AN ABSTRACT OF THE THESIS OF

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Title: Investigation of Film Forming Properties of β-chitosan from Jumbo Squid Pens (*Dosidicus gigas*) and Improvement of Water Solubility of β-chitosan

Abstract approved:

Yanyun Zhao

The objectives of this project were to investigate the critical factors impacting the physicochemical and antibacterial properties of β-chitosan based films derived from jumbo squid (*Dosidicus gigas*) pens, and to evaluate the feasibility of improving water solubility of β-chitosan through Maillard reaction. The studies examined the effect of molecular weight (1,815 and 366 kDa), acid (formic, acetic, propionic, and lactic acid), and plasticizer (glycerol and sorbitol) on the film properties, as well as reducing sugar (fructose and glucosamine) and heat treatment (high temperature short time (HTST), low temperature long time (LTLT)) on water solubility of chitosan. Results on β-chitosan were compared with α-chitosan in both studies.

Tensile strength (TS) and elongation (EL) of β-chitosan films were influenced by molecular weight (Mw), acid and plasticizer types ($P < 0.05$). High molecular weight (Hw) β-chitosan films had an overall TS of 44 MPa, 53% higher than that of low

molecular weight (Lw) β-chitosan films (29 MPa) across all acid types used. The mean TS of β-chitosan acetate and propionate films (43 and 39 MPa) were higher ($P < 0.05$) than that of β-chitosan formate and lactate films (34 and 29 MPa). Films incorporated with plasticizer (32 MPa) had lower TS than those without plasticizer (48 MPa). Mean EL of Hw β-chitosan films was 10% versus approximately 4% in Lw β-chitosan films. Formate and acetate films had higher EL than that of propionate film. Glycerol and sorbitol increased ($P < 0.001$) EL 151% and 106% compared with the films without plasticizer, respectively. Water vapor permeability (WVP) of the films was affected by acid and plasticizer. Formate films (34 g mm/m^2 d KPa) had higher WVP than other acid films. Adding plasticizer increased (11% to 31%) WVP of propionate films except the Lw β-chitosan propionate film with sorbitol. The antibacterial activity of Lw β-chitosan and α-chitosan films delayed ($P < 0.05$) the proliferation of *E. coli*, where lactate films showed the strongest effect. The growth of *L. innocua* at 24 h was completely ($P < 0.05$) inhibited by chitosan films except Hw β-chitosan acetate film.

A soft and cotton-like water soluble chitosan with mesopores was acquired after freeze-drying the Maillard reacted chitosan-sugar solution. The yield of β-chitosan-derivatives (8.48%) was 1.21 times higher than that of α-chitosan products (7.00%) ($P < 0.01$). Heat treatment only affected the yield of chitosan-glucosamine derivatives. Sugar type did not indicate any impact on the yield of the chitosan-derivative products in general ($P > 0.05$). The solubility was affected by sugar type ($P < 0.01$) only occurred in the β-chitosan products prepared with LTLT ($P<0.05$), where β-chitosan-fructose derivatives (9.56 g/L) had higher solubility than the glucosamine (5.19 g/L).LTLT

treatment had given all chitosan-derivatives a higher solubility (8.44 g/L) than HTST (3.83 g/L) did ($P<0.001$).

The results from this study demonstrated the feasibility of creating β-chitosan based film from jumbo squid pens with similar mechanical, water barrier and antibacterial properties compare to α-chitosan films as a food wrap and controlled the properties with several important factors, and developing water soluble chitosan through Maillard reaction that possess the potential as functional substance in a wider range of applications.

Investigation of Film Forming Properties of β-chitosan from Jumbo Squid Pens (*Dosidicus gigas*) and Improvement of Water Solubility ofβ-chitosan

by

Jeremy L. Chen

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CONTRIBUTION OF AUTHORS

Dr. Yanyun Zhao assisted with direction of research of chapter 3 and 4, the experimental design of each research topic and the writing of each chapter.

TABLE OF CONTENTS

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

LIST OF FIGURES

LIST OF TABLES

Investigation of film forming properties of β-chitosan from jumbo squid pens (*Dosidicus gigas*) and improvement of water solubility of β-chitosan

Chapter 1 :Introduction

Chitin (the origin of chitosan) is the second most abundant polysaccharide after cellulose that naturally distributed on earth and is ordinarily found in lower end species like fungi, insect, and crustaceans (Lee and others 2008). Chitosan is commonly produced from chitin hydrolysis in hot alkaline medium (i.e. deacetylation reaction) (Broussignac 1968; Kurita and others 1993).The alkaline hydrolysis process removed the acetyl groups (-$COCH_3$) in *N*-acetyl-D-glucosamine units in chitin and converted to D-glucosamine units with free amine groups (-NH_2) to acquire chitosan that are highly soluble in selective dilute acids. Chitin is known to exist in three polymorphism forms, α, β, and γ which differ in the arrangement of the pile of molecular chains, whereas the crystalline structures of chitin are presented differently based on the raw materials (Jang and others 2004). Interestingly, β-chitosan derived from deacetylated squid chitin was reported as a versatile polysaccharide due to the much greater affinity to various solvents and higher reactivity than crustaceans derived α-chitosan (Kurita and others 1993).

As a food polysaccharide, chitosan not only has excellent gelling and film-forming ability like pectin, but also possesses various unique properties including antioxidant, antimicrobial, and chelating ability due to the existence of amino groups on each D-glucosamine (the monomer consisted of the chitosan polymer chain).Comparing to α-chitosan obtained from crustacean shells, related works of β-chitosan derived from squid pens were limited and mostly from the specie of *Loligo*, and the focus of the studies were mainly on the optimization of deacetylation process of preparing the β-chitosan from

chitin (Tolaimate and others 2003; Chandumpai and others 2004; Lamarque and others 2004; Santhosh and others 2010).Jumbo squid (*Dosidicus gigas*) had taken the third place of squid catch (12.8%, 406,356 tons) worldwide and several studies had demonstrated the potential usage of β-chitosan from other squid species in the aspect of film-forming and antibacterial properties (Shimojoh and others 1996; Uriarte-Montoya and others 2010; Arias-Moscoso and others 2011).

Chitosan is insoluble in most organic solvents, but readily soluble in dilute aqueous acids at pH below 6.0 (Pillai and others 2009) because of the presence of free amino groups (Kurita 2006). Chitosan was not able to dissolve in neutral or high pH solvent system where its pKa value was reported in a range of 6.3-6.5 (Sorlier and others 2001). Several strategies were developed to improve the solubility of α-chitosan based on arrangement principles, namely homogeneous phase reaction (Sannan and others 1976), chitosan molecular weight reductions (Hirano and others 1985; Ikeda and others 1993; Chang 1996), and chemical medications (Qin and others 2006; Feng and Xia 2011). However, a relatively higher cost and the use of synthetic chemical reagent are among the major concerns for the commercialization of water soluble chitosan products. Maillard reaction is a chemical process without using synthetic chemicals that involves amino and carbonyl groups of various molecules, and usually requires heat (Jokic and others 2004).Since chitosan contains amino groups along the polymer chain, Maillard reaction was discovered as an alternative chemical modification method to produce high soluble chitosan by blending reducing sugar such as fructose into heated chitosan solution (Yang and others 2002; Chung and others 2005).

The current study was aimed to understand the film forming and antibacterial properties of β-chitosan from squid pens (*Dosidicus gigas*) and to improve the water solubility of β-chitosan through Maillard reaction.The work was divided into five chapters, including this introduction.The second chapter was a comprehensive review of β-chitosan, first retrospect the chitosan history, properties and commercial applications and then reviewed the potential methods of improving the solubility of chitosan. The third chapter examined the effect of chitosan molecular weight (1815 and 366 kDa), type of acid (1% formic, acetic, propionic acid, 0.5% lactic acid) and plasticizer (0, 25% glycerol or sorbital w/w chitosan) on the mechanical, water barrier, and antibacterial properties of β-chitosan films. The fourth chapter investigated the influence of different heat treatments (high temperature short time (HTST), 121 °C for 15 min; low temperature long time (LTLT), 65°C for 2 or 6 days) and reducing sugars (fructose (F), glucosamine (GS)) on the solubility of β- and α- chitosan. The final chapter highlighted the important findings of the β-chitosan work and provided suggestions for future research and potential food applications.

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Chapter 2 : Literature Review

2.1 Chitosan (i.e. α- and β-type): overview and properties

2.1.1 History of chitosan

Chitin (the origin of chitosan) is naturally distributed as the second most abundant bio-polymer on earth behind cellulose and is ordinarily discovered in lower end species such as fungi, insects, and crustaceans, yet not in mammal(Lee and others 2008). Chitin is an "old" polymer that has been found in fossil insects with chemically detectable remains back to the Oligocene period (25 million years ago)(Stankiewicz and others 1997).

The first explicit depict of chitin was not until 1811, attributed to a French scientist Henri Braconnot, who observed that a certain material (chitin) derived from mushrooms was resistant to sulfuric acid and named it "fungine" (Braconnot 1811). In 1823, the name "chitin" was designated by Odier from the Greek word "chiton" that implicated "coat of mail" referring to the cuticle, who found the same substance as fungine from the cuticle of beetles (Oider 1823). In 1843, Payen indicated that chitin differs from cellulose owing to the existence of nitrogen (Khor 2001), and later in 1876 Ledderhose acquired a crystalline material (glucosamine) from the acid hydrolysis of chitin which varied from glucose in replacing one hydroxyl by an amine group (Ledderhose 1876). In 1859, the discover of "modified chitin" (chitosan) was attributed to Rouget who reported that the chitin compound became soluble in dilute organic acid solutions after boiling chitin in concentrated potassium hydroxide in water (Rouget 1859). It was not until 1894 that Hoppe-Seiler renamed "modified chitin" as "chitosan" (Hoppe-Seyler 1894).

From early to mid-1900s, chitin and chitosan research had been apparently accelerated. By 1916 a researcher indicated that certain bacteria contain chitin (Van Wisselingh 1916), and by 1930 the chemical character (i.e. (1→4) linkage and its β-D conformation) of chitin was confirmed by X-ray diffraction showing considerable similarity to patterns of cellulose which the crystallographic results were later summarized in a book in 1942 (Meyer and Mark 1930; Meyer 1942). In the early 1930s more works showed that some bacteria possess enzymes which degrade chitin (Karrer 1930; Johnson 1932). Meanwhile, Rammelberg confirmed more chitin sources not only form fungi but also crab shells (Rammelburg 1931).

The first patent on chitin and chitosan were filed in 1936 in the United State by Rigby who was an employee of Du Pont de Nemours & Co (Rigby 1936a; Rigby 1936b; Rigby 1936c; Rigby 1936d; Du Pont de Nemours and Co 1936a; Du Pont de Nemours and Co 1936b; Du Pont de Nemours and Co 1936c; Du Pont de Nemours and Co 1936d; Rigby 1937). The patent described from large-scale methods of isolating chitin from crustaceans shells to manufacturing chitosan based films, emulsions, and filaments. At the same time, other researchers were continuing the work of the occurrence of chitin in enormous type of microorganisms (Smith 1936; Zobell and Rittenberg 1937). Studies were also proceeding on the physical and chemical properties of chitin and chitosan (Yonge 1932; Shoruigin and Hait 1934; Shoruigin and Hait 1935; Clark and Smith 1936; Castle 1936; Thor 1939). Expect 1940s which plagued by World War II, the published work related to chitin and chitosan had increased each decade beginning from the 1930s. In 1951, the first book related to chitin and chitosan was written by Richards which was 140 years after the discovery of Braconnot (Richards 1951). In 1977, the first

comprehensive and interdisciplinary book on chitin was published by an Italian professor, Muzzarelli (Muzzarelli 1977).

2.1.2 Structure of chitosan

Chitosan (a partially *N*-deacetylated derivative of chitin) is a polysaccharide consisted of D-glucosamine and *N*-acetyl-glucosamine units, connected via β-(1→4) glycosidic linkages. When the overall portion of D-glucosamine units is more than the *N*-acetyl-D-glucosamine units within the chitin polymer (i.e. degree of deacetylation (DDA) of chitin is above 50%) throughout the deacetylation process, the product becomes soluble in selective diluted acid by salt formation and is defined as chitosan (Muzzarelli and Muzzarelli 2005; Rinaudo 2006). The distribution of the D-glucosamine and *N*-acetyl-D-glucosamine units was suggested to be random and the repeating units were not blocked together within the chitosan molecular (Sashiwa and others 1989; Varum and others 1992). Most commercial grade chitosan product comprise 75-95% D-glucosamine and 5-25% *N*-acetyl-glucosamine units (Domszy and Roberts 1985). The structure of chitosan is very similar to cellulose with the only difference that at the C-2 position of each glucose ring of chitosan contains an amine ($-NH_2$) group instead of a hydroxyl (-OH) group.

Chitosan is known to exist in three polymorphism forms as chitin, α, β, and γ which differ in the arrangement of the piles of molecular chains, whereas the crystalline structures of chitin are presented differently based on the raw materials(Jang and others 2004). Generally, the individual chains are assumed to possess an essentially linear structure which adopts one full helical (a twofold-screw, cellulose-like) conformation every 10.1-10.5 Å along the chain axis(De Angelis and others 1998). For each polymer chain, a distinct “left” and “right” direction is assigned owing to each glycosidic unit in

the linear chain is chiral and all units are linked by an oxygen atom which connects one glycosidic unit in the C1 position to C4 of an adjacent one.

The most common allomorph that chitin and chitosan occur is α conformation (Fig. 2.1), where each unit cell is "orthorhombic" with a $P2_12_12_1$ space group (Ramakrishnan and Prosad 1972) and the individual chain segment is packed in an antiparallel fashion (i.e. adjacent chains arranged in opposite directions) inside a polymer sheet along the *c* axis, although parallel arrangement existed between two consisted sheets of chains along the *a* axis, while *b* represents the fiber axis. The packaging structure of α-form chitosan is highly stabilized by inter-sheet, intra-sheet, and intrachain hydrogen bonds in the directions of three unit cell (Lamarque and others 2004).

A less common allomorph exhibited by chitin and chitosan is β conformation (Fig. 2.2), in which possess "monoclinic" unit cells with a $P2_12_12_1$ space group (Gardner and Blackwell 1975) and the individual chain oriented in a parallel fashion. Since all the linear chains are parallel along the *a* and *c* axes (*b* indicates the fiber axis), no hydrogen bonds exist between two successive chain segments along the *c* axis. The least common allomorph is γ conformation, where two chains oriented in one direction and another chain runs in the opposite way (Jolles and Muzzarelli 1999). The distinct allomorphs were examined by X-ray diffraction patterns and NMR spectrophotometers (Takai and others 1989; Jang and others 2004)

2.1.3 Source of chitosan

Similar to cellulose in plants, chitin (precursor of chitosan) widely distributes in nature in a wide variety of species from microorganisms to sea animals, which perform as

a reinforcement substance for cell wall in lower animals and plants in cuticular and exoskeletons of invertebrates like insects, crabs, and shrimps (Table 2.1). Commercial chitosan products from chitin are principally produced from shell wastes of shrimps, crabs, lobsters and krills generating by the seafood industries due to the readily accessibility with considerable quantities, where estimated chitosan use in year 2000 was approximately 2,073 tons (Kurita 2006) (Table 2.3). Generally, chitin (15-40%) exists in the arthropod skin or shells as a composite with 20-40% proteins and 20-50% calcium carbonate that are the two major substances, while pigments and various metal salts are the minor components (Kurita 2006). Other promising sources for chitin and chitosan production include oysters, clams, krill, crayfish, jellyfish, algae, insects, and fungi (Kurita 2001).

Like chitin, chitosan is also naturally existed worldwide but limited to specific species, such as cell walls of some fungi (*Zygomycetes*), green algae (*Chlorella* sp.), yeast, protozoa, and cutricles of insect (Singla and Chawla 2001; Pochanavanich and Suntornsuk 2002). Advances in fermentation technology with controlling process indicated that fungi (*Aspergillus niger*) cultivation may supply as an attractive alternative source of chitosan (Teng and others 2001; Pochanavanich and Suntornsuk 2002). Nevertheless, chitosan produced from various source is slightly different, whereas the amino groups in chitosan isolated from crustacean chitin are uniformly distributed along the linear chain, a chitosan derived from fungal cell walls with a similar degree of deacetylation may own amine residues which are grouped into clusters (Raafat and Sahl 2009).

Comparing to shrimp cuticle, squid pen also contains high content of chitin (20-40%) which is classified as β-form but with negligible amount of calcium carbonate, pigments and other minerals (Kurita 2006) (Table 2.2).β-chitin is distinguished from the common α-chitin from crustacean shells based on the distinct crystalline structure, where β-chitin possess a weaker intermolecular forces between the successive chain segments and is quite attractive as an alternative source of chitin with some unique characteristics different from α-chitin(Jang and others 2004).Based on the crystalline structure of chitin indicated by Blackwell and Rudall (Rudall 1963; Rudall 1967), α-chitin possesses forceful hydrogen bonding by intersheet and intrasheet, whereas β-chitin has weaker intrasheets hydrogen bonding. As a result, β-chitin has much higher reactivity than α-chitin in alkaline deacetylation and the crystalline structure of β-chitin was readily destroyed throughout the process comparing to α-form (Kurita and others 1993). Although β-chitin and chitosan is less abundant than α-ones from crustacean source, the ease and mild isolation process from the raw squid pen material and remarkable affinity toward solvent such as organic acids and water suggested the potential and commercial value of β-chitosan(Kurita and others 1993). β-chitin also exists in *Aprodite* chaetae, progonophore tubes, lorica of sessile ciliate, and diatom spinces (Kurita 1997).

In summary, occurrence source of chitin and chitosan can mainly classify into three categories, namely sea animals, insects, and microorganisms; where sea animals include annelida, mollusca, coelenterate, crustaceans (lobster, crab, shrimp, prawn, and krill), and squids; insects contain scorptions, sqiders, brachiopods, ants, cockroachs, and beetles; microorganisms include green algae, yeast (β-type), fungi (cell walls), Mycelia Penicillium, brown algae, spores, Chytridiaceae, Ascomydes, and Blastocladiacease

(Mathur and Narang 1990). In addition, commercial sources of chitin and chitosan in the 1990s are particularly from shell wastes of crustaceans, while krills, some cell wall of fungi, and Mycelia Penicillium were suggested as future potential sources.

2.1.4 Chitosan preparation

Chitosan derived from deacetylated chitin may be approached by several methods, including alkaline deacetylation (Chang and others 1997), enzymatic deacetylation (Martinou and others 1995), intermittent water washing (Mima and others 1983), flash treatment (Focher and others 1990), and organic solvent used (Batista and Roberts 1990). Among the methods mentioned above, alkaline deacetylation process has been mostly used.

Chitosan is produced from the chitin hydrolysis in hot alkaline medium (i.e. deacetylation reaction), in which KOH or NaOH at high temperature are commonly used as strong alkaline reagents (Broussignac 1968; Kurita and others 1993). The deacetylation process removed the acetyl groups (-$COCH_3$) in *N*-acetyl-D-glucosamine units in chitin and converted to D-glucosamine units with free amine groups (-NH_2) to acquire chitosan product that are highly soluble in selective dilute acids. Characteristics of the final chitosan product in terms of molecular weight and degree of deacetylation are based on the used reagents and treatment conditions (Tolaimate and others 2003).For α-chitin from shrimp shells, the typical deacetylation conditions were suggested by using 40-50 % NaOH at 80-100 ℃ for 6-12 h (Tolaimate and others 2003).

The source of the raw material is also a critical factor in the development of the *N*-deacetylation reactions of chitin and the degree of deacetylation of resulting chitosan,

where α- and β-chitin are mainly found in crustacean shells and squid pens, respectively (Rhazi and others 2000). Since β-chitin (squid chitin) chains are arranged in a parallel fashion with relatively weak intermolecular force and loose package comparing to α-chitin (Gardner and Blackwell 1975) (Fig. 2.3), the deacetylation process of β-chitin are able to manipulate under relatively mild conditions by using alkali medium at lower temperature (Kurita and others 1993; Tolaimate and others 2000) which indicates β-chitosan possesses a much greater affinity to various solvents and higher reactivity than α-chitosan (Kurita and others 1993). For example, Kurtia and others (1993) showed the degree of deacetylation (DDA) of squid pen chitosan reached as high as 70% with 30% NaOH at 100 ℃in 2 h, whereas DDA of shrimp chitosan was only 20%. Recently, the optimal deacetylated conditions for seeking high molecular weight and high degree of deacetylation of β-chitin were suggested by adopting 40-50 % NaOH at 90 ℃ for 6 h with one step or three separate steps (2 h+ 2 h + 2 h) (Jung and Zhao 2011). Also, squid pens have low mineral content, eliminated the demineralization step in the preparation of β-chitosan (Tolaimate and others 2003; Lamarque and others 2004; Jung and Zhao 2011).

