree of inhibition, one to two logs, in this study compared to that of Wang (1992), up to five logs, could be the result of binding of the chitosan to proteins in the TSB despite the higher temperatures used in Wang's study. Addition of chitosan to the TSB caused formation of a cloudy precipitate, which increased with increasing chitosan percentage. Precipitate also was evident in nutrient broth but not to the extent that it was in TSB. Hansen and Gill (2000) have also reported this

phenomenon in an in vitro antimicrobial study in TSB with the compound protamine against *Listeria monocytogenes* and *E. coli*. Like chitosan, protamine is a cationic compound with antimicrobial properties, but it is not a polysaccharide. Hansen and Gill (2000) noted that the addition of protamine formed a precipitate in TSB and the protamine available in the solution dropped, most significantly above pH 6.0. However, the authors also reported that protamine displayed the best antimicrobial activity toward *Listeria* at pH levels of 6.5 and higher, despite the higher precipitate and thus, lower amounts of free protamine. The authors theorized that the protamine was more able to bind to the cell surface of *Listeria* at higher pH levels, regardless of interactions with the broth. Chitosan is a different compound than protamine, but a study of water-soluble chitosan in TSB at higher pH levels or in nutrient broth may demonstrate more antimicrobial activity than with the environmental parameters used in this study. In addition, the binding displayed by chitosan to proteins in the TSB may have ramifications when applied to a food system high in protein. The chitosan may bind to proteins in the food, which may reduce its antimicrobial efficacy. Support for this argument was reported by Jo *et al.* (2001) and Lin and Chao (2001), both of whom added water soluble chitosan oligomers to sausage products and reported no significant aerobic plate count reductions. Conversely, Darmadji and Izumimoto (1994) reported a two-log reduction in aerobic plate counts in ground beef that had 1% chitosan (most likely a water-soluble derivative) added to it. The differences in these studies possibly may be attributed to chitosan addition level. Jo *et al.* (2001) and Lin and Chao (2001)

added chitosan to the sausage formulations at levels of 0.2 and 0.1%, respectively, whereas Darmadji and Izumimoto (1994) added chitosan at a 1% concentration.

Efficacy of chitosan against *E. coli* in phosphate buffer was reported by Tsai and Su (1999) to decrease with decreasing temperature. Conversely, Tsai *et al.* (2000) reported that chitoligosaccharides were more effective in inoculated sterilized milk at 4°C than at 37°C. The contrasting results of these two studies indicate that the antimicrobial efficacy of chitosan varies with different substrates. Although this factor was not tested in this study, it is possible that chitosan may have been more effective against *Listeria* at temperatures higher than 4°C.

The 24 hour degraded, water-soluble, chitosan exhibited the least antibacterial activity. Similar results were observed by both Jeon and Kim (2000) and Jeon *et al.* (2001), who reported decreasing antibacterial activity with increasing degree of degradation. Jeon and Kim (2000) and Jeon *et al.* (2000) also reported an increase in antibacterial activity against *E. coli* with increasing chitosan percentage, regardless of degree of degradation. This was also the case in this study. Rhoades and Roller (2000) reported a slight increase in antimicrobial activity in slightly degraded chitosan, but not greatly so. The authors reported more highly degraded chitosan oligomers were less effective than undegraded chitosan. In this study, we did not observe this effect against *Listeria* but did in the study with *Pseudomonas aeruginosa.*

Jeon *et al.* (2001) reported that many types of bacteria were killed by chitosan and chitosan oligomers. Gram-positive strains were slightly more

affected by the chitosan compared to gram-negative strains. The authors also reported that *Pseudomonas* was the most resistant to the antimicrobial effects of chitosan and chitosan oligomers. In this study, the gram-negative *Pseudomonas* were more affected by the chitosan treatment than the gram-positive *Listeria*. Since the two studies were conducted in different broths, no conclusion regarding susceptibility based on gram status can be assumed.