2.1.5 General properties of chitosan

As a food polysaccharide, chitosan not only occurs excellent gelling and film-forming ability like pectin, but also possesses various unique properties including antioxidant capacity, antimicrobial properties, and chelating ability due to the existence of amino groups on each D-glucosamine (the monomer consisted of the chitosan polymer chain).However, the solubility of native chitosan is restricted to certain range of pH and its applications may be limited without further modifications.Moreover, the inherited properties of chitosan such as chitosan type, molecular weight, degree of deacetylaiton,

distribution of amine along the polymer chain, have demonstrated to contribute to these properties directly and details will be discussed in the following sections.

2.1.5.1 Solubility

Chitosan is insoluble in pure water, alkali and organic solvents, but is readily soluble in most organic acid aqueous media at pH below 6 (Pillai and others 2009) due to the presence of the primary aliphatic amino groups of chitosan are regards as a strong base with a pKa value of 6.3 (Park and others 1983). Hence, at low pH, amines on each D-glucosamine residue are partially protonated (i.e. from $–NH_2$ to $–NH_3^+$) and become positively charged which driven chitosan as a cationic water-soluble polyelectrolyte owing to the repulsion between positively charged chains that allows water molecules to diffuse. In contrast, when pH increases above 6, the amines become deprotonated (i.e., the chitosan molecular loses its charge) and become insoluble. The soluble-insoluble transition exists at the pKa value of chitosan which ranges from 6.3 to 6.5 (Sorlier and others 2001). Since the pKa value is highly depending on the degree of *N*-acetylation, the solubility of chitosan is according to the degree of deacetylation and the method of deacetylation (Cho and others 2000), where degree of deacetylation could be affected by time and temperature of deacetylation, alkali concentration, ratio of chitin to alkali medium, prior treatments adopted to chitin, and particle size of chitin (Pillai and others 2009).

At low pH environment, chitosan can readily form quaternary nitrogen salts with organic acids (Pillai and others 2009) such as formate, acetate, lactate, citrate, pyruvate, malate, and citrate, which are soluble in water (Rinaudo and others 1999; Kim and others 2006). Formic acid was found to be the best solvent to dissolve chitosan with a large

range of concentration from 0.2 to 100% (Kienzlesterzer and others 1982), while 1% acetic acid (as a reference) is the most commonly used solvent which may cause chitosan depolymerization at high temperature when concentrated as β-glycosidic linkages hydrolysis occurs (Rinaudo and others 1999). Acid concentration also is an important factor to impart desired property (Mima and others 1983), while solubility is based on the ionic concentration and salting-out effect. The acid amount needed is related to the chitosan quantity to be dissolved (Rinaudo and others 1999), where required proton concentration is at least to be equal to the amine concentration involved. As a result, the solubility of chitosan is quite a difficult parameter to control when it involves in a complex solvent system of controlling factors (Rinaudo 2006).

2.1.5.2 Film-forming ability

The linear chitosan polymer has excellent film and coating forming capacity owing to the β-(1→4) glycosidic linkages between the sugar monomers consisting of the chain.This biodegradable film is usually acquired by evaporation of dilute organic acid mixture with the chitosan polymer (i.e., transferring hydrocolloids from aqueous suspension leading to change its phase by solvent evaporation) (Demargerandre and Domard 1994). Generally, α-chitosan films were reported to be transparent, flexible, tough, durable, and hard to tear with several permeable properties and most of the mechanical properties of the films were comparable to various commercial polymers with medium strength such as cellulose (Butler and others 1996a). However, the elongation of α-chitosan films was much less compared to plastic synthetic films which ideally would be in a range of 250 to 300 % (Brody and Marsh 1997). The elongation characteristics of α-chitosan films can however be ameliorated by implanting plasticizers such as polyols

which were indicated to be the most effective plasticizers for hydrocolloid-based films since its structure is similar to the polymer matrix (Sothornvit and Krochta 2005).

For food applications, chitosan edible films were mostly made from dilute carboxylic acids such as formic, acetic, propionic, and lactic acid (Caner and others 1998; Park and others 2002; Kim and others 2006), where the mechanical and barrier properties (i.e. water vapor permeability) of these α-chitosan films could be controlled by selecting the film forming compositions such as chitosan molecular weight and degree of deacetylation, solvent type, adjusting the pH, and adding plasticizer agents, etc.Chitosan films possessed moderate water permeability were suggested to be used to extend the shelf life of fresh produce and foodstuffs with high water activity (Kittur and others 1998). Like α-chitosan based films, β-chitosan film characters such as tensile strength, elongation at break, and water vapor permeability were also affected by chitosan molecular weight and degree of deacetylation with similar trendsas α-form (Shepherd and others 1997; Chung and others 2005a; Santos and others 2006; Da Silva and Santos 2007). However, to the best of our knowledge, the influence of solvent type and plasticizer on β-chitosan based films are still unknown.

2.1.5.3 Antioxidant property

Antioxidants are molecules that delay or inhibit the oxidation of oxidizable compounds, where it can be further classified as two types, namely primary and secondary antioxidants.Primary antioxidants are characterized by the phenolic groups present in the molecule and counteract during the earlier stage of oxidative reaction. Secondary antioxidants possess the capability to chelate metal ions involved in catalyzing the oxidative reaction. Chitosan was indicated to act as a secondary antioxidant (Labuza

and Breene 1989; Peng and others 1998; Mitani and others 1992; Agullo and others 2003; Xing and others 2005).

Although the exact mechanism of antioxidative activity is still unknown, various hypotheses were proposed. One is that it is believed that amino and hydroxyl groups that bonded on C-2, C-3, and C-6 positions in chitosan are able to react with the free radicals to create more stable macromolecular radicals (Labuza and Breene 1989; Peng and others 1998; Park and others 2004). For example, chitosan performs high inhibition capacity against linolenic acid peroxidation, where 83.7% activity was react with hydroxyl radicals (Feng and others 2007). The other indicated that the chitosan scavenging capacity is related to the fact that the hydrogen ion from $NH^{+}3$ group (ammonium ions) can react with the free radicals to form a stable component (Xie and others 2001). The $NH^{+}3$ was formed by the amino group absorbing a hydrogen ion from the solvent solution. In sum, several authors have considered chitosan as a hydroxyl radical scavenger (Xie and others 2001; Feng and others 2007) and possess metal-bonding abilities (Xue and others 1998).

Antioxidant activity of chitosan depends on the deacetylation degree and the type of substituted group (Je and Kim 2006), where in one study demonstrated the most effective reactive oxygen species scavenging effect was found to be the 90% deacetylated *N*-aminoethyl chitosan which possessed the highest percentage of free amino groups. The results indicated that the amino groups are the major factor effecting the free radical scavenging activity, yet the introduction of an amine on the C-6 position did not show any effect.Moreover, the higher the degree of deacetylation of chitosan, the higher the

scavenging activity (Park and others 2004). Molecular weight of chitosan is also strongly related to its antioxidant activity, where results demonstrated that low molecular weight chitosans were more pronounced than higher ones for DPPH radical (Tomida and others 2009). The difference in antioxidative capacity may be ascribed to the effects of intramolecular hydrogen bonding occur in the chitosan polymer, where considerable hydrogen bonding are formed between N_2-O_6 and O_3-O_5. High molecular weight chitosans own a more compact structure that enable the overall intramolecular hydrogen bonding effect stronger. The strong effect of intramolecular hydrogen bonds declines the available amino and hydroxyl groups. In contrast, low molecular weight chitosan has a less compact structure that makes the intramolecular hydrogen bonding less effective. For instance, it has shown that the scavenging activity is dependent on molecular weight where 1-3 kDa exists the strongest effect (Park and others 2003) in the antioxidative activity study of chitooligosaccharides,.Similar trend was also observed while the low viscosity chitosan indicated the highest antioxidative effect (Lin and Chou 2004).

2.1.5.4 Antimicrobial property

The antimicrobial activity of chitosan is mainly depended on its polycationic nature.Chitosan becomes more soluble toward solvent and exhibits better antimicrobial activity than chitin since the glucosamine monomer on the C2 position is positively charged when the environmental pH is below the pKa of chitosan and its derivatives(Chen and others 1998).

Even though the exact antimicrobial action of chitosan has precluded a complete understanding, several mechanisms were proposed.Physical state and molecular weight

of chitosan and the types of microorganism imparted antibacterial action in distinctive modes (Kong and others 2010).

Firstly, chitosan mainly acted on the exterior membrane of the bacteria where electrostatic interaction between positively charged chitosan polymer and the predominantly negative charged components of the microorganism surface (i.e. cell surface proteins and lipopolysaccharide of Gram-negative bacteria) leads to the leakage of cell proteinaceous and other intracellular components (Young and others 1982; Papineau and others 1991; Sudarshan and others 1992).At low concentration (< 0.2 mg/mL), the protonate chitosan was indicated binding to the anionic bacterial surface leading agglutination, when at higher concentrations, enormous cationic chitosan polymer may render a net positive charge to the bacterial membrane maintaining them in suspension (Papineau and others 1991; Sudarshan and others 1992). UV absorption research demonstrated chitosan leads substantial losses of proteinic component to the *Pythium oaroecandrum* at pH 5.8 (Helander and others 2001; Liu and others 2004).

Secondly, low molecular weight chitosan and ultrafine chitosan nanoparticles may penetrate the microorganism cell wall toward the nuclei and combine to the DNA, therefore interfer mRNA synthesis and DNA transcription (Sudarshan and others 1992).In contrast, high molecular weight chitosan and solid chitosan including large-size nanoparticles probably interact with the cell wall of the bacteria and alter cell permeability (Leuba and Stossel 1985), or form an non-permeable barrier around the cell that blocks the essential solutes to transport into the cell tissues (Eaton and others 2008).

Thirdly, the characteristic of cell surface was closely related to the antibacterial activity of chitosan.Gram-negative bacteria such as *E. coli*, has an outer membrane including lipolysaccharide that provides a hydrophilic surface around the cell, while the lipid components contain anionic groups such as phosphate and carboxyl, which stabilizes the lipolysaccharide layer by electrostatic interactions with divalent cations.On the other hand, gram-positive bacteria possess peptidoglycan and teichoic acid in the cell wall, where teichoic acid is an essential polyanionic (glycerol phosphate) polymer and responsible for the integrity of the cell wall.Generally, chitosan has shown stronger antimicrobial effect against gram-positive bacteria than that on gram-negative bacteria (Jeon and others 2001; No and others 2002).However, contradict results suggested that gram-negative bacteria have a higher negative charge on the cell surface comparing to gram-positive bacteria, resulting in more chitosan adsorption, thus higher antibacterial effect against gram-negative bacteria(Chung and others 2004).

2.1.5.5 Chelating properties

Besides possessing ion exchange properties, chitosan has good ability to form complex with various transition metals and some of those from group 3-7 of the periodic table (Muzzarelli 1973). However, chitosan polymer is not able to absorb metals from group 1 or 2. The formation of chitosan/heavy metal complexes are suggested to involve interactions between the $–NH_2$ and/or the –OH groups via dative bonding (i.e. donation of the nonbonding pair of electrons from the nitrogen and/or the oxygen of the hydroxyl groups to a heavy metal ion). Interestingly, the interaction of the first row transition metal ions with chitosan is followed with appearance of color in most complexes, namely red with titanium, orange with metavanadate and hexavalent chromium, yellowish-brown

with divalent iron, yellowish green with trivalent iron, green with trivalent chromium and nickel, blue with copper, pink with cobalt (Agboh and Qin 1997).

In solid state, cupric ion forms one of the strongest metal/chitosan complexes (Kentaro and others 1986; McKay and others 1986; Domard 1987). A mechanism of copper/chitosan complex formation at pH less than 5 was proposed (Domard 1987) concur with X-ray data based on chitosan-copper stretched films (Ogawa and others 1984). Lately, the mechanism of copper/chitosan complex fabrication in dilute solution was reconfirmed according to the pH and copper content and two distinct complexes were proposed: $[Cu\ (-NH_2)]^{2+}$, $2OH^-$, H_2O and $[Cu\ (-NH_2)]^{2+}$, $2OH^-$ (Rhazi and others 2002). As a result, the physical state of chitosan (i.e., powders, films, fibers) affects the chelation ability and better chelation occurs in higher degrees of deacetylation of chitosan. Moreover, the chelation is also associated with the $–NH_2$ distribution (Kurita and others 1979) and the degree of polymerization (DP) of oligo-chitosans, where the complex begin to form since DP is above 6 (Rhazi and others 2002). The affinity of chitosan to metal ions in 0.1 M potassium chloride solution was observed in the order of Cu>Ni>Zn>Co>Fe>Mn (Muzzarelli 1973), where affinity of chitosan for cations absorbed on film was followed by Cu> Hg>Zn>Cd>Ni>Co~Ca(Rhazi and others 2002).

2.1.6 β-chitosan and its recent studies

β-chitosan derived from deacetylated squid (*Ommastrephes bartrami*) chitin was reported as a versatile polysaccharide due to the much greater affinity to various solvents and higher reactivity than α-chitosan (Kurita and others 1993).The difference between α- and β-chitin and chitosan are generally based on the crystalline structure of the molecule.The β-chitin chains are arranged in a parallel arrangement with relatively weak

intermolecular forces and loose package (Gardner and Blackwell 1975), while the α-structure is aligned in an antiparallel fashion responsible for a stronger intermolecular hydrogen bonding (Blackwell and others 1980; Jang and others 2004). Accordingly, the deacetylation process of β-chitin can be operated under mild conditions by using alkali solution at relatively low temperature (Kurita and others 1993; Tolaimate and others 2000).Moreover, squid pens have low mineral content, eliminated the demineralization step in the preparation of β-chitosan (Tolaimate and others 2003; Lamarque and others 2004), where these characteristics of β-chitosan are potential for manufacture process in commercial scale.

Comparing to α-chitosan obtained from crustacean shells, related works of β-chitosan derived from squid pens were limited and mostly from the specie of *Loligo* and the focus of the studies were mainly on the optimization of deacetylation process of preparing the β-chitosan from chitin (Tolaimate and others 2003; Chandumpai and others 2004; Lamarque and others 2004; Lamarque and others 2005; Lamarque and others 2007; Santhosh and others 2010).To the best of our knowledge, no studies had further demonstrated the potential usage and application of the β-chitosan products that produced from *Loligo* squid pen which only had approximately 9% of world cephalopod catch.In contrast, *Illex argentines* and *Todarodes pacificus,* which were the first (16.1%, 511,087 tons) and second (15.9%, 504,438 tons) largest harvested squid species in the world (Marine Resources Service (United Nations) and others 2005), respectively. Moreover, the characteristic of β-chitosan films prepared by these two species were comparable to common α-chitosan films from crustacean in regard of barrier and mechanical properties (Chung and others 2005a; Santos and others 2006).However, no work has indicated the

optimized deacetylation condition for each specific squid species to obtain the β-chitosan products in the studies above.

Jumbo squid (*Dosidicus gigas*) had taken the third place of squid catch (12.8%, 406,356 tons) worldwide and several studies had demonstrated the potential usage of β-chitosan from other squid species in the aspect of film-forming and antibacterial properties (Shimojoh and others 1996; Uriarte-Montoya and others 2010; Arias-Moscoso and others 2011).Moreover, a comprehensive study in our laboratory has evaluated the deacetylation and depolymerization of β-chitin from jumbo squid pens and suggested the optimal treatment conditions to obtain a wide range of molecular weights and degrees of deacetylation of β-chitosan (Jung and Zhao 2011).

2.2 Chitosan applications

2.2.1 Commercialized applications

As a versatile food polysaccharide, the commercial applications of chitosan were not only limited to dietary supplements, but also to several specific environmental and medical applications owing to its capacity to interact with definite targets such as hazard metals and microorganisms (Table 2.4). The estimate use of chitosan for commercial applications worldwide from nutraceuticals to medical devices was estimated to be a total of 2073 tons in 2000 (Table 2.3). Several unique commercial scale applications related to chitosan such as waste water treatment, cosmetic and personal care, would healing, agriculture, and dietary supplements were discussed in the sections below to give an insight of this marvelous hydrocolloid.

2.2.1.1 Waste water treatment

The chemical contamination of industrial waste waters that are related to toxic impurities such as heavy metals and pesticides may cause serious ecological and health problems by accumulating through the food chain, therefore the demand for purification of these effluents is strongly needed prior to the discharge or use (Jeuniaux 1986; Knorr 1991). Conventional technologies for removing the metals from industrial waste pollutants could be expensive and ineffective, while metals are available under low concentrations (Volesky 1987; Deans and Dixon 1992). Consequently, the use of environmental safe and commercially available biopolymers like chitosan to remove water impurities is a great interest (Crini 2005). These biopolymers, which possess a number of functional groups such as amino and hydroxyl moieties, may be utilized to extend the efficacy of transition metal uptake to parts per billion levels through the chelation ion exchange (Deans and Dixon 1992).

Currently, the main commercial application for chitosan is industrial waste water treatment (Asano and others 1978). Chitosan have been used for purification of waste water owing to its high sorption capacity toward a great range of transition metals and coagulating colloidal matter (Jeuniaux 1986; No and Meyers 2000). In Japan, an approximately 500 tons of chitosan in 1986 were used in water purification (Hirano 1989). In the United States, the use of commercially flaked chitosan for potable water treatment has been approved by the U.S. Environmental Protection Agency (USEPA) up to a maximum level of 10 mg/L (EPA Polyglucosamine exemption from the requirement of tolerance 1980). The present of NH_2 groups in chitosan occur to form coordinate covalent bonds with metal ions. Chitosan powder and dried films are more promising in

chelating metal ions because it would release most of its free amines under the environment condition above the pKa of the amino group of chitosan (Tirmizi and others 1996).

Several factors affect the metal ion complexing ability of chitosan. First, the accessibility of NH_2 groups to water and pollutants have been suggested as an important parameter in the metal sorption process (Guibal 2004; Roussy and others 2005; Guibal and others 2006). The accessibility is particularly controlled by the crystallinity of the chitosan products which relies on the sample origin and also the treatment during extraction. Secondly, the distribution of the NH_2 groups along the chitosan polymer chains influences the adsorption process, while study indicated that metal chelating rate of chitosan with the same degree of deacetylation was greater when homogeneous hydrolysis was applied to produce the samples (Kurita and others 1979). Thirdly, molecular weight of chitosan affects the coagulant efficiency:the coagulant effectiveness was increased by increasing the deacetylation degree of chitosan under the same molecular weight; a decline of efficiency was observed when molecular weight increased even if the deacetylation degree was greater (i.e. from 77 to 86%) (Huang and others 2000). Moreover, study found that increase of chitosan molecular weight has a better potency of coagulation, but the efficiency was not proportional to the molecular weight (Bough and Landes 1978). Fourthly, under low ionic strength (i.e. deionized water) conditions and at pH close to neutral are able to ameliorate the coagulation and flocculation of organic suspensions while applying higher degree of deacetylation and lower molecular weight chitosan samples (Guibal and others 2006). In contrast, the degree of deacetylation and molecular weight of chitosan were almost unaffected under

higher ionic strength (i.e., tap water) and acidic pH environment. Finally, the purity of chitosan is another critical aspect that influences the potential usage in waste water treatment since it impacts the accessibility of the NH_2 groups to the impurities. In sum, chitosan products with high deacetylation degree and low crystallinity are generally preferred to be utilized for water purifications.

2.2.1.2 Cosmetics and personal care

Chitosan and its derivatives are reported as an ingredient in various cosmetics, nail lacquers, toothpaste, lotions, hand and body creams, and hair-care products (Mark and others 1985; Synowiecki and Al-Khateeb 2003). These bio-polysaccharides were also investigated as components of cosmetic formulations especially focusing on sensitive skin applications. The moisture-holding effect of chitosan is based on molecular weight and degree of deacetylation, while high molecular weight chitosan increase the protection against sun irradiation by increasing the water resistance of emulsions (Shahabeddin and others 1990; Wachter and Stenberg 1996; Horner and others 1997). In cosmetic cream, 0.1% chitosan can increase the availability of bioactive, lipophilic ingredients like vitamins that superior penetrate the skin outer layer accompanying with fibroblast activations and improved collagen deposition. Moreover, the film-forming and antiseptic properties of chitosan prevent the skin from conceivable microbial infections. Also, glucosamine from chitosan affects the excellent development of glycosaminoglycan and glycoprotein structures in the skin extracellular matrix. For dental fluids and toothpastes chitosan products, chitosan lower the dentin permeability in the hydrogel forms which can seal dentinal tubules and inhibit microbial infection yet keep the beneficial diffusion

of ions and water (Paw-lowska 1997; Mohire and Yadav 2010), where the effect can be altered by the chitosan buffering ability.

The first studies of chitosan as a personal care ingredient for beauty industry were stared in the 1980s by German firm Wella, Inc., a largest hair cosmetics manufacturer. The first commercialized application of chitosan in cosmetics was applied in a hair-care product by Fine Cosmetics Co. of Japan in 1986, where chitosan offers protection of mechanical hair damage and occurs antielectrostaic ability on hair owing to the high-performance moisturizing and film-forming capacities (Mathur and Narang 1990). This chitosan hair care product is acquired by the hypochlorite oxidation of chitin, which possess cosmetic properties similar to a sparse natural polysaccharide, hyaluronic acid, and has been applied as a substitute. The difference between hyaluronic acid and chitosan only exist in the presence a distinct functional group in position 6 of the hexose, where carboxylic group presents in the former and $–CH_2OH$ occurs in the latter.

2.2.1.3 Wound healing

A scientific basis for chitosan efficacy in the wound healing promotion was first reported in 1978 (Balassa and Prudden 1978). The need of rapid wound healing is strongly desirable for patients, especially for people having diabetes as they possess extremely show healing rate. Chitin, chitosan and its derivatives would safely apply to animals and humans with various forms that are available for medical applications such as finally divided powder, porous beads, nonwoven fabrics, gauges, lyophilized soft fleeces or gels, laminated sheets, and transparent films (Singh and Ray 2000). Water soluble chitin derivatives in isotonic saline can be executed intramuscularly or intravenously (Singh and Ray 2000).

Substantial biochemical evidence linking "*N*-acetylglucosamine" with the hexamine metabolism was assumed to originate and cross-link wound collagen. On the other hand, the physiological matter of "glucosamine" has been stressed by several authors, in which glucosamine mainly occurs in the detoxification function of liver and kidneys and own the anti-inflammatory, antireactive, antihypoxic, and hepatoprotective activities (Zupanets and others 1990; Setnikar and others 1991). D-glucosamine also possessed natural killing activity in cancer patients (Matheson and others 1984). *N*-acetylglucosamine, which is the repeating monomer units of chitin derivative, is a predominant substance of dermal tissue and its existence is necessary for scar tissue repair. Virtually, glycoproteins, which involve numerous amount of *N*-acetylglucosamine, are one of the major proteins separated in the early phase of wound healing. Chitin derivatives was suggested to degrade easily by lysozemes that naturally present in wound fluid and may boost wound healing through a controlled delivery of *N*-acetylglucosamine (Carlozzi and Iezzoni 1966).

Several wound healing mechanisms of chitin and chitosan were proposed and discussed below.

For chitin derivatives, a conceivable mechanism in wound healing is the present of glucosaminoglycan which functions in the collagen structural organization. The glucosaminoglycan components of wound tissue affect in the giving strength and structure of the lately formed collagen in the granulating tissue of the healing wound. Another mode of action where chitin derivatives may influence the wound healing process due to their ability to stimulate the inflammatory substances needed for wound healing. Macrophage cell is well known to play an important role in the healing process,

while the absence of macrophages has suggested to result impaired would healing in macrophage-deficient animals (Leibovich and Ross 1975).

For chitosan derivatives, it is believed that chitosan are hydrolyzed by lysozyme to oligomers in vivo which activate macrophages to generate interferon, tumor necrosis, and interleukin-1. The macrophages, active by chitosan oligomers, also produce *N*-acetyl-β-glucosamidase which catalyzed *N*-acetylglucosamine, D-glucosamine, and substituted glucosamines production. These amino sugars are able to incorporate into glycosaminoglycan and hyaluronates under the action of interleikin-1, thus leading the ordered deposition of collagen. Recent studies have indicated that chitosan and its derivatives support blood coagulation, act as a biocide, prevent abnormal fibroblastic reacritives, and wound healing accelerators during tissue regeneration and reorganization processes (Zikakis 1984; Nishimura and others 1986; Muzzarelli and others 1988; Muzzarelli and others 1990; Biagini and others 1991a; Biagini and others 1991b; Minami and others 1992).

Commercial chitosan products for veterinary wound healing propose were in a significant progress and a Japanese company, Sunfive Inc has developed and marketed wound healing product in names of "Chitopak TM C" (chitosan-cotton) and "Chitofin TM S" (chitosan suspension), where the 3M company has marketed Tegasorb™ as a chitosan wound healing product (i.e. excipient) for human usage (Illum 1998). Moreover, a wound-healing dressing is manufactured on a pilot scale by Kendall Company, a branch of Colgate Palmolive in the United States, and is approved by Food and Drug Administration (FDA) to use for clinical trials (Mathur and Narang 1990).

2.2.1.4 Agriculture

Chitosan obtained from the cell walls of some fungi or crustacean shells exists antimicrobial effect on phytopathogenic fungi and bacteria, and elicit plant resistance to viroid, viral , or fungal infections (Pospieszny and others 1996; Struszczyk and others 1996; Pospieszny and Mackowiak 1997). Nevertheless, low molecular weight chitosan oligomers lost their capacity inhibiting microbial growth yet still are able to protect plants from pathogens (Matheson and others 1984) which indicates chitosan oligomers are able to induct the natural resistance of plants against microbial infections.

Chitosan suppresses the proliferation of bacteria and bacterial infection, and stimulates the natural defenses in plants based on the mechanism of "octadecanoid pathway" (Doares and others 1995). The resistance against fungal infections is accounted to the hydrolytic destruction of their cell walls via plant β-glucanase and chitinase and the release of chitosan which elicits phytoalexin synthesis (Ikeda and others 1996). Phytoalexin is considered as a potential suppressor of fungal growth. The antimicrobial capacity of chitosan and its derivatives is based on their average molecular weight, the susceptibility to enzymatic degradation, and the release of oligomers that are soluble in water (Struszczyk and others 1996). Moreover, the excellent antiviral activity was shown while applying microcrystalline chitosan and its derivatives, especially in salts form.

In agricultural field applications, chitosan is performed as an antimicrobial control agent. Spraying of aqueous chitosan solution on bean plant significantly protected the spices against virus infections (Walker-Simmons 1983). The addition of chitin and chitosan to soil is efficient in eliminating certain plant diseases yet encourage the growth of some chitinolytic microorganisms that are dominant in the soil (Toyoda and others

1996). As a result, this restricts the growth of plant pathogens both in plant vascular system and soil via the hydrolysis of fungal cell walls by chitinolytic enzymes which secreted via antagonist. Chitosan and its derivatives are also favorable for the intensification of seed germination in the case of wheat, cucumber, and pea seeds(Kauss and others 1989; Santos and others 1991; Ikeda and others 1996).In 1991, a practice (proved by the U.S. Environmental Protection Agency) of chitosan used in agriculture has been adopted by 11 states (Jeuniaux 1971; Nordtveit and others 1991).

2.2.1.5 Dietary supplements and foods worldwide that contain chitosan

Commercially, chitosan is used as dietary supplement owing to its hypocholesterolemic and hypolipidemic abilities lowering the cholesterol and lipid levels in *vivo* studies of humans(Maezaki and others 1993; Wuolijoki and others 1999; Bokura and Kobayashi 2003; Gades and Stern 2005; Liao and others 2007) or animals(Sugano and others 1978; Nagyvary and others 1979; Jennings and others 1988; Fukada and others 1991; Chiang and others 2000; Yao and others 2008), where the reduction in levels of cholesterol and other lipids in both humans and animals were indicated in liver tissue or blood serum. For humans, chitosan appears to be active even at doses as low as 1.2 g per day resulting in significant reductions in serum cholesterol (Wuolijoki and others 1999; Bokura and Kobayashi 2003).

The mechanism by which chitosan reduces cholesterol levels is still not distinctly understood. It is known that chitosan ingestion has an influence on the bile acids which is used by the body to emulsify water-insoluble components (e.g. cholesterol) of the stomach (Fukada and others 1991), where these water-insoluble contents cannot transport through the small intestinal wall without an emulsified process (Mansbach and others

1975). In *vitro*, chitosan was reported to entrap bile acids with approximately one-half or equal ability of cholestyramine which is a strong synthetic ion exchange resin (Sugano and others 1980; Lee and others 1999). Chitosan has been indicated lowering the emulsified cholesterol concentration in the intestines (Ebihara and Schneeman 1989) though a currently unknown mechanism and it was assumed that chitosan has bound to some cholesterol-containing micelles as it precipitates in the small intestine (Vahouny and others 1983). A study suggested that, in *vitro*, chitosan-bound bile acid micelles is formed, resulting in the assimilation of cholesterol, bile acid, monoglycerides, and fatty acids (Nauss and others 1983), while another study discovered that chitosan prohibited the pancreatic lipase activity (Han and others 1999).

Chitosan has an additional remark being as an unspecific substrate towards various lipases in a *vitro* study (Muzzarelli 2000), as a result, soluble chitosan may further suppress the lipase activities against lipids by acting as an alternative substrate.The insoluble chitosan-bound bile acid salts were demonstrated to collect lipids by hydrophobic interaction (Faldt and others 1993; Kim and Chun 1999; Lee and others 2000).It is important to note that bile acids, once were sequestered by chitosan, were no longer available to act as emulsifiers in a correct form to emulsify lipids necessarily during its digestion.It is known that pancreatic lipases necessitate a specific dimension of the oil droplets in the emulsion to hydrolyze triglycerides. As a result, their ability as emulsifiers would cause inadequate emulsions when the bile acid becomes scanty and therefore lowering the hydrolysis of triglycerides (Weng and others 1999; Miled and others 2000; Lombardo 2001).The existence of bile salts not only activates the bile salts-dependent lipases but also provide the emulsion required to the pancreatic lipases for

enzymatic activity: several models have been proposed (Rubin 1994; Muzzarelli 1996; Fernandez-Lopez and others 2002).In sum, as soon as the bile salt was blocked due to chitosan intake, lipases become unable to function adequately and lipid assimilation may decline sharply in the organism.

In the market, foods that contain chitin or chitosan can either be found in natural products or processed foods.For natural foods, chitin-containing organisms are commonly harvested through fishing or farming practices.*Agaricus bisporus* mushroom, for example, contains approximately 1% chitin (Temeriusz 1975) and can be readily purchased from most groceries in the United States.For processed foods, chitosan was added deliberately as an ingredient during the manufacturing process in certain countries such as Japan and Korea.In 1986 less than 100 tons of chitosan were utilized as a food additive (Hirano 1989) in Japan, where no commercial food products were using "chitin" as a food ingredient.In Japan, dietary chitosan-containing vinegar, noodles, potato chips, and cookies were produced commercially and were considered to possess hypocholesterolemic and hypolipidemic effect as an additional value (Brine and others 1992; Hirano 1989), where Japanese Health Department had approved chitosan as a functional food ingredient in 1983 (Weiner 1992). Moreover, Korea Food and Drug Administration also authorized the use of chitosan as food additives in 1995 (KFDA 1995). In the United States, chitosan was approved as a feed additive by the Food and Drug Administration (US FDA) back in 1983 (Knorr 1986).

It is worth to note that chitin and chitosan was regarded by the Codex Alimentarius Commission in 2003 yet not currently listed in the General Standard for Food Additives and it is not approved as a food ingredient in Europe (Aranaz and others 2009). Although

chitosan has obtained the GRAS (generally recognized as safe) status according to the scientific procedures for food usage in general including poultry and meat by the US FDA in 2005, its full-fledged usage in food formulations as a functional food still requires official clearance.

2.2.2 Other potential applications

2.2.2.1 Ophthalmology

Chitosan have included all the needed characteristics of developing an ideal contact lens, which are optical clarity, gas permeability, partially towards oxygen, mechanical stability, wettability, sufficient optical correction, and immunological compatibility (Dutta and others 2002). Contact lenses produced from partially depolymerized and purified squid pen chitosan through spin casting are clear and tough with desirable physical properties like modulus, tear strength, tensile strength, elongation, oxygen permeability, and water content. The antimicrobial, wound healing, and superior film-forming properties of chitosan leading this biopolymer become a suitable material developing ocular bandage lenses (Markey and others 1989). Nevertheless, extensive preclinical and clinical safety tests following by Food and Drug Administration (FDA) guidelines are required prior to commercialization of chitosan-based contact lenses (Allan 1985).

2.2.2.2 Water-resistant adhesive

Chitosan was demonstrated to mimic the function of the mussel-produce adhesive protein by reacting with dopamine to form a cross-linked hydrogel via tyrosinase catalyzed reactions(Yamada and others 2000) owing to the fact that chitosan possesses primary amino residues with moderately low pKa range of 6 to 6.5 (e.g., more reactive

than amino acid like lysine with relatively high pKa of 10.5) (Rinaudo and others 1999; Muzzarelli and Ilari 1994).The attempt to mimic these natural adhesive glue is mainly due to its salient characteristics which do not require either elevated temperatures or specific surface preparation for strong adhesion (Peshkova and Li 2003).Furthermore, these protein glues may generate strong bonds on wet surfaces even with a wide range of temperatures and salinities (Waite and others 1989; Waite 1990a).

The biological analogy based on the cuticular sclerotization process in insects (i.e., hardening of the insect shell) was profited by mimicking the water-resistant adhesive protein (Yamada and others 2000).During sclerotization, the enzyme tyrosinase is considered to oxidize low molecular weight sclerotizing precursors (i.e. *N*-acetyldopamine) (Sugumaran 1987; Andersen and others 1996) and the end products (o-quinones) from this reaction will experience subsequent nonenzymatic cross-linking reactions with proteins to generate the "quinone tanning" substances being a part of the hardened outer integument.Interestingly, study suggested that terrestrial animals may utilize low molecular weight cross-linking substitutes, while marine animals exploited crossing-linking materials mainly based on high molecular components (i.e., proteins) to prevent the lose of secreted water-soluble compounds in the marine environment (Waite 1990b).Although the exact quinone chemistry of the sclerotization process is still unknown, studies indicated that the most critical cross-linking sites on the proteins appear to be histidine residues (Schaefer and others 1987; Christensen and others 1991).Most likely, histidine residues were more reactive comparing to the amines presented in lysine due to the histidine residue possessed a lower pKa.

Instead of using protein to facilitate quinone reactions in sclerotizaion process to form mussels-like adhesive glue, chitosan with substantial amino groups also has the ability to react with o-quinones which obtain from oxidizing low molecular weight 3,4-dihydroxyphenethylamine (dopamine) by tyrosinase leading to a "quinone-tanned" chitosan (Yamada and others 2000).In related studies, researchers had provided supportive evidences that these "quinone-tanned" chitosan that generated via a tyrosinase catalyzed reactions of dopamine also behaved as gels (Muzzarelli and Ilari 1994; Muzzarelli and others 1994; Kumar and others 2000; Yamada and others 2000).In addition, these chitosan/dopamine/tyrosinase systems were further determined by Yamada and others (2000) to use as a water-resistant adhesive for glass, where these amino or phenolic functional groups can react to each other by means of the phenol-oxidative enzyme to generate forceful adhesive bonds (Waite 1985; Waite 1987; Waite 1990b).For wood adhesive applications, a chitosan/phenolics/laccase system was suggested to produce a natural based adhesive that provide relatively strong adhesive strengths to generate wood composites (Peshkova and Li 2003).

2.2.2.3 Food emulsifier

Under acidic condition (i.e. pH < 6.3), chitosan become soluble in aqueous system and its location at the interface of two phases is a splendid predisposition being as an antimicrobial ingredient in food emulsions due to the fact that chitosan has a positive ionic charge and possesses both reactive amine and hydroxyl groups that gives the capacity to bind with the negatively charged protein (Zivanovic and others 2004).However, the antimicrobial capacities of chitosan in aqueous may be insufficiently performed in the complex food systems where chitosan activities may alleviate due to the

interactions with other constituents (Oh and others 2001).In spite of the fact that emulsions in oil phase with high concentrations do not support microbial growth, the emulsions may include spoilage and pathogenic microorganisms that proliferate in the non-lipid phase.Mayonnaise, an oil-in-water emulsion, has been chosen as a food model system to conduct on chitosan use to improve and stabilize the emulsification in its preparation and was suggested to use chitosan as an emulsifier in commercial mayonnaise preparation (Lee 1996; Kim and Hur 2002).It is worth to note that, unlike other polysaccharides, chitosan provides additional stabilization owing to its hydration forces (Del Blanco and others 1999) and chitosan functions only in acidic solvent system to perform possible utility as an emulsion stabilizer and thickener (Filar and Wirick 1978).

Unlike acid soluble chitosan, water-soluble chitosan itself did not possess significant antimicrobial ability against both yeast and bacterial (Qin and others 2006). Nevertheless, water-soluble chitosan-glucosamine derivative prepared by Maillard reaction was demonstrated to have the metal-ion chelating ability and antibacterial capacity (Chung and others 2005b; Chung and others 2006) where this derivative appeared to act more effectual than other chitosans or it derivatives as a natural biocide agent (Chung and others 2005c).In the past, Maillard reaction was also applied to protein (i.e. β-lactoglobulin) to enhance its antioxidant and antimicrobial activity and other functional properties (Chevalier and others 2001; Miralles and others 2007)

2.2.2.4 Chitosan based edible films and coatings

Chitosan is apparently the most exploited polysaccharide with antimicrobial capacities and may be applied as edible films and coatings to improve and extend shelf-life of fresh and processed foods (No and others 2007; Vargas and others 2008) due to

their biodegradable and ecofriendly nature (Kittur and others 1998; Tharanathan 2003). A patent by Rigby in 1936 reported the earliest attempt of forming chitosan films (Rigby 1936d; Du Pont de Nemours and Co 1936b) and later in 1950s patents was granted to use chitosan films to extend shelf-life of foods (Guilbert and others 1996). Chitosan films have been studied on fishes (Jeon and others 2002) and fruits (Srinivasa and others 2002; Srinivasa and others 2004), where chitosan coatings have been evaluated in a wide range of food items, including fruits (Pen and Jiang 2003; Park and others 2005; Wang and others 2007; Chien and others 2007a; Chien and others 2007b; Campaniello and others 2008; Gonzalez-Aguilar and others 2009), vegetables (Eissa 2007; Waimaleongora-Ek and others 2008; Fuchs and others 2008; Simoes and others 2009), cheeses and eggs (Coma and others 2003; Duan and others 2010; Cao and others 2009; Del Nobile and others 2009; Caner 2005; No and others 2005; Kim and others 2008; Kim and others 2009).

Several mechanisms have been proposed in respect to the capability of chitosan for extending shelf-life of foods. These may include controlled moisture and atmosphere transfer between food and the environment, controlled respiration rate and oxygen partial pressure reduction in the package to decrease the metabolism rate of fresh produce, controlled release of chemical agents such as biocides and antioxidants, and controlled solute transports and temperature (Kester and Fennema 1986; Labuza and Breene 1989). In addition, the inherit antimicrobial activity of native chitosan film/coating is also one of the most critical properties to improve shelf-life and safety of food which let chitosan stand out from other hydrocolloids.

2.3 Important factors affecting chitosan-based film properties

Overall, the characteristics of chitosan films varied from one study to another mainly due to the various formulations. However, itis generally agreed that the property of the chitosan films are commonly influenced by the molecular weight and degree of deacetylation of chtiosan, solvents, amounts and types of plasticizers used (RemunanLopez and Bodmeier 1996; Begin and Van Calsteren 1999).