Pseudomonas in Nutrient Broth and Trypticase Soy Broth

Pseudomonas aeruginosa was not inhibited in TSB compared to NB. The difference in antimicrobial efficacy based on broth choice was substantial. Potential explanations for this difference were discussed in the previous section.

Pseudomonas aeruginosa was reported by Jeon *et al.* (2001) to be the most resistant organism to the antimicrobial effects of chitosan at 37°C. That study was conducted in TSB, and undegraded chitosan still resulted in a 68% kill, as measured spectrophotometrically. The authors did not mention any interference caused by chitosan precipitate. In this experiment with *Pseudomonas*, significant log reductions, up to four logs, compared to the controls were observed.

Helander *et al.* (2001) reported that the antibacterial properties of chitosan toward gram-negative bacteria could be attributed to binding on the outer membrane of the bacteria, as evidenced by electron micrographs. In this study, binding of the chitosan to the proteins in the TSB probably left less of the

chitosan to bind to the proteins in the outer membrane of the *Pseudomonas*, reducing the efficacy of the chitosan.

Degree of deacetylation was reported by Liu *et al.* (2000) to affect the antimicrobial action of chitosan, which increased with increasing degree of deacetylation. The authors theorized that the reason for this effect was an increase of available NH_2 groups and therefore, NH_3^+concentrations, which bind to the negative portions of the bacterial cell membrane. The chitosan used in this study had a degree of deacetylation of approximately 80%. If the degree of deacetylation had been higher, the chitosan may have been even more effective against *Pseudomonas*, conversely, it may not have been as easily degraded by alpha amylase, as discussed previously.

The pH of the broth may also play a role in the antimicrobial efficacy of chitosan. The reported pK_a of chitosan is 6.3 and Liu *et al.* (2000) reported the highest antibacterial activity at pH 6.3 because of the high amount of NH_3^+. The authors also reported the antibacterial activity of the chitosan declined at pH values below 6.3 because the amount of H^+ ions increased and competed for binding sites on the bacterial membrane. This contradicts the findings of Wang (1992) who found chitosan was ineffective against a variety of bacteria at pH 6.5, which is slightly above the pK_a of chitosan. Sudarshan *et al.* (1992) reported water soluble chitosans were equally antimicrobial toward both gram-positive and negative bacteria at pH values of 5.8, but were ineffective at pH levels of 7.0. This study was conducted at pH 5.5, below the pK_a of chitosan. An obvious

extension of this research would be to test the antibacterial effects of chitosan, especially the fully degraded chitosan, at higher pH levels.

CONCLUSIONS

This study indicates that chitosan definitely possesses antimicrobial properties and can be degraded easily by commercially available enzymes. However, the food matrix and method of application is very important to achieve the desired effect. More research is required to determine appropriate food products and chitosan forms to add to those products.

Salmon Product Applications

Future research of chitosan's effects on salmon products should include an analysis of lipid oxidation and color change over time in addition to the other analyses already conducted. Another study with salmon trim should be conducted either with a pre-solubilized chitosan or a water soluble chitosan (without acetic acid) or chitosan derivative as solubility is an important factor influencing the efficacy of chitosan. It would also be interesting to test chitosan products in other foods, perhaps ones not as high in protein as the proteins may interfere with chitosan's efficacy in a food system. A study of chitosan in food systems in conjunction with other food preservatives such as nisin or sodium benzoate may also be of interest since there may be synergistic effects.

Enzyme Degradation

A natural extension of the work already completed in this study would be to quantify the end products of the enzymatic breakdown by alpha amylase. This may be accomplished in a number of ways including high performance liquid

chromatography. Additionally, it would be of interest to develop a method that uses alpha amylase to continuously produce water soluble or degraded chitosan.