2.3.1 Molecular weight and degree of deacetyaltion of chitosan

Molecular weight and degree of deacetyaltion are the two most critical factors impacting the physicochemical, permeability, and antimicrobial properties of chitosan films (Jeon and others 2002; Kim and others 2007; Butler and others 1996b; Begin and Van Calsteren 1999; Hwang and others 2003; Chung and others 2005a; Chen and Hwa 1996; Santos and others 2006). Shrimp and squid chitosan (α- versus β-) based films were tested for mechanical properties, water vapor permeability, and swelling in water to determine the effect of chitosan molecular weight and deacetylation degree on these properties (Santos and others 2006).For both α- and β-chitosan films, decreasing the molecular weight of chitosan impaired mechanical properties by decreasing entanglement density, crosslinking degree and looser network formation.Also, the decrease of chitosan molecular mass lowed water vapor permeability of the films, which may be related to the increasing excluded volume with the increased molecular weight leading to a more effective polymer chains arrangement within the film matrix and a lower interstitial space degree in the films.On the others hand, increasing the deacetylation degree of chitosan led to an increase of intermolecular interactions (i.e. hydrogen-bonding) among the linear polymer chains, as a result, forming a more compact polymer network.Thus, the

mechanical properties of films were improved accompanying with lower water vapor permeability and swelling in water.

2.3.2 Plasticizers

The nature of chitosan films developed by pure chitosan polymers tend to be rigid and brittle. Adding food-grade plasticizers to film-forming solution can alleviate the problem to obtain a more favorable mechanical properties(Domjan and others 2009). The primary role of plasticizers (usually are low molecular weight non-volatile substances) is to increase the process ability and flexibility of polymers by reducing the second order transition temperature, the glass transition temperature (*Tg*) (Adeodato Vieira and others 2011).

Generally, biopolymers and plasticizers are both hygroscopic, thus moisture content of film is influenced by ambient environments. Moreover, water is the major solvent for developing the natural hydrocolloid film-forming solution. Water molecules can reduce the *Tg* and increase the free volume of the biopolymer, functioning as a plasticizer (Cheng and others 2006; Karbowiak and others 2006). Certainly, water is the most common and powerful natural plasticizer of hydrocolloid-based films, its plasticization action on various biomaterials has been widely reported (Gontard and others 1993; McHugh and Krochta 1994; Jangchud and Chinnan 1999; Sobral and others 2002; Kristo and Biliaderis 2006; Suyatma and others 2005).

For chitosan-based films, polyols such as glycerol, ethylene glycol, polyethylene glycol, and propylene glycol was studied to determine the effect on film mechanical and surface properties regarding the plasticizer volatility (i.e., the less volatile the

plasticization, the better for its application) (Suyatma and others 2005). As a result, glycerol and polyethylene glycol are more suitable than ethylene glycol and propylene glycol and a 20% (w/w) plasticizer concentration was sufficient to develop flexible chitosan film with good stability for 5 months storage. Furthermore, glycerol and polyethylene glycol were confirmed to be good candidates as plasticizers for the rigid and brittle chitosan films with differentmechanisms (Domjan and others 2009), in which polyethylene glycol may act as an external plasticizer, while glycerol rather performs as an internal plasticizer.

2.3.3 Chitosan solvents

Chitosan is insoluble in most organic solvents, but readily soluble in dilute aqueous acids at pH below 6.0 (Pillai and others 2009) because of the presence of free amino groups (Kurita 2006).The primary amino groups of chitosan are regarded as a strong base with a pKa value of 6.3 (Park and others 1983).At low pH, amines on each D-glucosamine residue are protonated and become positively charged which driven chitosan as a cationic water-soluble polyelectrolyte.Some nontoxic monocarboxylic (formic, acetic, propionic, and butyric) and L- or D-lactic acid(Kurita 2006), as well as dicarboxylic acid (oxalic, succinic, malic, and adipic) (Chen and others 2008) are frequently used to prepare chitosan solutions (Kurita 2006).In addition, a type II chitosan acid complex which possessed a relaxed 2-fold helix conformation (hydrated crystal) can be formed by applying monocarboxylic and L- or D-lactic acids as solvents (Kawada and others 2001).

The effect of different types of organic acid solvents on α-chitosan based films was investigated in several studies (Caner and others 1998; Park and others 2002; Kjm and

others 2006). Food-grade organic solvents such as formic, acetic, propionic, lactic, citric, and malic acid were evaluated in these studies. Differences ($P < 0.05$) in mechanical and barrier properties such as tensile strength, elongation at break, and water vapor permeability were observed when using various acid types as chitosan solvent. For example, Kim and others (2002) indicated that chitosan acetate films showed lower water vapor permeability (0.85×10^{-5} g • m/m^2 • h • Pa) and higher integrity with higher tensile strength values (13.6 MPa), yet lower elongation at break (59.1%) comparing to other organic acids (formic, propionic, and lactic acid). Furthermore, the acids used to dissolve chitosan may play significant role in the antibacterial activity of chitosan (No and others 2002). However, no literature has evaluated the use of different acids on the film forming and antibacterial properties of β-chitosan based films.

2.3.4 Solubility

Chitosan was not able to dissolve in neutral or high pH solvent system where its pKa value was reported in a range of 6.3-6.5 (Sorlier and others 2001). As a result, the utilization of chitosan was limited to its solubility in a narrow pH range, which is a huge obstacle for its applications.

2.4 Improvement of chitosan solubility

Several strategies were developed to improve the solubility of α-chitosan based on arrangement principles, which include homogeneous phase reaction, chitosan molecular weight reductions, and chemical modifications and details are discussed below.

2.4.1 Homogeneous phase reaction

A homogeneous phase reaction (Sannan and others 1976) which controlled the deacetylation process produced water-soluble α-chitosan, yet the production yield was not high (Kurita and others 1991). Moreover, the solubility of partially *N*-acetylated α-chitosans is drastically affected by slight dissimilitude in preparation conditions regardless of similar substitution degrees around 50%. Synthetic chemical reagents such as pyridine and acetic anhydride were used among all preparative methods.

2.4.2 Chitosan molecular weight reduction

Reducing chitosan molecular weight through physical, chemical and enzymatic methods could modify solubility. Physical methods involved the shear-force and ultrasonic variants which reduced the α-chitosan molecular weights down to 1.1×10^5 and 1.4×10^5(Chang 1996). Kurita and others (2002) reported that these physical methods are not difficult to perform, however, resulted chitosan solubility was in various and unstable due to the random reactions and fast degradation rates throughout the process.

Chemical method of acid hydrolysis which generally used acetic acid as a solvent can break down chitosan polymers formed by thousands of N-acetylgucosamines into a unit of six, as a result, readily dissolute at neutral pH (Hirano and others 1985). No and others indicated that almost all biological and/or chemical functionality was lost while the α-chitosan molecular weight was low as 28 kDa.

Enzymatic preparation of water soluble chitosan using chitosanase, lysozyme or papain had higher solubility than other methods (Ikeda and others 1993; Nordtveit and others 1996; Terbojevich and others 1996). Nevertheless, a relatively high cost of these

enzymes had impeded the commercialization of water-soluble α-chitosan products. As a result, commercial water soluble α-chitosan products were mostly chitosan chlorides or glutamates salt thatwhich were not prepared by enzymatic method (Weerakkody and others 2011).

2.4.3 Chemical modification

Chemical modification was the technology to improve α-chitosan solubility by attaching hydrophilic functional groups to the polymer chain (Holme and Perlin 1997). Recently, many α-chitosan derivatives had been developed by chemical modification process such as O-fumaryl-chitosan (Feng and Xia 2011), free-amine chitosan(Jang and others 2002), N-acetylation(Qin and others 2006; Feng and others 2007), trimethylated and triethylated 6-amino-6-deoxy chitosan(Sadeghi and others 2008), carboxymethylated chitosan(Liu and others 2001; Song and others 2011) and quaternized chitosan(Guo and others 2006). Although some of the water soluble α-chitosan mentioned above had increased antibacterial (Feng and Xia 2011; Liu and others 2001) or antioxidant properties (Feng and others 2007), the preparation of a complex solvent system is typically needed and therefore become difficult and unfavorable to control.

2.4.4 Millard reaction: a feasible achievement for food application

The Millard reaction is a chemical process that involves amino and carbonyl groups of various molecules and usually required heat (Jokic and others 2004). Since chitosan contained amino groups along the polymer chain, Maillard reaction was discovered as an alternative chemical modification method to produce high soluble chitosan by blending mono- or disaccharides into heated chitosan solution (Chung and others 2005b; Chung

and others 2006; Yang and others 2002). Maillard reaction was recognized as a mildness reaction with appropriate controllability without using any synthetic chemicals and ease operation comparing to other chemical modification process (Tessier and others 2003). The results demonstrated that Maillard reaction was a feasible method to generate water-soluble α-chitosan commercially. Interestingly, the water soluble chitosan derivative was indicated to have a higher antibacterial activity than native chitosan (Chung and others 2005b; Kosaraju and others 2010; Kanatt and others 2008).

2.5 Conclusion

β-chitosan derived from squid pen possess a much greater affinity to various solvent and higher reactivity with a easier sample preparation than α-chitosan from crustacean shell. Studies to determine its properties in various forms are necessary to understand and develop the potential applications. Limited information is available on β-chitosan (*Dosidicus gigas*) based film and the critical factors that impact its performance in terms of physicochemical and antibacterial properties simulaneously. Hence, a designed protocol is essential to evaluate the properties of β-chitosan based films and identify the important factors to optimize the formulation.

Moreover, Maillard reaction are recognized as an ease and novel method to improve the chitosan solubility and more suitable for food applications. However, only few studies had developed water soluble chitosan via Maillard reaction and determine the critical factors that alter its yield and solubility. It is worthy to discover the solubility and yield of water soluble chitosan as a guidance of developing the product in a commercial scale. Accordingly, this project also investigated the effects of reducing sugar and heat treatment on the solubility of β- and α- water soluble chitosan.

Table2.1 Sources of chitin and chitosan

Sea animals	Insects	Microorganisms
Annelida	Scorpions	Green algae
Mollusca	Spiders	Yeast (β-type)
Coelenterata	Brachiopods	Fungi (cell walls)[b]
Crustaceans:	Ants	Mycelia Penicillium[b]
Lobster[a]	Cockroaches	Brown algae
Crab[a]	Beetles	Spores
Shrimp[a]		Chytridiaceae
Prawan[a]		Ascomydes
Krill[b]		Blastocladiaceae
Squid[b]		

[a] Current (1990) sources

[b] Future potential sources when demand increases

Source: adapted from Mathur and Narang (1990).

Table 2.2 Contents of chitin and calcium carbonate

Source	Chitin (%)	CaCO3 (%)
Crab cuticle	15-30	40-50
Shrimp cuticle	30-40	20-30
Krill cuticle	20-30	20-25
Squid pen	20-40	Negligible
Clam/oyster shell	3-6	85-90
Insect cuticle	5-25	Negligible
Fungi cell wall	10-25	Negligible

Source: adapted from Kurita (2006).

Table 2.3 Estimated chitosan usage for the year of 2000 (tons)

Market	North America	Europe	Asia	Other	Total
Nutraceuticals (dietary supplements)	500	125	250	125	1000
Flocculation (water treatment)	125	25	200	50	400
Foods (preservation)	0	0	125	25	150
Oligosaccharides	0	0	150	0	150
Agriculture	25	0	75	25	125
Cosmetics	25	25	50	0	100
Textiles (hygienic)	0	0	50	0	50
Pulp and paper	25	0	25	0	50
Feed	10	0	25	10	45
Medical devices	1	1	1	0	3
Total	711	176	951	235	2073

Adapted from Kurita (2006).

Table2.4 Commercialized chitin/chitosan-based products worldwide

Application	Product	Manufacturer
Biodegradable suture	Chitin fiber	Yunichika Inc., Kyoto, Japan
Skin Care	Chitin liquid (CM-chitin)	Ichimarn Farukosu Inc., Gifu, Japan
Artificial skin	Chitosan-collagen composite	Katakurachikkarin Inc., Tokyo, Japan
Hair care	Depolymerized chitosan	Wella Inc, Germany
Personal care	Evalson R	Chito-Bios, Ancona, Italy
Personal care	TEECOMT chitosan toothpaste	Winning Medical Inc. Taichung, Taiwan
Burn therapy	Nonwoven chitin fabric	Yunichika Inc., Kyoto, Japan
Dietary foods	Noodles containing chitosan	NihonKayaku Inc, Tokyo, Japan
Wound healing	ChitoFlex® hemostatic dressing	HemCon Medical Technologies Inc., Oregon, USA
	ChitoGauze™ & GuardaCare™	
	3M™ Tegasorb™ hydrocolloid dressing	3M Inc., Minnesota, USA
Nutracetical product	Chitosan gold tea (tea bag)	Kitto Life, Seoul, Republic of Korea
	KL chitosan oligosaccharide 100 (capsule)	Kitto Life, Seoul, Republic of Korea
	ChitoClear® (food ingredient)	Primex, Siglufjordur, Iceland
	Chitosan HD™ (high density) (capsule)	Whole Health Products, Colorado, USA
	Chitosan Plus (capsule)	Doctor's Trust
	Chitosan Plus (capsule)	Universal Nutrition
	Diet chitosan (capsule)	Source Naturals
	FBlock (capsule)	Absolute Nutrition, Connecticut, USA
	Chitosan diet formula (capsule)	Optimum Nutrition, Inc, USA
	Chit-O-Power™	Chitopower

Source: From Muzzarelli and others (1986), Brine and others (1992), Sjak-Braek and others (1992), Nicol (1991), Illum(1998),Rasmussen and Morrissey(2007) and HemCon Medical Technologies Inc. (2012).

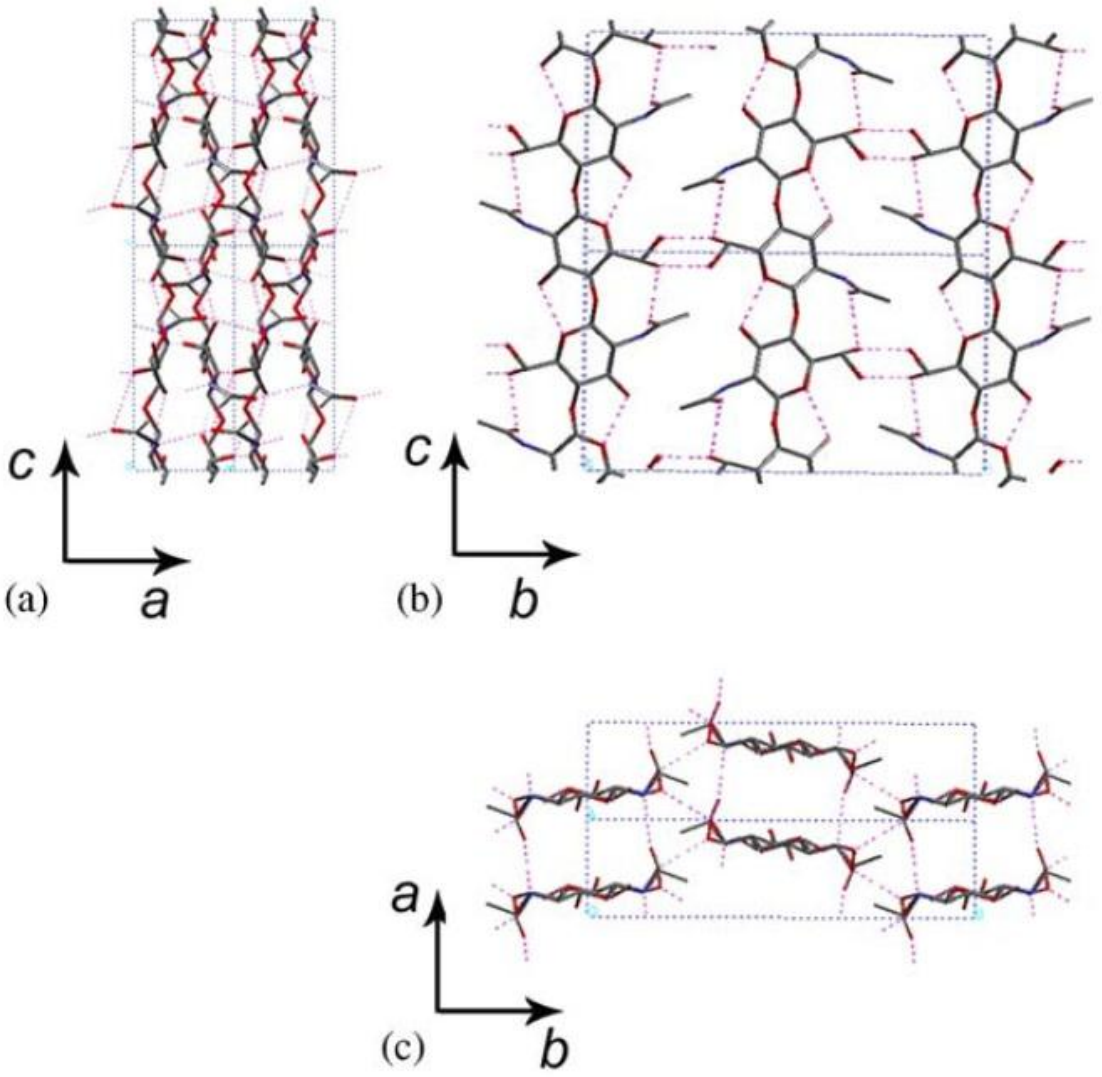

Figure 2.1 Structure of α-chitin (a) *ac* projection; (b) *bc* projection; (c) *ab* projection. The structure contains a statistical mixture of 2 conformations of the –CH_2OH groups. Source: adapted from Rinaudo (2006).

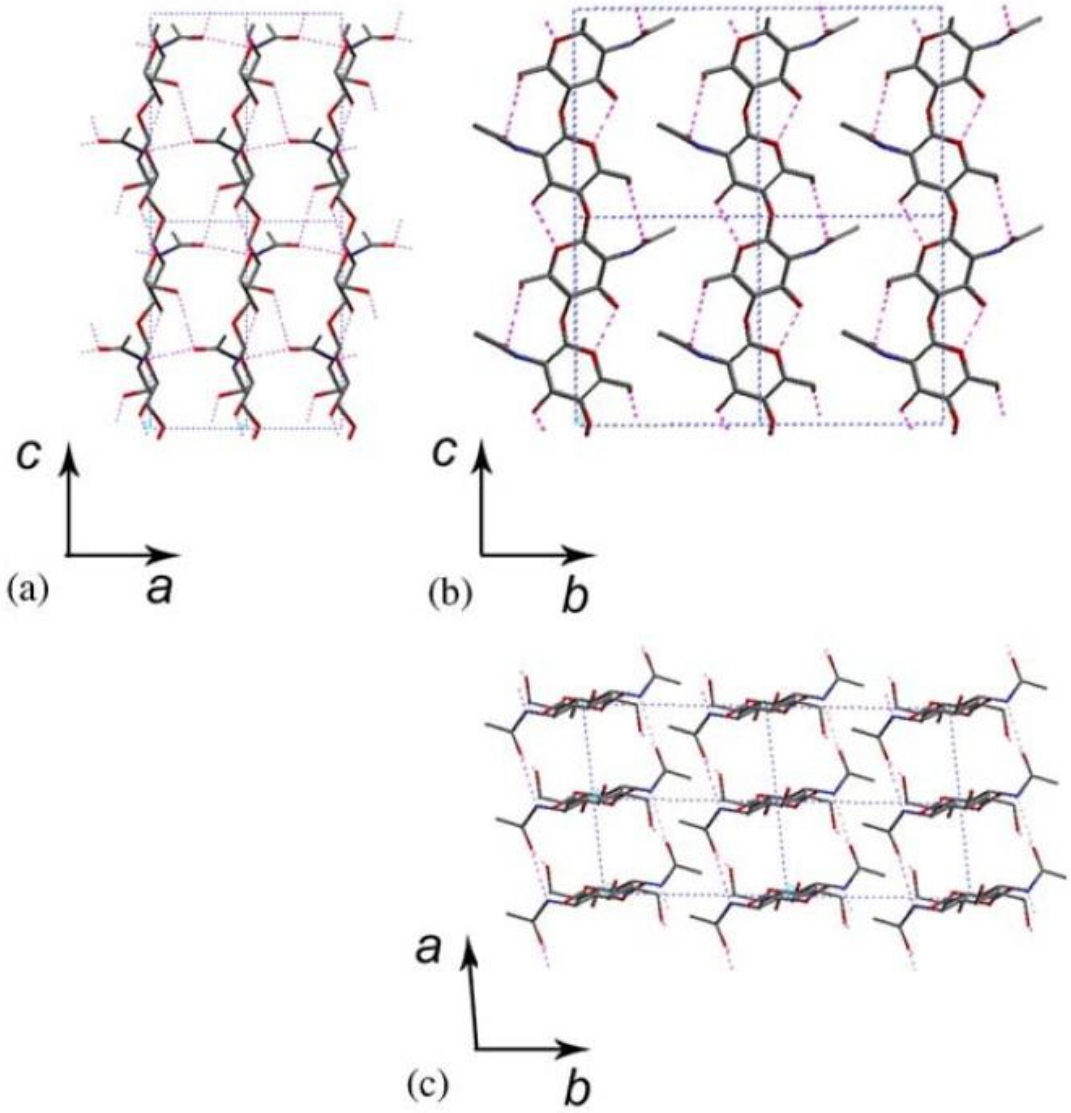

Figure 2.2 Structure of anhydrous β-chitin: (a) *ac* projection; (b) *bc* projection; (c) *ab* projection. Source: adapted from Rinaudo (2006).

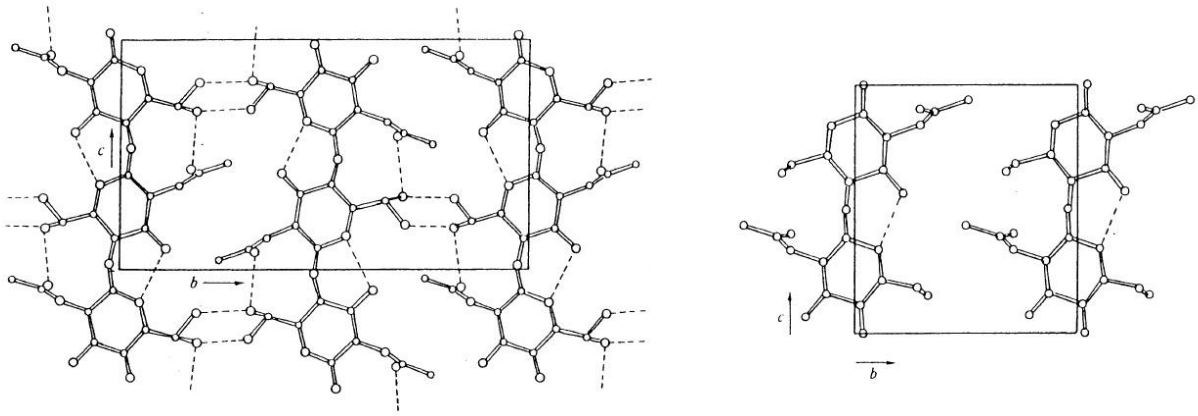

Figure 2.3 Arrangements of chitin molecules in α-chitin (left) and β-chitin (right) (adapted from Blackwell and others (1980))

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Chapter 3 : Effect of molecular weight, acid and plasticizer on the physicochemical and antibacterial properties of β-chitosan based films

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Abstract

Effects of chitosan molecular weight (1,815 and 366 kDa), type of acid (1% acetic, formic, and propionic acid, or 0.5% lactic acid) and plasticizer (0, 25% glycerol or sorbital w/w chitosan) on the mechanical, water barrier, and antibacterial properties of β-chitosan films were investigated.Tensile strength (TS) of high molecular weight (Hw) films was 53% higher than that of low molecular (Lw) ones, acetate and propionate films had the highest TS (43 and 40 MPa) among tested acids, and plasticizer reduced film TS 34%. Film elongation at break (EL) was higher in Hw films than in Lw ones, in which formate and acetate films were the highest (9 and 8%), and plasticizer increased the film EL 128%. Molecular weight of chitosan did not influence water vapor permeability (WVP) of the films. Acetate and propionate films had lower WVP than other acid types of films, and plasticizer increased film WVP about 35%. No difference was found between glycerol and sorbitol films in terms of film mechanical and water barrier properties. Lw β-chitosan films showed significant antibacterial activity against *E. coli* and *L. innocua.* This study demonstrated that β-chitosan films are compatible to α-chitosan films in physicochemical properties and antibacterial activity, yet with simple sample preparation.

Keywords: β-chitosan films, monocarboxylic acid, plasticizer, mechanical properties, water vapor permeability, antibacterial activity

Practical Application:

β-chitosan based edible films at molecular weight of about 300 kDa showed great antibacterial activity against Gram-positive and Gram-negative bacteria. The films have similar mechanical and water barrier properties to α-chitosan based films at the similar molecular weight, but simple sample preparation procedures and more attractive color. The release of active chitosan fragment from the film matrix acts as an antibacterial agent, making β-chitosan films suitable as intelligent food wraps or coatings for a wide range of food products to control moisture loss and prevent surface bacterial growth.

Introduction

Chitosan, a linear polysaccharide composing of β-1,4-D-glucosamine, has excellent film forming capacity due to the β-(1→4) linkages between the monomers consisting of the chain. This biodegradable film is usually acquired by evaporation of dilute acid mixture with the polymer (Demargerandre and Domard 1994). There are three forms of chitosan, α, β and γ based on their crystalline structure, in which α form has been mostly studied (Kurita 2001). Interest in β-chitin and chitosan has been increased due to greater affinity to various solvents and higher reactivity than α-chitosan (Kurita and others 1993).

The difference between α- and β-chitin and chitosan are generally based on the crystalline structure of the molecule.β-chitin chains are arranged in a parallel arrangement with relatively weak intermolecular forces and loose package (Gardner and Blackwell 1975), while the α-structure is aligned in an antiparallel fashion responsible for a stronger intermolecular hydrogen bonding (Blackwell and others 1980; Jang and others 2004). Consequently, the deacetylation process of β-chitin can be operated under mild conditions using alkali solution at relatively low temperature (Kurita and others 1993; Tolaimate and others 2000). Moreover, squid pens have low mineral content, eliminated the demineralization step in the preparation of β-chitosan (Tolaimate and others 2003; Lamarque and others 2004).

We have previously evaluated the deacetylation and depolymerization characteristics of β-chitin from jumbo squid (*Dosidicus gigas*) pens and identified the optimal treatment conditions to obtain β-chitosan with high degree of deacetylation (DDA) and a wide range of molecular weight (Mw) for various potential applications (Jung and Zhao 2011). This study aimed to develop edible films from β-chitosan derived from jumbo squid pens

and evaluated their functionalities. To our best knowledge, no study has investigated the effect of acid type and plasticizer on β-chitosan films.

Chitosan is insoluble in most organic solvents, but readily soluble in dilute aqueous acids at pH below 6.0 (Pillai and others 2009) because of the presence of free amino groups (Kurita 2006). The primary amino groups of chitosan are regarded as a strong base with a pKa value of 6.3 (Park and others 1983). At low pH, amines on each D-glucosamine residue are protonated and become positively charged which driven chitosan as a cationic water-soluble polyelectrolyte. Some monocarboxylic (formic, acetic, propionic, and butyric) and L- or D-lactic acid are frequently used to prepare chitosan solution (Kurita 2006). In addition, a type II chitosan acid complex which possessed a relaxed 2-fold helix conformation (hydrated crystal) can be formed by applying monocarboxylic and L- or D-lactic acids as solvents (Kawada and others 2001). However, no literature has evaluated the use of different acids on the film forming properties of β-chitosan based films.

Plasticizers are usually necessary in the edible film formulations by increasing film pliability and flexibility (Garcia and others 2000) through interfering with the hydrogen bonding between chitosan polymers. Water, polyols, lipids, and oligosaccharides are the types of plasticizers widely used in polysaccharide film. However, polyols were indicated to be the most effective plasticizers for hydrocolloid-based films since its structure is similar to the polymer matrix (Sothornvit and Krochta 2005). Since the linear chain of β-chitosan polymer is same as α-chitosan, but aligned in a different arrangement, the plasticized effect of adding polyols are expected to perform in a similar manner when

chitosan dissolves completely into the solvent. For this reason, glycerol and sorbitol which both contain multiple hydroxyl groups were chosen as plasticizers in this study.

The antibacterial activity of chitosan is one of the most unique properties compared to other food hydrocolloids used in making edible films (Dutta and others 2009; Campos and others 2011). Chitosan has been demonstrated its capability against fungi, both Gram-positive and Gram-negative bacteria due to its polycationic nature (No and others 2002; Ganan and others 2009; Kong and others 2010). However, no literature has investigated the antibacterial performance of β-chitosan films, although a few studies evaluated the antibacterial activity of β-chitosan in solutions (Shimojoh and others 1996; Lin and others 2009). Moreover, up to date, the β-chitosan film studies were limited to the squid species of *Nototodarus sloani,Todarodes pacificus* and *Illex argentinus*(Shepherd and others 1997; Chung and others 2005; Santos and others 2006), no β-chitosan film developed from jumbo squid (*Dosidicus gigas*) pens have been investigated. As it is well known that the source of chitosan material, Mw, and solvent type are critical factors impacting the film performance (Kong and others 2010), it motivated this study to investigate the basic film functionalities and antibacterial property of β-chitosan films prepared by different Mw of chitosan, different acid solvents and plasticizers.

Materials and Methods

Materials

β-chitin derived from jumbo squid (*Dosidicus gigas*)was provided by Dosidicus LLC (Lacey, WA). Chitin was deacetylated and depolymerized following the procedures developed in our lab to obtain β-chitosan with high Mw of 1,815 kDa (Hw) and low Mw of 366 kDa (Lw) and DDA >90% (Jung and Zhao 2011). Commercial α-chitosan with 300 kDa and 88% DDA was purchased from Primax ehf (Siglufjordur, Iceland).Acetic acid was purchased from Fisher Scientific (Fair Lawn, NJ), and formic acid, propionic acid, DL-lactic acid, sodium hydroxide, acetone, and methyl alcohol were all from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Glycerol was acquired from IBI Scientific (Peosta, IA) and sorbitol from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Hydrochloric acid and sodium chloride were from EMD Chemicals Inc. (Gibbstown, JN), and toluidine blue indicator solution and potassium polyvinyl sulfate titration solution (N/400) were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

Preparation of film forming solutions (FFSs) and their physicochemical properties

Chitosan FFSs were prepared by dissolving 1% chitosan in four different carboxylic acids solution, 1% formic, acetic, and propionic acid, or 0.5% DL-lactic acid with or without the addition of 25% plasticizer (glycerol or sorbitol) (w/w chitosan) in the mixture. The FFS formulations were decided based on our preliminary studies of using the least amount of materials while providing desirable film functionality. All FFSs were stirring for 24 h at ambient temperature (25±2 ℃) and filtered through nylon cloth under vacuum to remove insoluble residues.

The pH of the FFSs was measured using a pH meter (Model 125, Corning Science, Medfield, MA), total soluble solids (TTS) content was determined using a Brix meter

(Check-Brix, RA-250HE, Kyoto Electronics Manufacturing Co., Ltd., Kyoto, Japan), and the viscosity was measured using a Brookfield digital rheometer(DV-III+, Brookfield Engineering Laboratories, Inc., Middleboro, MA) with RV2 spindle at shear rate (0.5 sec^{-1}). All measurements were done at room temperature (25±2 ℃).

Film preparation

About 96.4±0.3 g of FFS was cast on the leveled Teflon-coated glass plate (170 × 170 mm) and dried under ambient temperature (25±2 ℃) for 48 h. Dried films were pulled out and cut into 70×70 mm^2 pieces for determining water vapor permeability and 25×86 mm^2 for evaluating mechanical properties. Film pieces were conditioned in a self-assembled chamber at 25 ℃ and 50% RH for 48 h before the measurements.

Physicochemical, mechanical, water barrier and microstructure properties of films

A caliper micrometer (No. 293-776-30, Mytutoyo Manufactureing C. Ltd., Japan) was used to measure film thickness on samples used for mechanical property and water vapor permeability. Five measurements were conducted at each film specimen randomly at different locations and the mean values were recorded. Film density was calculated by dividing film weight by film volume, in which the film volume was obtained by multiplying film thickness by film area. Moisture content of the films was determined by the percentage weight loss of film samples after drying in a forced-air oven at 100 ℃ for 24 h. All the measurements were done with three replications of two film specimens for each replication.

WVP was determined according to ASTM E96 (ASTM 2000) using a cup method at 25 ℃ and 100%/50% RH gradient (Park and Zhao 2004). WVP correction method (Gennadios and others 1994) was used to adjust for resistance of the stagnant air gap between the surface of water and the film. Three replications of two film specimens from each type of sample were applied for WVP measurement.

Tensile strength (TS) and percent elongation at the break (EL) of the films were determined according to ASTM D882 (ASTM 2001) and analyzed using a texture analyzer (TA.XT2i, Texture Technologies Corp., Scarsdale, NY) by following the same procedures as Park and Zhao (2004). Three replications of five film specimens from each type of film sample were tested.

Microstructure of the films was evaluated using a Nikon Eclipse E400 microscope (Nikon Co., Tokyo, Japan) equipped with an extended digital camera (Q imaging, Surrey, BC, Canada) (Deng and Zhao 2011). The surfaces of films were observed at a magnification of 40X.

Antibacterial activity of films

The antibacterial activity of β-chitosan based films against Gram-negative (*E. coli* ATCC 25922) and Gram-positive (*L. innocua* ATCC 51742) bacteria were investigated using a modified method from Duan and others (2008). A 0.03 g of β-chitosan film strips which has a film surface area of ~5 to 8 cm^2 with relatively uniform thickness (Table 3.4) was placed inside a clear glass bottle (60 mL/56.70 g), in which 12 mL of bacterial culture was added with an initial microbial concentration of approximately 10^5 CFU/mL.

Bacterial culture without film sample was considered as a control. All glass bottles were placed under room temperature and shaken at 100 rpm (Lab-Line Orbit Environ-shaker 18 No. 3527, Lab-Line Instruments, Inc., Melrose Park, IL). All films were completely immersed in the inoculum and fluctuating while shaking. Bacterial cultures (1 mL) with or without tested film sample were consecutively taken out from the same glass tube at 0, 4, 8, and 24 h and diluted with phosphate-buffered saline for the enumeration of *E. coli* and *L. innocua* that were plated on TSA and BHI agar, respectively, and incubated at 37 ℃for 24 h before counting colonies. Duplicate test of each film formulation was conducted for each bacterial strain.

Experimental design and statistical analysis

A complete randomized three-way factorial design with 24 combinations of chitosan FFSs was applied.Treatment factors included 2 chitosan molecular weight (1815 and 366 kDa), 4 acids (1% FA, 1% AA, 1% PA, or 0.5% LA), and 2 plasticizers (25% glycerol or sorbitol) with a control (without plasticizer).Three replications were performed for each experiment.The general linear models (GLM) procedure was performed to differentiate the differences among different films using SAS statistical software 9.02 (SAS Inc., Cary, N.C., U.S.A.).All results were reported as mean ± standard deviation.Tukey-Kramer multi-comparison test in analysis of variance (ANOVA) ($P<0.05$) was used to compare multiple means.

Results and Discussions

Physicochemical properties of film forming solutions (FFSs)

Mw of β-chitosan and acid type both affected the pH values of FFSs ($P < 0.0001$), and there was an interaction ($P < 0.0001$) between these two factors (Table 3.1). Hw β-chitosan FFSs had lower pH values than those of Lw β-chitosan FFSs regardless of acid and plasticizer used (Table 3.3). The pH values of the different acid types of FFSs were different ($P < 0.0001$) (Table 3.3), followed the order of formic acid (2.90) < acetic acid (4.11) < propionic acid (4.43) < lactic acid (4.80). Park and others (2002) also observed the pH of 2% chitosan FFSs in citric, lactic, malic and acetic acid followed the same order. Plasticizers (0, 25% glycerol or 25% sorbitol) had no significant effect on the pH of FFSs, probably due to the neutral pH of the plasticizers.

TSS of FFSs was affected by the type of acid and plasticizer ($P < 0.0001$) (Table 3.1). TSS of 1% propionic acid FFS was the highest (2.0%), and that of 0.5% lactic acid FFS was the lowest (1.8 %) (Table 3.3). Propionic acid has higher Mw than other tested acids, thus given a higher TSS value than other two monocarboxylic acids FFSs. In addition, more solids might dissolve in the solution since the anhydrous crystal of propionic acid salt has the lowest crystallinity (71%) among all tested acid salts (Kawada and others 2001). Overall, Mw of chitosan had no effect ($P > 0.05$) on TSS of FFSs expect the group prepared by propionic acid, in which an interaction ($P < 0.05$) between Mw and acid type existed.

Viscosity of FFSs was affected by Mw, acid type, and plasticizer, and there was an interaction between Mw and acid type ($P < 0.05$) (Table 3.1). Mean viscosity of Hw β-chitosan FFS was higher (2126 MPa•s) ($P < 0.05$) than that of Lw β-chitosan FFS (18 MPa•s). The interaction between Mw and acid type indicated that within the two Mw groups, viscosity responded differently to different acids. For Hw β-chitosan FFS, acetic

acid FFS had the lowest viscosity of 1970 MPa•s, but there was no difference among other acid types with a range of 2126 to 2222 MPa•s. Lw β-chitosan FFSs prepared by lactic acid had higher viscosity (20 MPa•s) than other acids FFSs, again no difference among other acid FFSs. For FFSs without plasticizer, viscosity of acetic acid FFS remained the lowest (1003 MPa•s), and no difference among other acid types of FFSs (1113 - 1160 MPa•s). As a result of the interaction between Mw and plasticizer, sorbitol was the only plasticizer that lowered ($P < 0.05$) the viscosity of Hw β-chitosan FFS (2061 MPa•s) comparing to the FFSs without plasticizer (2191 MPa•s), where the viscosity of FFSs with glycerol (2128 MPa•s) had no difference from the control.

Physicochemical properties of films

Overall, film thickness was influenced by Mw, acid, and plasticizer ($P < 0.0001$), and there was an interaction ($P<0.0001$) between Mw and acid (Table 3.2). Hw β-chitosan film with lactic acid and plasticizer was the thickest (0.048 mm) among all tested films (Table 3.3). This result may be explained as increasing the hygroscopic nature (i.e. lactic acid and plasticizer) of the film increased its thickness especially with polymer matrix that provides strong entanglement interactions (Suyatma and others 2005). Suyatma and others (2005) suggested that samples with higher hydrophilicity attribute to the plasticizer hygroscopocity. Lactic acid films were thicker than other acid films (0.038 mm), but no difference among other acid types of films, again probably because the hydroscopic nature of lactic acid bonded more water molecular within the film. Formic acid film was the thickest film among Lw β-chitosan films ($P <0.05$). Note that Lw β-chitosan formic acid films was no longer transparent as other films, and the surface of the film was rough

as reflected in the film microstructure (Fig. 3.1). This may indicate that larger crystalline chitosan acid salts are formed, thus increased the film thickness. Overall, films prepared with sorbitol were about 21% thicker than those without plasticizer. However, the thickness of films with monocarboxylic acid did not increase significantly by adding plasticizer expect the group of Hw acetate film (Table 3.3).Similar trend was observed by Martinez-Camacho and others (2010)that adding 20% sorbitol (w/w chitosan) into Lw α-chitosan (480 kDa) acetate film increase the film thickness from 40 μm to 60 μm. They concluded that increased film thickness may be caused by the compact chitosan chains during film formation process. In contrast, adding glycerol only increased film thickness in those prepared by Hw β-chitosan and lactic acid as both factors may tightly trap water molecules.