In Vitro Study

This study should include another analysis of the effects of chitosan against *Listeria innocua* in nutrient broth to determine whether the broth played a role in the results already obtained as it did in the *Pseudomonas aeruginosa* study. Additionally, it would be of interest to study the enzymatically produced water soluble chitosan at the pK_a of chitosan and slightly below and above this pH value as well as in broth at an unadjusted pH. A determination of the minimal inhibitory concentration of chitosan required would be useful as well. A study at elevated temperatures would also be necessary to determine if temperature played a role in the results obtained in this study.

REFERENCES

Alakomi, H.L., Skytta, E., Saarela, M., Matilla-Sandholm, T., Latva-Kala, K. and Helander, I.M. 2000. Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. Applied and Environmental Microbiology. 66(5): 2001-2005.

Ashie, I.N.A., Smith, J.P. and Simpson, B.K. 1996. Spoilage and shelf life extension of fresh fish and shellfish. Critical Reviews in Food Science and Nutrition. 36: 87-121.

ASTM. 2000. Petroleum products, lubricants, and fossil fuels. In: Annual Book of ASTM Standards Section 5. Philadelphia: American Society for Testing and Materials. p 188-196.

Baek, M.H.C., Lim, D.S. and Taikl, S. 2001. Effect of chitosan addition on textural properties of sweet potato starch noodle. Food Science and Biotechnology. 10(2): 161-165.

Bal'a, M.F.A. and Marshall, D.L. 1998. Organic acid dipping of catfish fillets: effect on color, microbial load, and *Listeria monocytogenes*. Journal of Food Protection. 61(11): 1470-1474.

Botta, J.R., Lauder, J.T. and Jewer, M.A. 1984. Effect of methodology on total volatile basic nitrogen determination as an index of quality of fresh Atlantic cod (*Gadus morhua*). Journal of Food Science. 49: 734-736.

Bronstein, M.N., Price, R.J., Strange, E.M., Melvin, E.F., Dewees, C.M. and Wyatt, B.B. 1985. Storage of dressed Chinook salmon in refrigerated freshwater, diluted seawater, seawater and ice. Marine Fisheries Review. 47: 68-72.

Bullard, F.A. and Collins, J. 1978. Physical and chemical changes in pink shrimp held in CO_2-modified refrigerated seawater compared to pink shrimp held in ice. Fisheries Bulletin. 76: 73-76.

Capita, R., Alonso-Calleja, C., Garcia-Fernandez, C. and Moreno, B. 2001. Efficacy of trisodium phosphate solutions in reducing *Listeria monocytogenes* populations on chicken skin during refrigerated storage. Journal of Food Protection. 64(10): 1627-1630.

Chang, D.S., Cho, H.R., Goo, H.Y. and Choe, W.K. 1989. A development of food preservative with the waste of crab processing. Bulletin of the Korean Fish Society. 22: 70-78.

Chang, K.L.B., Chang, J., Shiau, C.Y. and Pan, B. 1998. Biochemical, microbiological, and sensory changes of sea bass (*Lateolabrax japonicus*) under partial freezing and refrigerated storage. Journal of Agricultural and Food Chemistry. 46: 682-686.

Chen, C.S., Liau, W.Y. and Tsai, G.J. 1998. Antibacterial effects of N-sulfonated and N-sulfobenzoyl chitosan and application to oyster preservation. Journal of Food Protection. 61(9): 1124-1128.

Chen, M.C., Yeh, G.H.C.and Chiang, B.H. 1996. Antimicrobial and physiochemical properties of methylcellulose and chitosan films containing a preservative. Journal of Food Processing and Preservation. 20: 379-390.

Cho, H.R.C., Lee, D.S., Jeong, W.D., Lee, E.T. and Woo, E. 1998. Utilization of chitosan hydrolysate as a natural food preservative for fish meat paste products. Korean Journal of Food Science and Technology. 30(4): 817-822.

Choi, W.Y., Kim, K.M. and Park, H.J. Preparation of edible coating emulsions for fresh products. Institute of Food Technologists Annual Meeting, June, 2000, Anaheim, CA.

Cuero, R.G., Duffus, E., Osuji, G. and Pettit, R. 1991. Aflatoxin control in preharvest maize: Effects of chitosan and two microbial agents. Journal of Agricultural Science. 117: 165-169.

Darmadji, P. and Izumimoto, M. 1994. Effect of chitosan in meat preservation. Meat Science. 38: 243-254.

Deuchi, K., Kanauchi, O., Imasato, Y. and Kobayashi, E. 1995. Continuous and massive intake of chitosan affects mineral and fat-soluble vitamin status in rats fed on a high-fat diet. Bioscience, Biotechnology and Biochemistry. 59: 1211-1216.

Ehira, S. and Uchiyama, H. 1986. Determination of fish freshness using the K value and comments on some other biochemical changes in relation to freshness. In: Kramer, D.E., Liston, J. editors. Seafood Quality Determination. New York: Elsevier. p 186-209.

Einen, O. and Thomassen, M.S. Starvation prior to slaughter in Atlantic salmon (*Salmo salar*) II. White muscle composistion and evaluation of freshness, texture and colour characteristics in raw and cooked fillets. Aquaculture. 169: 37-53.

Emborg, J., Laursen, B.G., Rathjen, T. and Dalgaard, P. 2002. Microbial spoilage and formation of biogenic amines in fresh and thawed modified atmosphere-packed salmon (*Salmo salar*) at 2°C. Journal of Applied Microbiology. 92: 790-799.

Erikson, U., Beyer, A.R. and Sigholt, T. 1997. Muscle high-energy phosphates and stress affect K-values during ice storage of Atlantic salmon (*Salmo salar*). Journal of Food Science. 62(1): 43-47.

Food and Drug Association. 2001. "GRAS Notices Received in 2001." http://vm.cfsan.fda.gov/~rdb/opa-gn01.html (7/3/2001).

Gelman, A., Glatman, L., Drabkin, V. and Harpaz, S. 2001. Effects of storage temperature and preservative treatment on shelf life of the pond-raised freshwater fish, silver perch (*Bidyanus bidyanus*). Journal of Food Protection. 64(10): 1584-1591.

Ghaouth, A.E., Arul, J., Ponnampalum, R. and Boulet, M. 1991a. Chitosan coating effect on storability and quality of fresh strawberries. Journal of Food Science. 56(6): 1618-1620.

Ghaouth, A.E., Arul, J. and Ponnampalum, R. 1991b. Use of chitosan coating to reduce water loss and maintain quality of cucumber and bell pepper fruits. Journal of Food Processing and Preservation. 15: 359-368.

Gildberg, A. and Stenberg, E. 2001. A new process for advanced utilization of shrimp waste. Process Biochemistry. 36: 809-812.

Gomez-Guillen, M.C., Montero, P., Hurtado, O. and Borderias, A.J. 2000. Biological characteristics affect the quality of farmed Atlantic salmon and smoked muscle. Journal of Food Science. 65(1): 53-57.

Ha, S.M.L., Lee, C., Kim, Y.C. and Ok, K. 1999. Properties of chitosan hydrolysates and their influence on the quality of shortened cake. Food Science and Biotechnology. 8(2): 113-117.

Haard, N.F. 1992. Technological aspects of extending prime quality of seafood: a review. Journal of Aquatic Food Product Technology. 1: 9-26.

Hadlett, H.M., and Raab, C.A. 1990. Consumer perceptions of fresh fish quality. Journal of the American Dietetic Association. 98(8): 1109-1111.

Hansen, L.T. and Gill, T.A. 2000. Solubility and antimicrobial efficacy of protamine on *Listeria monocytogenes* and *Eschericia coli* as influenced by pH. Journal of Applied Microbiology. 88: 1049-1055.

Healy, M.G., Romo, C.R. and Bustos, R. 1994. Bioconversion of marine crustacean shell waste. Resources, Conservation and Recycling. 11: 139-147.