MC of the films was also affected by Mw, acid type, and plasticizer, and there was interaction among each of these factors ($P < 0.05$) (Table 3.2). Lw β-chitosan films prepared by formic, acetic, and propionic acid had 10 to 43% higher MC than those of Hw β-chitosan films ($P < 0.001$). In contrast, Lw β-chitosan film prepared by lactic acid had lower MC than that of Hw β-chitosan films ($P < 0.05$). Films prepared by formic and acetic acid had higher MC than those using propionic and lactic acid. Park and Zhao (2004) showed similar MC of α-chitosan film, in which MC of films with glycerol and sorbitol were 22% and 20%, respectively, while those without plasticizer were17% because plasticizer helped hold water within the chitosan film matrix.

Film density was affected by acid ($P < 0.0001$) and plasticizer ($P<0.05$), and again there was an interaction between Mw and acid (Table 3.2). Overall, lactic, acetic and propionic acid films had higher density than formic acid film. Siripatrawan and Harte

(2010) suggested that density of chitosan film increased as increase of intermolecular interactions, such as hydrogen or covalent bonding within the linear chitosan structure. The higher amount of hydroxyl groups in lactic acid may form greater intermolecular forces, given a higher film density. Similar film density was reported in α-chitosan lactate film with 25% glycerol and α-chitosan acetate film with 30% glycerol (Park and Zhao 2004; Siripatrawan and Harte 2010). The result may indicate that α- and β-chitosan molecules exist similar interactions between anions and polyols in the film matrix to give an alike film density. Hw β-chitosan film with formic acid had ~33% higher film density ($P < 0.0001$) than that of Lw β-chitosan film, while Hw β-chitosan films with acetic and propionic acid had 6-8% lower film density than Hwβ-chitosan formate film ($P < 0.05$). Adding sorbitol increased film density (1.32 g/cm^3) 6% comparing to those without the plasticizer (1.25 g/cm^3)($P < 0.05$), while adding glycerol did not change ($P > 0.05$) film density.

Mechanical properties of the films

Tensile strength (TS)

TS of β-chitosan films were affected by Mw, acid type, and plasticizer ($P < 0.0001$) and there were interactions ($P < 0.001$) between each individual factor. Hw β-chitosan films had an overall TS of 44 MPa, 53% higher than that of Lw β-chitosan films (29 MPa) across all acid types used (Table 3.2). This similar trend was observed in α-chitosan films prepared with different acids (Nunthanid and others 2001; Park and others 2002; Hwang and others 2003), which may be explained as increased chitosan Mw lead toentanglement

network formation during film forming process, thus increased the mechanical strength of the films (Nunthanid and others 2001).

The mean TS of β-chitosan acetate and propionate films (43 and 39 MPa) were higher ($P < 0.05$) than that of β-chitosan formate and lactate films (34 and 29 MPa) (Table 3.5). It was observed that Lw chitosan formate film without plasticizer does not have good film integrity, probably due to the low molecular weight of chitosan and formate. Therefore, it may not have enough entanglement force to form good film matrix. These results were consistent with the study by Caner and others (1998), in which 3% α-chitosan film prepared by 1% formic, acetic, or lactic acid had the lowest TS value. Kim and others (2006) also suggested that chitosan acetate and propionate films have higher TS than that of formate and lactate films. This may be explained as the hydroxyl group in lactic acid instead of hydrogen in monocarboxylic acids may induce electrolyte instability in the solution. Begin and Van Calsteren (1999) stated that α-chitosan lactate films lost its strength due to the large counter ion of lactic acid. Moreover, Park and others (2000) used a light scattering method to demonstrate the relationship between organic acid solvent and α-chitosan film properties in terms of Mw and molecular dimension of chitosan. Their results showed that chitosan dissolved in acetic acid has a larger Mw than that dissociated in citric, lactic, and malic acid solutions, and suggested that chitosan dimer formed in acetic acid solution has a relatively strong intermolecular interaction to give a tighter film structure.

Furthermore, Demargerandre and Domard (1994) demonstrated that α-chitosan formate film behaves differently from films prepared by other carboxylic acid. They suggested that formic acid yields amides with amines upon dehydration and does not

form anhydride during drying process. Kawada and others (2001) also indicated the difference of α-chitosan formic acid salt from α-chitosan acetic, propionic, and L-lactic acid salts through their crystalline behavior. The anhydrous crystal of formic acid salt has the highest crystallinity of 82%, while that of acetic acid and propionic acid salts was 74% and 71%, respectively. The different crystalline behavior of α-chitosan acid salts may help explain the differences observed in TS of β-chitosan films in this work, where chitosan formate film was more brittle than those produced by acetic and propionic acids due to the potential higher crystalline behavior; no difference in TS between chitosan acetate and propionate films owning to their similar crytallinity; and chitosan lactate film may produce a less tight structure since no crystal acid salt was formed during the dehydrate process.

Films incorporated with plasticizer had lower TS than those without plasticizer (Table 3.5). The mechanism of glycerol on chitosan films has been clearly demonstrated by Domjan and others (2009) using solid-state NMR spectroscopy. They indicated that solution-cast partial deacetylated chitosan film exists with an amorphous structure, and a significant amount of carbonyl groups does not participate in the formation of hydrogen bonds. Domjan and others (2009) believed that glycerol acts as an internal plasticizer in this matter, while external plasticizer, such as polyethylene glycol (PEG) creates only weak second-order bonds and could migrate in the polymer causing recrystallization of the material and loss of elasticity. Films with sorbitol were expected to give a lower TS comparing to those containing glycerol since sorbitol exists more hydroxyl groups in its structure to provide a higher plasticize effect. However, no difference was observed in TS of films prepared with sorbitol or glycerol in this study, probably because sorbitol also

acts as an internal plasticizer that has the similar mechanism as glycerol due to its linear carbon structure with hydroxyl groups along the polymer chain.

Interactive effects existed within different treatment factors. Adding plasticizer into Hw β-chitosan films with lactic acid lowered ($P < 0.05$) the mean TS value. However, no decrease was detected within the Lw β-chitosan films. Moreover, there was no difference in TS of Hw and Lw β-chitosan films prepared by lactic acid and glycerol.

Elongation (EL)

EL of β-chitosan films were influenced by Mw, acid and plasticizer, and there was an interaction between Mw and acid ($P < 0.05$) (Table 3.2). Mean EL of Hw β-chitosan films was 10% versus ~4% in Lw β-chitosan films. Previous studies in α-chitosan films also demonstrated increased EL with increased chitosan Mw (Hwang and others 2003; Chen and Hwa 1996). This study further confirmed that the entanglement network of Hw chitosan plays an important role in modifying mechanical properties of the films, in which if only hydrogen bonds are the main attribution to resist external force, an inverse relationship between TS and EL of polysaccharide films should be observed.

Formate and acetate films had higher EL than that of propionate film, but no difference in lactate film from other films (Table 3.5). In general, an inverse relationship between TS and EL was observed for all acid types of films except the acetate film that remained the highest value in both TS and EL. The α-chitosan lactate films were reported to be the most resilient among all other acid types used (Caner and others 1998; Park and

others 2002; Kim and others 2006). Kim and others (2006) reported that EL of α-chitosan lactate films are the highest (252%) followed by propionate (78%), acetate (59%), and formate (41 %) films. In our preliminary works, β-chitosan lactate films produced with 1% lactic acid solution was observed to be too moist, thus 0.5% lactic acid solution was used to give a similar film texture to other films. As a result, lactate film did not show a significant higher EL value among other acid types. The interactive effect between Mw and plasticizer indicated that adding plasticizer to lactate films increase ($P < 0.05$) film EL for Hw β-chitosan films from 4% up to 17%. However,lactate film with Lw chitosan and plasticizer did not affect ($P > 0.05$) film EL.Glycerol and sorbitol increased ($P<0.0001$) EL 151% and 106% compared with the control, respectively, but no difference in EL of films prepared by glycerol (9%) or sorbitol (8%)(Table 3.5).Interestingly, incorporating plasticizer in β-chitosan formic and acetic acid films did not change ($P > 0.05$) film EL. As mention above, the higher potential crystallization of chitosan formic and acetic acid salt might form a tighter film structure than other acid type, thus limiting the development of new hydrogen bonds in the matrix.

According to Rinaudo (2006), the different semi-crystalline structures of α- and β-chitosan were divinable between each other and its native form as demonstrated by X-ray and electron diffraction. To our knowledge, no study has illustrated the crystallographic of any types of β-chitosan organic acid salts. It is well known that no inter-sheet hydrogen bond is formed in β-chitin crystalline structure, and the sheet itself was tightly bound with number of intra-sheet hydrogen bonds. As a result, a unique semi-crystalline pattern for β-chitosan acid salt is expected to behave distinctly in film mechanical properties mainly due to enhanced reactivity of β-chitosan toward solvent than α-form

under similar DDA. However, results in this study showed similar mechanical properties of β-chitosan films to α-chitosan films from previous works (Caner and others 1998; Park and others 2002; Kim and others 2006). Moreover, researches had demonstrated that β-chitin is able to convert to α-chitin irreversibly through various chemical treatments (Hackman and Goldberg 1965; Saito and others 1997; Noishiki and others 2003), which suggested that the sugar residues consisted of the polymer backbone may be the same in both. Hence, the difference in mechanical properties of high molecular weight chitosan films might not be observed between α- and β-form due to more entanglement involved, but α- and β-chitosan films may behave distinctly at low molecular weight whereas more intermolecular forces such as hydrogen bonding and van der Waals forces help maintain the film integrity.

Water vapor permeability (WVP) of the films

WVP of the films were affected by acid and plasticizer, and there was an interaction between these 2 factors ($P < 0.001$) (Table 3.2). No difference ($P > 0.05$) in WVP values were detected in β-chitosan films prepared with different Mw.Previous studies also suggested that increasing α-chitosan Mw does not promote WVP of chitosan films (Park and others 1999; Hwang and others 2003).A similar film density between Hw and Lw β-chitosan films (1.29 and 1.27 g/cm^3) may attribute to a resemble open space within the film structure for water molecules to pass through.

Formate films had higher WVP than other acid films (Table 3.5). Although WVP values of β-chitosan films in this study were not directly comparable to α-chitosan film

from other studies (Rhim and others 1998; Kim and others 2006) owing to different DDA and pH of film forming solutions, same trend of WVP to the acid type was observed. Ritthidej and others (2002) received the same order of WVP in α-chitosan films prepared by the same types of acid used in this study except chitosan lactate films which were the highest in WVP. Interestingly, WVP values of different acid salt films followed the same order as the crystallinity of chitosan acid salts among monocarboxylic acid (Demarger-Andre and Domard 1994).

Overall, adding plasticizer increased (11-31%) WVP of propionate films except the Lw β-chitosan propionate film with sorbitol. However, plasticizer did not affect ($P > 0.05$) WVP of formic acid films. These results may be explained that plasticizer were able to enhance segmental mobility and had high affinity to water.

Film microstructure

Surface microstructure of the films without plasticizer under the 40x magnification was observed (Fig. 3.1). All film surfaces were compact with homogeneous appearance and continuous structures expect the Lwβ-chitosan formate film (E). Lw formate film was not transparent with rough surface and white film fragment, and did not show good film integrity. The micrographs of Hw formate (A) and Lw acetate (F) films showed the pattern of marbling with uniform distribution. Cross-linkage was observed in Hw acetate (B), Hw propionate (C), and α-acetate films (G), as demonstrated high TS in Hw acetate and propionate films. The micrograph of Hw lactate (D) films showed smooth surface without any structure pattern, corresponded to the fact that chitosan lactic acid salt was not found to possess crystalline behavior (Kawada and others 2001). In the future studies,

X-ray diffraction and FTIR spectroscope should be studied to provide additional information on the film microstructures.

Antibacterial activity of the films

The growth of *E. coli* was not inhibited by Hw β-chitosan films (Fig. 3.2).In contrast, all Lw β-chitosan and α-chitosan films delayed ($P < 0.05$) the proliferation of *E. coli*, where lactate films showed the strongest antibacterial property. Since propionic acid chitosan was found less effective in inhibiting bacterial growth than formic, acetic and lactic acid (No and others 2002), propionate films were not evaluated in this part of the study. *E. coli* culture solution containing α-chitosan acetate and Lw β-chitosan lactate films reached a 5-log reduction at 8 h, while Lw β-chitosan formate and acetate films showed the strongest antibacterial capability at 4 h, a 2-log reduction.The growth of *L.innocua* at 24 h was completely ($P < 0.05$) inhibited by chitosan films except Hw β-chitosan acetate film (Fig. 3.2), where a 4-log reduction was observed at 24 h.All films except Hw β-chitosan acetate film showed antibacterial effect ($P < 0.05$) against *L.innocua* at 8 h.At 4 h, only Lw β-chitosan films showed significant inhibition against *L.innocua* compared with the control.Nevertheless, α-chitosan acetate film did not significantly inhibit the growth of *L.innocua* at 4 h. Overall, Lw β-chitosan film showed good antibacterial effect against both *E. coli* and *L. innocua* and no difference ($P > 0.05$) in the antibacterial activity was observed between α- and Lw β-chitosan acetate film at all times.

Few reports havecompared theantibacterial activity of β-chitosan with α-chitosan. Shimojoh and others (1996) tested 10 to 426 kDa β-chitosan at 99% DDAagainst various oral *Streptococcus*, and observed that 220 kDa chitosan inhibits the growth of *S.*

*mutans*GS 5 even in a short incubation time of 15 s, thus suggested that increasing DDA of β-chitosan from 54% to 99% enhance the antibactericidal effect. Lin and others (2009) reported that by degrading β-chitosan from 120.4 kDa to 26.5 kDa at 90% DDA increases antibacterial efficacy against various Gram-positive and Gram-negative bacteria. Huang and others (2011) examined the effect of β-chitosan against *E. coli* and *S. aureus* at Mw of 11.93 to 20.82×10^5 and DDA of 71 to 92%, and indicated that the antibacterial capacity increased with decreasing Mw and increasing DDA. In this study, films prepared with Lw (~300 kDa) α- or β-chitosan showed a greater inhibition against both *E. coli* and *L.innocua*, agreed with the previous findings.

In addition, acids used to dissolve chitosan may play significant role in the bacterial activity of chitosan (No and others 2002). As mentioned previously, crystallization degree of various chitosan acid salts followed the order of formate (82%), acetate (74%) and propionate (71%) salt, while no crystal formation in lactate salt. Interestingly, the least antibacterial effect of Lw chitosan film against *E. coli* also followed this order. Fernandez-Saiz and others (2009) proved that the release of protonate glucosamine from the chitosan film into the bacteria culture solution are the main attribute to inhibit the bacteria growth due to the amine groups protonated. They demonstrated that the insoluble part of the acetate film without migrated chitosan fractions does not show any antibacterial activity. Since chitosan formate films may crystalize to form film matrix in a higher degree, less mobilize and active chitosan fragments were able to release from the film to interact with the cell surface and act as an antibacterial agent. On the other hand, chitosan lactate film might be able to release not only high amount of protonated glucosamine to inhibit the bacteria growth, but also deliver lactic acid progressively. The

changes in the integrity of chitosan-based film associated with the release of the functional substances from the film should be considered in the future studies to assist the interpretation of the antibacterial mechanism of chitosan-based films.

The deacetylation process of α- and β-chitin was proposed to give slightly different amorphous chitosan products under the same treatment condition (Lamarque and others 2004). For α-chitosan, the crystalline and amorphous domains of α-chitin which were insoluble and hydrated, respectively, were transformed to partially soluble Hw crystalline domain and highly deacetylated Lw soluble fraction after the deacetylation. For β-chitosan, the crystalline and amorphous domain of β-chitin was converted to totally Hw amorphous insoluble and Lw soluble fraction, respectively. As mention above, α- and β-chitosan may possess the same polymer backbone due to the fact that β-chitin could readily convert to α-chitin irreversibly. Moreover, based on the similar antibacterial activity of α- and β-chitosan, we believed that the soluble fraction of α- and β-chitosan may be function the same depending on the Mw and DDA, whereas the insoluble fractions of both chitosan might act differently. In addition, increasing deactylation time would provide more acid-soluble fractions and less insoluble parts of chitosan. Hence, regardless of which form, chitosan at similar Mw range with longer deacetylation period is expected to react similarly as demonstrated in this study.

Conclusions

Mw of β-chitosan, acid and plasticizer are critical factors affecting the physicochemical, mechanical, water barrier, and antibacterial properties of β-chitosan based films. As chitosan Mw increased, the entanglement network formation during film forming process may play the major role in increasing viscosity of FFSs and thickness

and mechanical strength of the films. Overall, β-chitosan acetate films possessed the most ideal film properties with high density, TS and EL, but lower WVP. Films incorporated with glycerol or sorbitol were not significantly different in terms of TS, EL, and WVP, probably because sorbitol may also act as an internal plasticizer with similar mechanism as glycerol owing to its linear carbon structure with hydroxyl groups along the polymer chain. Lw β-chitosan films significantly inhibited the growth of *E. coli* and *L. Innocua*, in which *L. Innocua* was more sensitive than *E. coli*. The β- and α-chitosan acetate film showed similar antibacterial activity at Mw of ~300 kDa.Lw β-chitosan films (~300 kDa) may be used as an intelligent package by release of active chitosan segment from the film matrix to food surface for controlling bacterial growth. It may be employed as antibacterial food wrap or coating that directly contact with the food surface. For the future studies, applications of β-chitosan wrap or coating on various food products should be evaluated. In addition, the anti-fungi property of β-chitosan films should be studied in comparison with α-chitosan based films.

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Table 3.1- Analysis of variance (ANOVA) tables (P = 0.05) for statistical significance and interactions among different treatment factors for β-chitosan film forming solutions.

Source of variation	df[a]	*F*-value		
		pH	TSS [b]	Viscosity
Linear terms				
Molecular Weight (M)	1	165.96*** [c]	0.03	12031.1***
Acid Type (A)	3	3264.16***	49.00***	8.50***
Plasticizer (P)	2	0.08	67.39***	3.81*
Interaction terms				
M × A	3	15.07***	2.94*	8.38***
M × P	2	2.98	0.12	3.87*
A × P	6	0.44	0.36	0.73
M × A × P	6	0.99	0.97	0.73

[a] df = degree of freedom.

[b] TSS = total soluble solids.

[c] The *F* value followed by *** represents the corresponding *P* value <0.001, by ** means *P*<0.01, and by * means *P* <0.05.

Table 3.2- Analysis of variance (ANOVA) tables (P=0.05) for statistical significance and interactions among different treatment factors for β-chitosan based films.

Source of variation	df [a]	*F*-value					
		Thickness	MC[b]	Density	TS[c]	EL [d]	WVP [e]
Linear terms							
Molecular Weight (M)	1	18.37*** [f]	15.99***	1.26	163.04***	68.16***	0.49
Acid Type (A)	3	14.41***	49.49***	10.46***	31.05***	3.19*	14.75***
Plasticizer (P)	2	40.22***	47.03***	3.44*	45.63***	16.94***	16.82***
Interaction terms							
M × A	3	9.62***	18.36***	19.79***	12.62***	2.82*	7.42***
M × P	2	2.37	1.15	1.03	15.50***	2.32	0.59
A × P	6	1.57	2.80*	1.04	3.28**	1.25	1.15

[a] df = degree of freedom.

[b] MC = moisture content.

[c] TS = tensile strength.

[d] EL = elongation at break.

[e] WVP = water vapor permeability.

[f] The *F* value followed by *** represents the corresponding *P*-value <0.001, by ** means *P*<0.01, and by * means *P* <0.05.