Helander, I.M., Lassila, E.L., Ahvenainen, R., Rhoades, J. and Roller, S. 2001. Chitosan disrupts the outer membrane of gram-negative bacteria. International Journal of Food Microbiology. 71: 235-244.

Hirano, S. 1997. Applications of chitin and chitosan in the ecological and environmental fields. In: Goosen, M.F.A. Applications of Chitin and Chitosan. Lancaster, PA: Technomic Publishing. p 31-50.

Hungerford, J.M. 1998. Fish and other marine products. In: AOAC. 16th ed. Gaithersburg, MD: AOAC. p 7.

Icekson, I., Pasteur, R., Drabkin, V., Lapidot, M., Eizenberg, E., Klinger, I. and Gelman, A. 1996. Prolonging shelf-life of carp by combined ionizing radiation and refrigeration. Journal of the Science of Food and Agriculture. 72: 353-358.

Ilyina, A.V., Tikhonov, V.E., Albulov, A.I. and Varlamov, V.P. 2000. Enzymatic preparation of acid-free-water-soluble chitosan. Process Biochemistry. 35: 563-568.

Ita, P.S. and Hutkins, R.W. 1991. Intracellular pH and survival of *Listeria monocytogenes* Scott A in trypticase soy broth containing acetic, lactic, citric, and hydrochloric acids. Journal of Food Protection. 64(1): 15-19.

Janak Kamil, Y.V.A., Jeon, Y.J. and Shahidi, F. Control of oxidation of cooked herring by chitosan. Institute of Food Technologists Annual Meeting, June, 2002. Anaheim, CA.

Jay, J. 2000a. Seafoods. In Chapt. 6 "Modern Food Microbiology," 6th ed. Gaithersburg, Maryland: Aspen Publishers, Inc. p 101-112.

Jay, J. 2000b. Low Temperature Preservation and Characteristics of Psychrotrophic Microorganisms. In Chapt. 16 "Modern Food Microbiology," 6th ed. Gaithersburg, Maryland: Aspen Publishers, Inc. p 323-339.

Jeon, Y.J. and Kim, S.K. 2000. Production of chitoligosaccharides using an ultrafiltration membrane reactor and their antibacterial activity. Carbohydrate Polymers. 41: 133-141.

Jeon, Y.J., Shahidi, F. and Kim, S. 2000. Preparation of chitin and chitosan oligomers and their applications in physiological functional foods. Food Reviews International. 16(2): 159-176.

Jeon, Y.J., Park, P.J. and Kim, S. 2001. Antimicrobial effect of chitoligosaccharides produced by bioreactor. Carbohydrate Polymers. 44: 71-76.

Jeon, Y.J., Janak Kamil, Y.V.A. and Shahidi, F. 2002. Chitosan as an edible invisible film for quality preservation of herring and Atlantic cod. Journal of Agricultural and Food Chemistry. 50: 5167-5178.

Jo, C., Lee, J.W., Lee, K.H. and Byun, M.W. 2001. Quality properties of pork sausage prepared with water-soluble chitosan oligomer. Meat Science. 59: 369-375.

Kim, C., Kim, S. and Choi, K. 1997. Synthesis and antibacterial activity of water-soluble chitin derivatives. Polymers for Advanced Technologies. 8: 319-325.

Kim, C.R. and Hearnsberger, J.O. 1994. Gram-negative bacteria inhibition by lactic acid culture and food preservatives on catfish fillets during refrigerated storage. Journal of Food Science. 59(3): 513-516.

Kim, D.H.L., Lee, C. and Chun, Y. 2000. Physiochemical and sensory properties of whipping cream with water soluble chitosan. Food Science and Biotechnology. 9(2): 111-115.

Kim, J.M., Huang, T.S., Marshall, M.R. and Wei, C.I. 1999. Chlorine dioxide treatment of seafoods to reduce bacterial loads. Journal of Food Science. 64: 1089-1093.

Kim, S.K., Park, P.J, Yang, H.P., and Han, S.S. 2001. Subacute toxicity of chitosan oligosaccharide in Sprague-Dawley rats. Arzneimittel-Forschung. 51(9): 769-774.

Knorr, D. 1991. Recovery and utilization of chitin and chitosan in food processing waste management. Food Technology. 45: 114-122.

Kosak, P.H. and Toledo, R.T. 1981. Effects of microbial decontamination on the storage stability of fresh fish. Journal of Food Science. 46: 1012.

Koutsoumanis, K. and Nychas, G.E. 2000. Application of a systematic experimental procedure to develop a microbial model for rapid fish shelf life determinations. International Journal of Food Microbiology. 60: 171-184.

Lee, J.S., and Kolbe, E. 1982. Microbiological profile of Pacific shrimp stored under refrigerated seawater spray. Marine Fisheries Review. 44(3): 12-17.

Lin, K. and Chao, J. 2001. Quality characteristics of reduced-fat Chinese-style sausage as related to chitosan's molecular weight. Meat Science. 59: 343-351.

Liu, X.F., Guan, Y.L., Yang, D.Z., Li, Z. and Yao, K.D. 2000. Antibacterial action of chitosan and carboxymethylated chitosan. Journal of Applied Polymer Science. 79: 1324-1335.

Lloyd, L.L., Kennedy, J.F., Methacanon, P., Paterson, M. and Knill, C.J. 1998. Carbohydrate polymers as wound management aids. Carbohydrate Polymers. 37: 315-322.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurement with the folin phenol reagent. Journal of Biological Chemistry. 193: 265-275.

Maezaki, Y., Tsuji, K., Nakagawa, Y., Kawai, Y., Akimoto, M., Tsugita, T., Takekawa, W., Terada, A., Hara, H., and Mitsuoka, T. 1993. Hypocholesterolemic effect of chitosan in adult males. Bioscience Biotechnology and Biochemistry. 57: 1439-1444.

Malle, P. and Poumeyrol, M. 1989. A new chemical criterion for the quality control of fish: trimethylamine/total volatile basic nitrogen (%). Journal of Food Protection. 52(6): 419-423.

Manzano, M.A., Aguilar, R., Rojas, E.I. and Sanchez, M.E. 2000. Postmortem changes in black skipjack muscle during storage in ice. Journal of Food Science. 65(5): 774-779.

Marshall, D.L. and Kim, C.R. 1996. Microbiological and sensory analyses of refrigerated catfish fillets treated with acetic and lactic acids. Journal of Food Quality. 19: 317-329.

Marshall, D.L. and Jindal, V. 1997. Microbiological quality of catfish frames treated with selected phosphates. Journal of Food Protection. 60: 1081-1083.

Maturin, L.J. and Peeler, J.T. 1998. Aerobic plate count. In: AOAC International. Bacteriological Analytical Manual. 8th ed. Gaithersburg, MD. p 3-4.

Muzzarelli, R.A.A. 1977. Chitin. Oxford: Permagon Press. p 139-150.

Muzzarelli, R.A.A., Tomasetti, M. and Ilari, P. 1994. Depolymerization of chitosan with aid of papain. Enzyme and Microbial Technology. 16: 110-113.

Muzzarelli, R.A.A., Xia, W., Tomasetti, M. and Ilari, P. 1995. Depolymerization of chitosan and substituted chitosans with the aid of a wheat germ lipase preparation. Enzyme and Microbial Technology. 17: 541-545.

Neter, J., Hunter, M., Nachtsheim, C. and Wasserman, W. 1996. Applied Linear Statistical Models. 4th ed. Chicago, Illinois: Irwin Publishing. 1408 p.

Nordtveit, R.J., Varum, K.M. and Smidsrod, O. 1994. Degradation of fully water-soluble, partially N-acetylated chitosans with lysozyme. Carbohydrate Polymers. 25: 253-260.