Table 3.3-pH, total soluble solids content, and viscosity of β-chitosan based film forming solutions. [a]

	pH			
Formulation	**Formic acid**	**Acetic acid**	**Propionic acid**	**Lactic acid**
Hw [b]				
Control	2.85±0.03$_{A(z)}$[d]	3.99±0.03$_{B(y)}$	4.33±0.04$_{B(x)}$	4.63±0.12$_{B(w)}$
Glycerol	2.86±0.03$_{A(z)}$	4.00±0.03$_{B(y)}$	4.38±0.04$_{B(x)}$	4.61±0.06$_{B(w)}$
Sorbitol	2.86±0.02$_{A(z)}$	4.02±0.03$_{B(y)}$	4.40±0.03$_{B(x)}$	4.68±0.05$_{B(w)}$
Lw [c]				
Control	2.94±0.04$_{A(z)}$	4.23±0.01$_{A(y)}$	4.51±0.01$_{A(x)}$	5.03±0.14$_{A(w)}$
Glycerol	2.93±0.05$_{A(z)}$	4.21±0.01$_{A(y)}$	4.49±0.02$_{A(x)}$	5.00±0.06$_{A(w)}$
Sorbitol	2.93±0.06$_{A(z)}$	4.23±0.03$_{A(y)}$	4.48±0.03$_{A(x)}$	4.88±0.17$_{AB(w)}$
	Total Soluble Solids (%)			
Formulation	**Formic acid**	**Acetic acid**	**Propionic acid**	**Lactic acid**
Hw				
Control	1.73±0.06$_{B\ (y)}$	1.73±0.06$_{B\ (y)}$	1.97±0.06$_{BC\ (x)}$	1.67±0.06$_{B\ (y)}$
Glycerol	1.87±0.15$_{AB(y)}$	1.90±0.10$_{AB(xy)}$	2.13±0.06$_{A(x)}$	1.83±0.06$_{AB(y)}$
Sorbitol	1.97±0.06$_{A(y)}$	2.00±0.00$_{A(y)}$	2.13±0.06$_{A(x)}$	1.83±0.06$_{AB(z)}$
Lw				
Control	1.73±0.06$_{B(yz)}$	1.80±0.00$_{B(xy)}$	1.87±0.06$_{C(x)}$	1.67±0.06$_{B(z)}$
Glycerol	1.90±0.00$_{AB(xy)}$	2.03±0.12$_{A(x)}$	2.03±0.06$_{AB(x)}$	1.80±0.10$_{AB(y)}$
Sorbitol	1.97±0.06$_{A(yz)}$	2.00±0.00$_{A(y)}$	2.13±0.06$_{A(x)}$	1.87±0.06$_{A(z)}$
	Viscosity (mPa • s)			
Formulation	**Formic acid**	**Acetic acid**	**Propionic acid**	**Lactic acid**
Hw				
Control	2267±88$_{A(x)}$	1989±102$_{A(y)}$	2211±19$_{A(x)}$	2300±58$_{A(x)}$
Glycerol	2244±51$_{A(x)}$	1933±240 $_{A(x)}$	2122.33±107$_{A(x)}$	2211±84$_{AB(x)}$
Sorbitol	2156±51$_{A(x)}$	1989±70$_{A(x)}$	2044.67±135$_{A(x)}$	2056±183$_{B(x)}$
Lw				
Control	16.7±0.0$_{B(x)}$	16.7±0.0$_{B(x)}$	16.7±0.0$_{B(x)}$	20.0±3.3$_{C(x)}$
Glycerol	16.7±0.0$_{B(y)}$	17.8±1.9$_{B(xy)}$	16.7±0.0$_{B(y)}$	21.1±1.9$_{C(x)}$
Sorbitol	16.7±0.0$_{B(y)}$	17.8±1.9$_{B(xy)}$	16.7±0.0$_{B(y)}$	21.1±1.9$_{C(x)}$

[a] The results are the mean of 3 replicates ± SD.

[b] Hw = high molecular weight β-chitosan (1815 kDa).

[c] Lw = low molecular weight β-chitosan (366 kDa).

[d]Means followed by the same superscript (A-C) in the same column and (w-z) in the same row are not significantly different (P > 0.05) based on Tukey-Kramer multiple comparison test.

Table 3.4- Thickness, moisture Content, and density of β-chitosan based films. [a]

	Thickness (μm)			
Formulation	**Formic acid**	**Acetic acid**	**Propionic acid**	**Lactic acid**
Hw [b]				
Control	32.00±2.00$_{A(x)}$[d]	30.67±0.47$_{B(x)}$	30.00±0.21$_{AB(x)}$	33.00±1.20$_{BC(x)}$
Glycerol	36.33±2.95$_{A(y)}$	34.67±4.43$_{AB(y)}$	31.67±1.77$_{AB(y)}$	47.67±1.72$_{A(x)}$
Sorbitol	40.33±6.55$_{A(x)}$	40.33±1.06$_{A(x)}$	37.33±2.37$_{A(x)}$	47.67±3.96$_{A(x)}$
Lw [c]				
Control	33.33±3.33$_{A(x)}$	30.67±3.42$_{B(x)}$	28.33±3.20$_{B(x)}$	29.67±1.61$_{C(x)}$
Glycerol	38.67±2.01$_{A(x)}$	33.33±3.66$_{AB(x)}$	30.33±3.97$_{AB(x)}$	31.67±3.19$_{BC(x)}$
Sorbitol	41.67±4.29$_{A(x)}$	32.67±2.85$_{AB(x)}$	34.33±4.33$_{AB(x)}$	38.67±3.33$_{B(x)}$
	Moisture Content (%)			
Formulation	**Formic acid**	**Acetic acid**	**Propionic acid**	**Lactic acid**
Hw				
Control	20.45±0.89$_{C(x)}$	19.71±0.48$_{CD(x)}$	10.31±2.64$_{C(y)}$	14.48±2.64$_{AB(y)}$
Glycerol	22.32±0.89$_{BC(x)}$	23.49±1.31$_{B(x)}$	15.80±3.88$_{AB(y)}$	22.23±1.97$_{A(x)}$
Sorbitol	20.13±0.17$_{C(xy)}$	21.27±0.57$_{C(x)}$	17.33±1.72$_{A(y)}$	18.19±1.31$_{AB(y)}$
Lw				
Control	22.76±0.60$_{BC(x)}$	19.26±0.62$_{D(xy)}$	16.90±1.54$_{AB(y)}$	10.17±4.11$_{B(z)}$
Glycerol	28.38±1.40$_{A(x)}$	26.65±0.48$_{A(xy)}$	22.48±2.48$_{A(xy)}$	18.23±2.55$_{AB(z)}$
Sorbitol	24.19±1.91$_{B(x)}$	23.36±0.53$_{B(x)}$	21.61±1.70$_{A(x)}$	13.74±4.52$_{B(y)}$
	Density (g/cm^3)			
Formulation	**Formic acid**	**Acetic acid**	**Propionic acid**	**Lactic acid**
Hw				
Control	1.33±0.05$_{A(x)}$	1.30±0.03$_{A(x)}$	1.22±0.04$_{B(x)}$	1.28±0.07$_{A(x)}$
Glycerol	1.37±0.07$_{A(x)}$	1.27±0.02$_{A(xy)}$	1.19±0.06$_{B(y)}$	1.29±0.03$_{A(xy)}$
Sorbitol	1.32±0.08$_{A(xy)}$	1.29±0.02$_{A(xy)}$	1.27±0.01$_{AB(y)}$	1.38±0.02$_{A(x)}$
Lw				
Control	0.87±0.27$_{B(y)}$	1.37±0.11$_{A(x)}$	1.33±0.09$_{AB(x)}$	1.31±0.09$_{A(x)}$
Glycerol	1.07±0.05$_{AB(y)}$	1.33±0.08$_{A(x)}$	1.29±0.02$_{AB(x)}$	1.33±0.04$_{A(x)}$
Sorbitol	1.09±0.00$_{AB(x)}$	1.41±0.05$_{A(x)}$	1.42±0.06$_{A(x)}$	1.42±0.30$_{A(x)}$

[a]The results are the mean of 3 replicates ± SD.

[b] Hw = high molecular weight β-chitosan (1815 kDa).

[c] Lw = low molecular weight β-chitosan(366 kDa).

[d]Means followed by the same superscript (A-D) in the same column and (x-y) in the same row are not significantly different (P > 0.05) based on Tukey-Kramer multiple comparison test.

Table 3.5- Mechanical properties and water vapor permeability (WVP) of β-chitosan based films [a]

	Tensile Strength (MPa)			
Formulation	**Formic acid**	**Acetic acid**	**Propionic acid**	**Lactic acid**
Hw [b]				
Control	51.52±4.62$_{A(y)}$[d]	63.44±2.56$_{A(x)}$	56.30±5.24$_{A(xy)}$	56.14±1.10$_{A(xy)}$
Glycerol	40.57±6.14$_{A(x)}$	48.11±9.50$_{B(x)}$	42.45±7.35$_{AB(x)}$	16.15±3.14$_{C(y)}$
Sorbitol	42.27±8.32$_{A(x)}$	44.54±4.99$_{BC(x)}$	42.72±3.08$_{AB(x)}$	21.41±2.73$_{BC(y)}$
Lw [c]				
Control	NA[4]	40.80±1.11$_{BCD(x)}$	37.41±6.77$_{BC(x)}$	28.38±6.62$_{B(x)}$
Glycerol	18.05±3.84$_{B(y)}$	29.58±5.41$_{D(x)}$	32.83±5.23$_{BC(x)}$	26.47±0.73$_{B(xy)}$
Sorbitol	19.77±2.20$_{B(y)}$	33.06±3.36$_{CD(x)}$	25.07±4.55$_{C(y)}$	23.95±0.44$_{BC(y)}$
	Elongation at break (%)			
Formulation	**Formic acid**	**Acetic acid**	**Propionic acid**	**Lactic acid**
Hw				
Control	3.92±1.09$_{A(y)}$	8.06±1.06$_{AB(x)}$	4.69±0.36$_{ABC(y)}$	3.94±0.21$_{B(y)}$
Glycerol	17.95±6.44$_{A(x)}$	12.53±4.60$_{A(x)}$	8.74±3.72$_{A(x)}$	16.95±3.90$_{A(x)}$
Sorbitol	13.78±12.05$_{A(x)}$	11.54±4.56$_{AB(x)}$	6.58±0.53$_{AB(x)}$	15.07±1.28$_{A(x)}$
Lw				
Control	NA[e]	2.80±0.46$_{B(x)}$	1.74±0.46$_{C(xy)}$	1.00±0.34$_{B(y)}$
Glycerol	5.00±0.56$_{A(x)}$	7.44±3.79$_{AB(x)}$	3.66±1.17$_{BC(x)}$	2.78±0.85$_{B(x)}$
Sorbitol	4.75±0.57$_{A(x)}$	5.34±2.79$_{AB(x)}$	2.29±0.32$_{BC(x)}$	2.22±0.65$_{B(x)}$
	WVP (g mm/m^2 d kPa)			
Formulation	**Formic acid**	**Acetic acid**	**Propionic acid**	**Lactic acid**
Hw				
Control	25.36±1.15$_{A(x)}$	24.89±1.26$_{A(xy)}$	22.14±0.60 $_{B(y)}$	23.72±1.15$_{B(xy)}$
Glycerol	31.38±3.60$_{A(x)}$	29.13±4.84$_{A(x)}$	26.16±1.69$_{AB(x)}$	34.95±4.52$_{A(x)}$
Sorbitol	31.56±6.20$_{A(x)}$	29.07±2.27$_{A(x)}$	29.07±0.65$_{A(x)}$	35.20±0.73$_{A(x)}$
Lw				
Control	NA	22.41±0.65$_{A(x)}$	23.03±2.36$_{B(x)}$	22.40±0.66$_{B(x)}$
Glycerol	41.40±11.09$_{A(x)}$	31.23±6.31$_{A(x)}$	25.60±2.71$_{AB(x)}$	29.36±1.70$_{AB(x)}$
Sorbitol	40.38±5.11$_{A(x)}$	28.03±3.46$_{A(y)}$	22.61±2.42$_{B(y)}$	32.07±3.74$_{A(xy)}$

[a]The results are the mean of 3 replicates ± SD.

[b] Hw = high molecular weight β-chitosan (1815 kDa).

[c] Lw = low molecular weight β-chitosan (366 kDa).

[d]Means followed by the same superscript (A-D) in the same column and (x-y) in the same row are not significantly different (P > 0.05) based on Tukey-Kramer multiple comparison test.

[e] NA = Not available

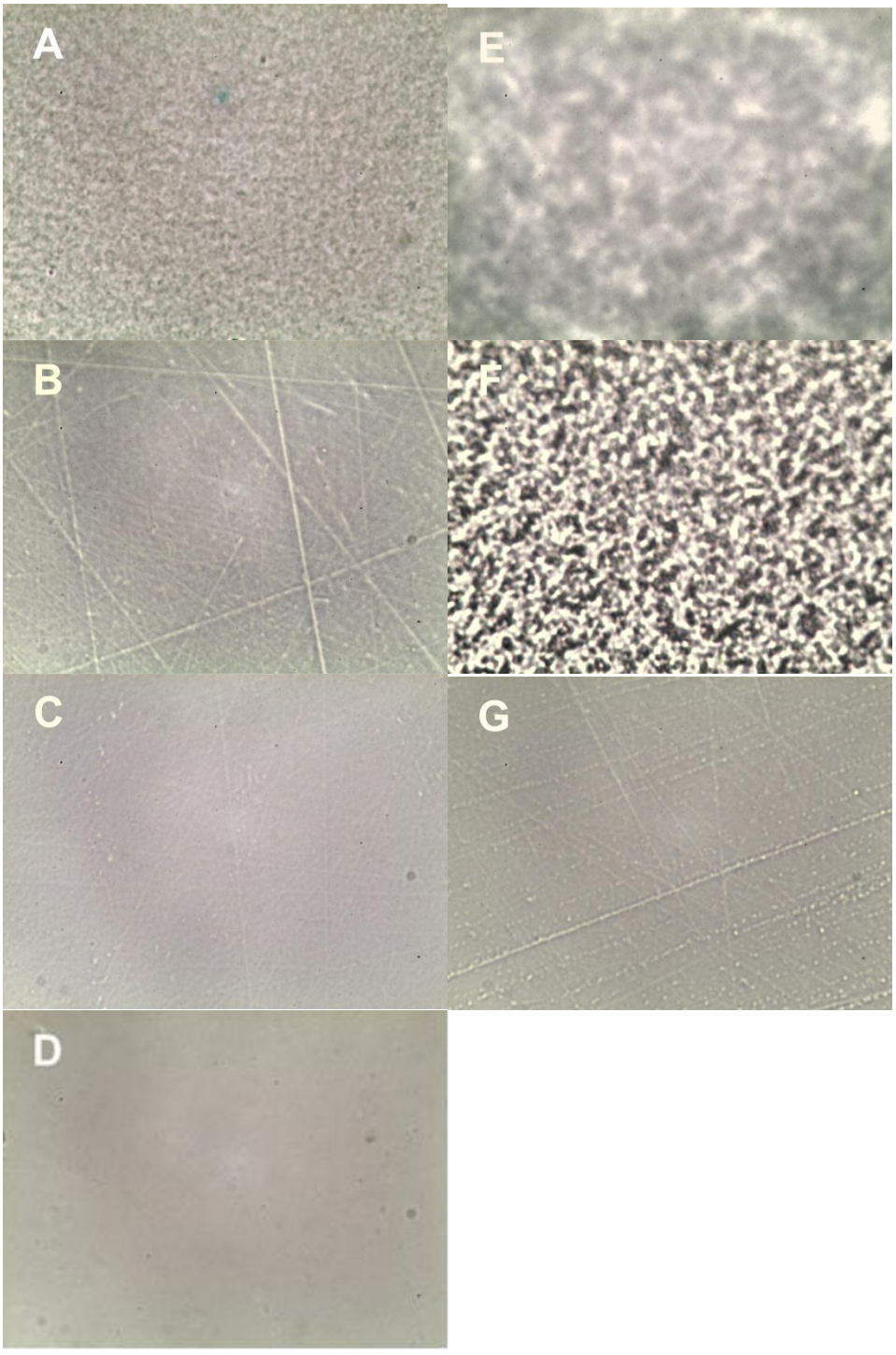

Figure 3.1- Surface microstructures of α- and β-chitosan based films viewed at magnification 40x;(A) Hw-FA; (B) Hw-AA; (C) Hw-PA; (D) Hw-LA; (E) Lw-FA; (F) Lw-AA; (G) αlpha-AA. Hw = high molecular weight β-chitosan (1,815 kDa); Lw = low molecular weight β-chitosan (366 kDa); FA = formic acid; AA = acetic acid; PA = propionic acid; LA = lactic acid. α-chitosan has molecular weight of 302 kDa.

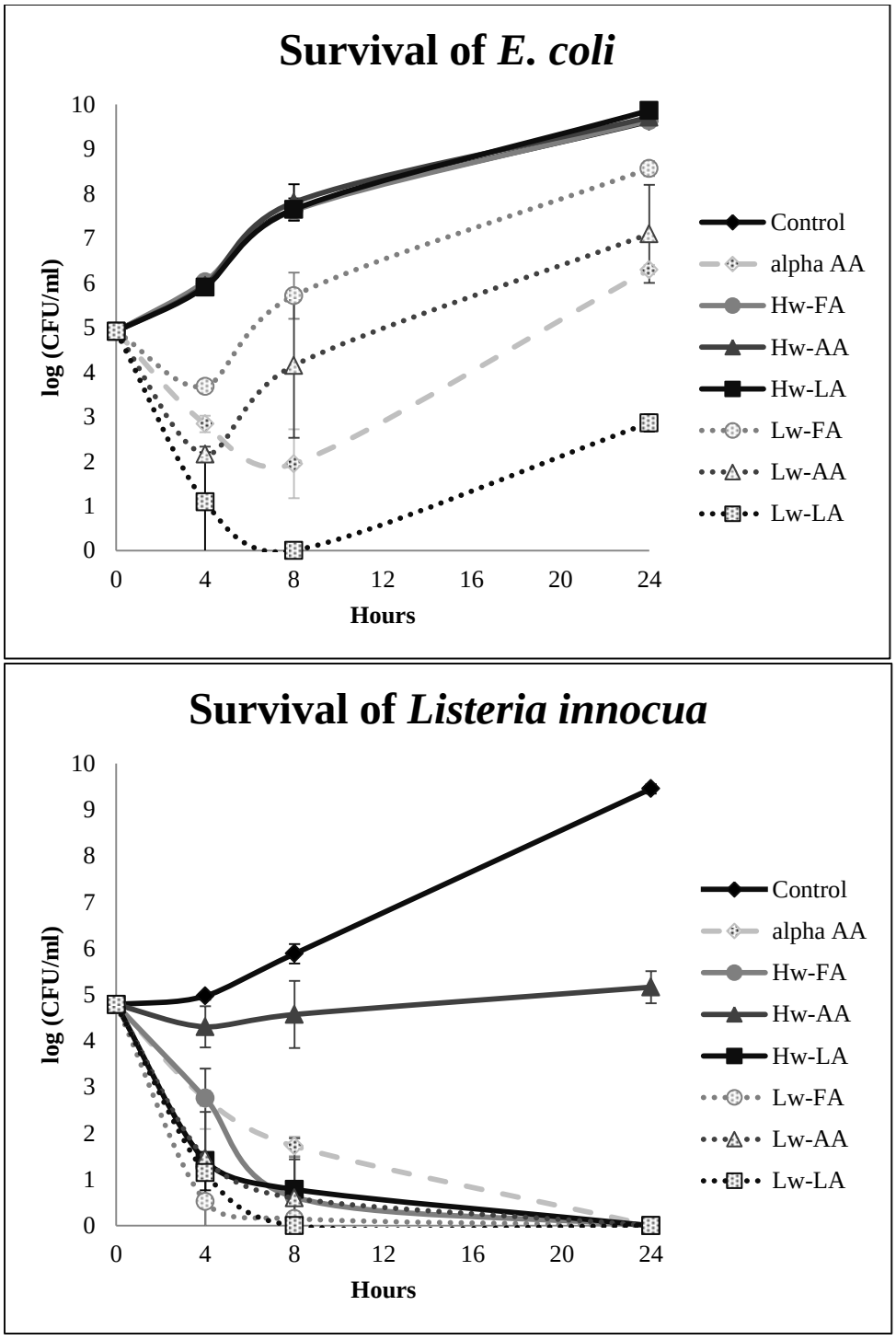

Figure-3.2 Antimicrobial activity of β-chitosan based films against E. coli ATCC 25922 and Listeria innocua ATCC 51742. The results are the mean of duplicates with standard deviation. Hw = high molecular weight β-chitosan (1,815 kDa); Lw= low molecular weight β-chitsosan (366 kDa); alpha = α-chitosan (302 kDa); FA= formic acid; AA= acetic acid; LA=lactic acid.