Ohtakara, A., Izume, M. and Mitumtomi, M. 1988. Action of microbial chitinases on chitosan with different degrees of deacetylation. Agricultural and Biological Chemistry. 52: 3181-3182.

Ouattara, B., Simard, R.E., Piette, G., Begin, A. and Holley, R. 2000. Inhibition of surface spoilage bacteria in processed meats by application of antimicrobial films prepared with chitosan. International Journal of Food Microbiology. 62: 139-148.

Pantaleone, D., Yalpani, M. and Scollar, M. 1992. Unusual susceptibility of chitosan to enzymic hydrolysis. Carbohydrate Research. 237: 325-332.

Park, S.M.Y., Kim, S.K., Ahn, H.J. and Hyun, D. 1999. Studies on the improvement of storage property in meat sausage using chitosan. Journal of the Korean Society of Food Science and Nutrition. 28(1): 167-171.

Peavey, S., Work, T. and Riley, J. 1994. Consumer attitudes toward fresh and frozen fish. Journal of Aquatic Food Products Technology. 3: 71-87.

Pittler, M.H., Abbot, N.C., Harkness, E.F., and Ernst, E. 1999. Randomized double-blind trial of chitosan for body weight reduction. European Journal of Clinical Nutrition. 53(5): 379-381.

Reddy, N.R., Solomon, H.M., Yep, H., Roman, M.G. and Rhodehamel, E.J. 1997. Shelf life and toxin development by *Clostridium botulinum* during storage of modified-atmosphere-packaged fresh aquacultured salmon fillets. Journal of Food Protection. 60(9): 1055-1063.

Regenstein, J.M., and Regenstein, C.E. 1991. Introduction to Fish Technology. New York: Van Nostrand Reinhold. 257 p.

Reppond, K.D., Collins, J. and Markey, D. 1983. Walleye Pollock: changes in quality when held on ice, slush ice, refrigerated seawater then stored as block of fillets at –18°C. Journal of Food Science. 48: 1552-1553.

Rhoades, J. and Roller, S. 2000. Antimicrobial actions of degraded and native chitosan against spoilage organisms in laboratory and media foods. Applied Environmental Microbiology. 66: 80-86.

Roller, S. and Covill, N. 1999. The antifungal properties of chitosan in laboratory media and apple juice. International Journal of Food Microbiology. 47: 67-77.

Roller, S. and Covill, N. 2000. The antimicrobial properties of chitosan in mayonnaise and mayonnaise based shrimp salads. Journal of Food Protection. 63: 202-209.

St. Angelo, A.J. and Vercellotti, J.R. 1992. Preparation and use of food grade N-carboxymethylchitosan to prevent meat flavor deterioration. In: Developments in Food Science. Amsterdam: Elsevier Scientific Publications. p 711-723.

Sagoo, S.K., Board, R. and Roller, S. 2002. Chitosan potentiates the antimicrobial action of sodium benzoate on spoilage yeasts. Letters in Applied Microbiology. 34: 168-172.

Shahidi, F. and Naczk, M. 1989. Chemical composition and chitin content of crustacean offal. In: Botta, R.J. Advances in fisheries technology and biotechnology for increased profitability: papers from the 34th Atlantic fisheries technological conference and seafood biotechnology workshop, August 27 to September 1, 1989, St. John's, NF, Canada. Lancaster, PA: Technomic Publishing Co. p 229-304.

Shiau, S. and Yu, Y. 1999. Dietary supplementation of chitin and chitosan depresses growth in tilapia, *Oreochromis niloticus* X *O. aureus*. Aquaculture. 179: 439-446.

Shin-ya, Y., Lee, M., Hinode, H. and Kajiuchi, T. 2001. Effects of N-acetylation degree on N-acetylated chitosan hydrolysis with commercially available and modified pectinases. Biochemical Engineering Journal. 7: 85-88.

Simpson, B.K., Gagne, N., Ashie, I.N.A. and Noroozi, E. 1997. Utilization of chitosan for preservation of raw shrimp. Food Biotechnology. 11: 25-44.

Skaugrud, O. and Sargent, G. 1990. Chitin and chitosan: crustacean biopolymers with potential. In: Keller, Sue, editor. Making Profits out of Seafood Wastes Proceedings of the International Conference on Fish By-Products Anchorage, Alaska-April 25-27, 1990. p 61-70.

Sudarshan, N.R., Hoover, D.G. and Knorr, D. 1992. Antibacterial action of chitosan. Food Biotechnology. 6: 257-272.

Suvanich, V., Ghaedian, R., Chanamai, R., Decker, E.A. and McClements, D.J. 1998. Prediction of proximate fish composition from ultrasonic properties: catfish, cod, flounder, mackerel and salmon. Journal of Food Science. 63(6): 966-968.

Suvanich, V., Marshall, D.L. and Jahncke, M.L. 2000a. Microbiological and color quality changes of channel catfish frame mince during chilled and frozen storage. Journal of Food Science. 65(1): 151-154.

Suvanich, V., Jahncke, M.L. and Marshall, D.L. 2000b. Changes in selected chemical quality characteristics of channel catfish frame mince during chill and frozen storage. Journal of Food Science. 65(1): 24-29.

Tanaka, Y, Tanioka, S., Tanaka, M., Tanigawa, T., Kitamura, Y., Minami, S., Okamoto, Y., Miyashita, M. and Nanno, M. 1997. Effects of chitin and chitosan particles on BALB/c mice by oral and parenteral administration. Biomaterials. 18: 591-595.

Tsai, G.J. and Su, W.H. 1999. Antibacterial activity of shrimp chitosan against *Escherichia coli*. Journal of Food Protection. 62(3): 239-243.

Tsai, G.J., Wu, Z.Y. and Su, W.H. 2000. Antibacterial activity of chitooligosaccharide mixture prepared by cellulase digestion of shrimp chitosan and its application to milk preservation. Journal of Food Protection. 63: 747-752.

Tsugita, T. 1989. Marine colloids biotechnology. In: Chitin/Chitosan and its Applications. Tokyo: Gihodo Publishing Co. p 287-297.

Wang, G.H., 1992. Inhibition and inactivation of five species of foodborne pathogens by chitosan. Journal of Food Protection. 55: 916-919.

Williams, S.K., Rodick, G.E. and West, R.L. 1995. Sodium lactate affects shelf life and consumer acceptance of fresh catfish (*Ictalurus nebulosus, marmoratus*) fillets under simulated retail conditions. Journal of Food Science. 60: 636-639.

Yaplani, M. and Pantaleone, D. 1994. An examination of the unusual susceptibilities of aminoglycans to enzymatic hydrolysis. Carbohydrate Research. 256: 159-175.

Ylitalo, R., Lehtinen, S., Wuolijoki, E., Ylitalo, P., and Lehtimaki, T. 2002. Cholesterol-lowering properties and safety of chitosan. Arzneimittel-Forschung. 52(1): 1-7.

Zhang, H. and Neau, S.H. 2001. In vitro degradation of chitosan by a commercial enzyme preparation: effect of molecular weight and degree of deacetylation. Biomaterials. 22: 1653-1658.

Zhang, H., Yuguang, D., Zingju, Y., Mitsutomi, M. and Aiba, S. 1999. Preparation of chitoligosaccharides from chitosan by a complex enzyme. Carbohydrate Research. 320: 257-260.

Zhou, Y., Martins, E., Groboillot, A., Champagne, C.P. and Neufeld, R.J. 1998. Spectrophotometric quanitification of lactic acid bacteria in alginate and control of cell release with chitosan coating. Journal of Applied Microbiology. 84: 342-348.

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While at the University of Maine, Todd has become a member of the Institute of Food Technologists, Northeast IFT, and Phi Tau Sigma honorary society. Todd is a candidate for the Master of Science degree in Food Science and Human Nutrition from the University of Maine in May, 2003.