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Chapter 4 : Investigation of Maillard reaction for developing water soluble β- and α-chitosan as affected by sugar type and different heat treatments

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Abstract

This study investigated the effects of different heat treatments (high temperature short time (HTST), low temperature long time (LTLT)) and reducing sugars (fructose (F), glucosamine (GS)) on the solubility of α- and β-chitosan derivative (i.e., water soluble chitosan (WSC)) using Millard reaction. A 1%α- and β-chitosan (154 kDa) was dissolved in 0.2M acetic acid with 1% sugar.Mixtures were heated to 121 °C for 15 min (HTST) or to 65 °C for 2 and 6 days (LTLT). Samples were dialyzed against deionized water with a dialysis membrane for 10 days. Reducing sugar and heat treatment both affected pH, TSS%, and Maillard reaction extent of the initial mixtures, where chitosan type only influenced pH and TSS%. Reducing sugar and heat treatment influenced solubility of WSC. Different yields of WSC were observed in α- and β-chitosan, in whichthe yield of β-chitosan-sugar derivatives (8.5%) was 1.21 times higher than that of α-chitosan products (7.0%). LTLT treatment resulted in a higher solubility (8.44 g/L) than HTST (3.83 g/L) did. Study indicated that α-chitosan-glucosamine and β-chitosan-fructose solutions treated by LTLT generated the highest yield and solubility of water soluble chitosan.

Keywords

β-chitosan, reducing sugar, water soluble chitosan, Maillard reaction, fructose, glucosamine

Introduction

β-chitosan, derived from squid pens, was discovered to be a versatile polysaccharide due to its higher reactivity than α-chitosan from the parallel arrangement of the linear polymer chain, i.e., less compact package(Kurita and others 1993). Kurita and others (1993) indicated that the loose crystal structure of β-chitin did not require a harsh deacetylation process as α-chitin, yet a mild condition even without the demineralization step. A lower production cost may be achieved through the lower chemical and energy consumption for preparing β-chitosan from squid pens. However, both α- and β-chitosan are considered as a weak polybase which naturally dissolves in specific organic acid solvents such as acetic acid, formic acid, propionic acid, and lactic acid (Kim and others 2006). Native chitosan is unable to dissolve in neutral or high pH solvent system where its pKa value was reported in a range of 6.3-6.5 (Sorlier and others 2001). As a result, utilization of chitosan was limited to its solubility in a narrow pH range, a huge obstacle especially for their food applications.

Several strategies were developed to improve the solubility of α-chitosan based on its arrangement principles.First, a homogeneous phase reaction (Sannan and others 1976) controlling the deacetylation process produced water-soluble α-chitosan, butwith a low production yield (Kurita and others 1991). Secondly, reducing chitosan molecular weight through physical, chemical and enzymatic methods could modify its solubility. Physical methods involved the shear-force and ultrasonic variants could reduce the α-chitosan molecular weights down to 1.1×10^5 and 1.4×10^5 Da (Chang 1996). Kurita and others (2002) reported that the physical methods are not difficult to perform, however, chitosan products were in various and unstable solubility due to the random reactions and fast

degradation rates throughout the process. Chemical method of acid hydrolysis which generally used acetic acid as a solvent can break down chitosan polymers formed by thousands of N-acetylgucosamines into a unit of six, readily dissolute at neutral pH(Hirano and others 1985). No and others(2002) indicated that almost all biological and/or chemical functionality was lost while the α-chitosan molecular weight was below 28 kDa. Enzymatic preparation using chitosanase, lysozyme and papain to get water soluble α-chitosan had demonstrated to have a higher solubility than other methods (Ikeda and others 1993; Nordtveit and others 1996; Terbojevich and others 1996). Nevertheless, a relatively higher cost of these enzymes had impeded the commercialization of watersoluble α-chitosan products. As a result, commercial water soluble α-chitosan products were mostly chitosan chlorides or glutamates slat prepared by different chemical modification methods(Weerakkody and others 2011).

Chemical modification improved α-chitosan solubility by attaching hydrophilic functional groups to the polymer chain (Holme and Perlin 1997). Recently, many α-chitosan derivatives had been developed by chemical modification process such as O-fumaryl-chitosan (Feng and Xia 2011), free-amine chitosan (Jang and others 2002), N-acetylation (Qin and others 2006;Feng and others 2007), trimethylated and triethylated 6-amino-6-deoxy chitosan (Sadeghi and others 2008), carboxymethylated chitosan (Song and others 2011;Liu and others 2001) and quaternized chitosan (Guo and others 2006). Although some of the water soluble α-chitosan productsmentioned above had increased antibacterial (Feng and Xia 2011; Liu and others 2001) or antioxidant properties (Feng and others 2007), the preparation of a complex solvent system is typically required and therefore become difficult and unfavorable to control.

Maillard reaction is a chemical process involving amino and carbonyl groups of various molecules and usually requires heat (Jokic and others 2004). Since chitosan contained amino groups along the polymer chain, Maillard reaction was discovered as an alternative chemical modification method to produce highly water soluble chitosan by blending mono- or disaccharides into heated chitosan solution (Chung and others 2005; Chung and others 2006; Yang and others 2002). Maillard reaction was recognized as a mild reaction with appropriate controllability without using any synthetic chemicals and ease to operatein comparison with other chemical modification processes(Tessier and others 2003). Maillard reaction may be feasible to produce watersoluble α-chitosan commercially. Interestingly, the water soluble chitosan derivative from Maillard reaction was reported to have a higher antibacterial activity than native chitosanbecause of the excellent surfactant properties of chitosan-sugar conjugate may destabilize the outer membrane and suppress the growth of bacteria cell(Nakamura and others 1992; Chung and others 2005; Kosaraju and others 2010; Kanatt and others 2008). Moreover, the higher antioxidative activity of chitosan-sugar derivatives may attribute to the present of OH or NH2 scavenge hydroxyl radicals through hydrogen transmission in the pyranose rings (Xie and others 2001). The greater radical-scavenging capacity of the chitosan-sugar derivatives can be accounted to the advanced Maillard reaction product melanoidins, which demonstrated high antioxidative activity via chain-breaking, oxygen-scavenging and metal-chelating mechanism (Silvan and others 2006).

To our best knowledge, none reported study has investigated the optimization process of preparing chitosan-sugar derivatives under different heat treatment preparation conditions. Moreover, the Maillard reaction occurs more rapidly at the initial stages

whenfructose exists due to the higher extent in the open-chain form than glucose. Also, glucosamine is the main composition unit of chitosan and the present of the extra amines in glucosamine may play an important role in the Maillard reaction. Therefore, the objective of this study was to determine the effects ofchitosan and sugar types,and heat treatment on the rheological characteristics, yield, and solubility of water soluble chitosan prepared through Maillard reaction with the outmost goal ofincreasing the solubility of chitosan with a simple manner and low cost for potential food applications.

Material and Methods

Materials

β-chitin derived from jumbo squid (*Dosidicus gigas*) pens was provided by Dosidicus LLC (Lacey, WA). Commercial α-chitosan derived from shrimp shells (154 kDa and 80% DDA) was obtained from Primax ehf (Iceland) without further purification. Acetic acid was purchased from Fisher Scientific (Fair Lawn, NJ). Fructose (i.e. D-fructose; Levulose; Fruit sugar) was purchased from Spectrum Chemical Mfg. Corp. (Gardena, CA). Glucosamine (i.e. D-(+)-glucosamine hydrochloride) was obtained from Sigma-Alorich Co. (St. Louis, MO). Sodium hydroxide, acetone, and methyl alcohol were from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Toluidine blue indicator solution and potassium polyvinyl sulfate titration solution (N/400) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Spectra/Por® dialysis membranewith molecular weight cut-off of 12,000-14,000 was purchased from Spectrum Laboratories

Inc. (Rancho Dominguez, CA). Whatman® Cellulose nitrate membrane filters 0.45 um were purchased from Whatman GmbH (Dassel, Germany).

Preparation of β-chitosan

β-chitin from squid pens was ground into powdersby using a disc miller (Glenmill Inc., Maywood, NJ)with a mesh size of 18, and then deproteinized twice with 5% NaOH, neutralized with 2% HCl, and then washed with water prior to drying at hot air oven under 100 ℃ overnight(Jung and Zhao 2011). After drying, deproteinized β-chitin was treated with 50% (w/w) NaOH at 90 ℃ in a water bath (Precision Scientific Inc., Chicago, IN)for 6 h. The ratio of squid pens and NaOH for deacetylation was 1:10. Samples were washed by deionized water until neutral (pH 7±1) after the deacetylation process, and then washed with methyl alcohol and acetone. The washed squid chitosan was placed in a convection oven (Precision Scientific Inc., Model STM 40, Chicago, IN) at 40 ℃ for 48 h until the moisture content was below 10%.

A low molecular weight of β-chitosan was prepared by an enzymatic depolymerization process using cellulase (Jung and Zhao 2011).A 2% (w/w) chitosan was dissolved in 5% (w/v) acetic acid solution, stirred for 24 h, followed by the addition of 10% (w/w) cellulase solution. To advance the degradation of chitosan polymer, the mixture was heated in a 42 ℃ water bath for 4 h. To inactivate the enzyme activity,the depolymerized chitosan mixture was placed into boiling water bath for 10 min and then in an ice bath for cooling. Precipitated chitosan was obtained by titrating 5N NaOH into the polymer mixtureuntil reaching pH 11. Chitosan in white suspension form was filtered

through nylon cloth under vacuum condition, washed vigorously by deionized water until neutral pH, and then placed in a 40 ℃ convection oven to dry for at least 48 h.

Determination of viscosity-average molecular weight

Ubbelohde Dilution Viscometer (Cannon Instrument Co., USA) with a capillary size of 0.58 mm was used to determine the intrinsic viscosity of chitosan.For molecular weight measurement, 10-40 mg of chitosan was dissolved in 10 ml of 0.1 M acetic acid/0.2M NaCl (Jung and Zhao 2011). Four different concentrations of chitosan in a range of 0.05% to 0.1% were used to determine the viscosity of the samples.The intrinsic viscosity was determined by the Huggins (reduced viscosity, ŋ sp/C~C) and Kraemer (relative viscosity, ŋ rel/C ~C) plots where the intercept represents the value when the concentration was 0(Mao and others 2004). Relative viscosity, reduced viscosity, and intrinsic viscosity were represented as:

$$\eta_{rel}=\frac{t}{t0} \quad (1)$$

$$\eta_{sp}=\eta_{rel}-1,\eta_{red}=\frac{\eta sp}{C} \quad (2)$$

$$[\eta]=(\frac{\eta sp}{C})_{c=0}=(\frac{\ln[\eta]\mathrm{rel}}{C})_{c=0} \quad (3)$$

where t was the measured flow time for each sample solution at a given time t; t0 was the flow time of the solution (0.1M acetic acid/0.2M NaCl) at t=0; and C represented the chitosan samples concentration in diluted solution, %.

Mark-Houwink-Sakurada (MHS) equation (4) was used to calculate the viscosity-average molecular mass of chitosan:$[\eta] = K(Mv)^a$ (4)

where K and a were the constants, $K=1.81 \times 10^{-3}$, a = 0.93(Broussignac 1968); and [η] was the intrinsic viscosity determined by the Huggins and Kraemer plots.

Analysis of degree of deacetylation (DDA)

The DDA of β-chitosan was determined by a colloidal titration method(Chang and others 1997). A 50mg of deacetylated chitosan was dissolved in 10 ml of 5% (v/v) acetic acid solution. The solution was diluted with 30 ml of deionized water and then transferred into a flask with an additional of 100 μL of toluidine blue indicator. The mixture was titrated by a 1/400 potassium polyvinyl sulfate (PPVS) and the amount of PPVS used to change the solution color from blue to violet was recorded. DDA was calculated as:

$$DDA(\%)=(X/161)/(X/161)+(Y/203) \quad (5)$$

$$X=\frac{1}{400}\times\frac{1}{1000}\times F\times 161\times V \quad (6)$$

$$Y=0.5\times\frac{1}{100}-X \quad (7)$$

where X was the weight of D-glucosamine residue, g; F was the factor of 1/400 PPVS; V was the volume of consumed PPVS, ml; Y was the weight of N-acetyl-D-glucosamine residue, g; and 161 and 203 in Equation (5) were the molecular weight of D-glucosamine and N-acetl-D-glucosamine (2-acetamido-2-deoxy-D-glucose), respectively.

Preparation of water-soluble β-chitosan

Three treatment factors were considered when preparing water-soluble chitosanusing Maillard reaction: types of chitosan (α- and β-form), types ofreducing sugar (glucosamine and fructose), and conditions of heat treatment (HTST at 121 °Cfor 15 min) and LTLT at 65 °Cfor 2 or 6 days). These treatment conditions were chosen based on the literatures that the optimal Maillard reaction conditions for α-chitosan-glucosamine and α-chitosan-fructose solutions were 65 °C with maximal mean average yields of 42% and 48% on a particular day of 2^{nd} and 6^{th}, respectively (Chung and others 2005). Moreover, water soluble chitosan prepared from chitosan-glucose mixture (1% chitosan to 1% glucose) through Maillard reaction was feasible by using autoclaving treatment for 15 min (Kanatt and others 2008). Fructose is an easy access sugar and has a greater extent in the open-chain than glucose that leads to more rapidly initial stage of the Maillard reaction, and glucosamine is the monomer that composes the structure of chitosan, thus was used in this study. A completely randomized three-wayfactorial design was implemented in this study.

A 1% (w/v) chitosan (α- and β-form) was dissolved in 0.2 M acetic acid solution (pH 3.49). After stirring for 3 h, glucosamine or fructose was dissolved in the chitosan solution to obtain a final sugar concentration of 1% (w/v). Chung and others (2005) indicated that 1% monosaccharide was sufficient to react with chitosan completely using the Maillard reaction.For HTST treatment, chitosan-sugar solutions were heated up to 121°C for 15 min using a Magna-Clave autoclave (Pelton & Crane, Charlotte, NC),while for the LTLT treatment,the chitosan-sugar mixtureswere reacted at 65°C for 2 and 6 days, respectively. All samples were placed in an ice bath after heat treatment to terminate

further reactions. Triplicate samples were centrifuged (8000 rpm, 15 min), the supernatant was dialyzed against deionized water with a dialysis membrane (molecular weight cut-off 12,000-14,000, Spectrum Laboratories Inc., GA, USA) for 4 days and finally freeze-dried.

Determination of reactive extent of Maillard reaction, yield, and solubility

The reactivity of the Maillard reaction was determined by a UV spectrophotometer (Shimadzu UV 160U) by measuring absorbance of 3 ml solutions from various chitosan-sugar solutions at 420 nm (Liu and others 2003).The yield ofchitosan-sugar derivative was presented as the ratio ofchitosan-sugar derivative to the total addedchitosan and sugar.Solubility of chitosan-sugar derivative was measured by dissolving 0.05 g sample into 5 ml distilled water and stirredfor 5 h.The chitosan solution wasthen filtered through a cellulose nitrate 0.45 um filter paper.Solubility was determinedby the weight change of filterpaper(Yalpani and Hall 1984).

Experimental design and statistical analysis

Acomplete randomized three-way factorial design with 2 chitosan types (α- and β-form), 2 reducing sugars (fructose and glucosamine), and 2 heat treatments (HTST and LTLT) was applied. Experiments were triplicated. The general linear models (GLM) procedure was applied to determine the differences among various water-soluble chitosan products by utilizing SAS statistical software 9.02 (SAS Inc., Cary, N.C., U.S.A.). All

results were reported as mean ± standard deviation. Tukey-Kramer multi-comparison test in analysis of variance (ANOVA) ($P<0.05$) was used to compare multiple means.

Results and Discussions

Physicochemical properties of chitosan-sugar solutions after Maillard reaction

pH

The pH values of chitosan-sugar solutions were affected ($P<0.001$) by type of chitosan, sugar, and heat treatment, and there are interaction ($P<0.001$) within all individual factors (Table 4.1). Overall, β-chitosan-sugar solutions had higher pH values (4.36) than those prepared by α-chitosan (4.30) (Table 4.3). Since β-chitosan derived from squid pens was indicated to have remarkable affinity toward organic acid solvents (Kurita and others 1993) based on its relatively weak intermolecular forces and loose package than α-chitosan (Gardner and Blackwell 1975), the overall partially protonated amines (i.e. from $–NH_2$ to $–NH_3^+$) of β-chitosan may be higher and are regarded as a stronger base than α-form at low pH, thus giving a higher pH value.

The overall pH value of chitosan-fructose solutions (4.39) were higher than that of chitosan-glucosamine ones (4.27) ($P<0.001$). It is worth to mention that the solubility of chitosan-fructose derivatives (7.00 g/L) (Table 4.4) was greater than that of chitosan-glucosamine ones (5.27 g/L) ($P<0.01$), which might explain that pH of the chitosan-sugar solution increased as more chitosan-sugar derivatives dissolved in the solvent.

HTST treatment had given a lower pH value (4.32) than LTLT (4.34) for all chitosan-sugar solutions ($P<0.001$). As discussed below, the solubility of chitosan-sugar

derivatives prepared by LTLT (8.44 g/L) were higher than that of HTST one (3.83 g/L), thus giving a higher pH value since chitosan polymer was recognized as a weak base.

Total soluble solids (TSS) %

TSS of chitosan-sugar solutions was influenced by sugar type ($P<0.05$) (Table 4.1), and the interaction between sugar type and heat treatment ($P<0.05$) only occurred in LTLT chitosan-sugar solutions, but not the HTST ones (Table 4.3). Generally, β-chitosan-sugar solutions had higher TSS value (2.63%) than α-chitosan-sugar solution (2.56%) (Table 4.3), which may be because the crystalline and amorphous domain of β-chitin was converted to totally amorphous insoluble and soluble fraction after the deacetylation, while α-chitin transformed to partially soluble crystalline and soluble fraction (Lamarque and others 2004). It is possible that more portion of reducing sugar was able to react readily with the amorphous domain of β-chitosan than the crystalline of α-chitosan, thus giving a higher detectable amount of TSS. However, no differences ($P>0.05$) were observed between the two different forms of chitosan solutions when same sugar and heat treatment were applied. A similar trend also occurred in different heat treatments. Overall, HTST treated samples possessed higher TSS value (2.63%) than LTLT treated ones (2.56%) ($P<0.05$), where interactions between sugar type andheat treatment suggested that no difference ($P>0.05$) was detected within the same chitosan-sugar mixture that applied with different heat treatment (e.g. α-chitosan-fructose with HTST (2.67%) vs. α-chitosan-fructose with LTLT (2.60%)) (Table4.3).

Maillard reaction extent

Sugar type and heat treatment both affected the extent of Maillard reaction of chitosan-sugar solutions ($P<0.001$), and the interactions occurred between heat treatment and type of chitosan and sugar, respectively ($P<0.001$) (Table 4.1). Chitosan-glucosamine solutions had a higher Maillard reaction extent (2.35) than that of solutions prepared by fructose (0.88) ($P<0.001$) (Table 4.3). This result was consistent with the study by Chung and others (2005), in which the maximum absorbance for chitosan-glucosamine and chitosan-fructose derivatives were 1.52 and 0.68 at 65 °C, respectively. The relatively high Maillard reaction rate of chitosan-glucosamine may attribute to the extra amino groups from the glucosamine units besides those from the chitosan itself (Chung and others 2005).

Heat treatment had a distinct impact ($P<0.001$) on the extent of Maillard reaction for each chitosan-sugar solution regardless of the chitosan type (Table 4.1), as indicated by the interaction between heat treatment and sugar type. For the same α-chitosan-sugar solutions, for example, the Maillard reaction extent in fructose added solutions with HTST (0.42) was lower ($P<0.05$) than that of LTLT-prepared ones (1.27). In contrast, glucosamine added α-chitosan solution with HTST (2.70) was higher ($P<0.05$) than the one treated with LTLT (1.97). Similar trend occurred within the β-chitosan solutions. Moreover, HTST has a higher impact on the Maillard reaction extent of chitosan-glucosamine solution than fructose, suggested that glucosamine was more sensitive to temperature. The present of the extra amines in glucosamine may play an important role in the Maillard reaction, giving a higher reaction rate especially under high temperature comparing to fructose. For the different sugar types, it is worth to note that the Maillard

reaction extent of chitosan-fructose solutions (0.88) treated at 65 °Cfor 6 days were still lower ($P<0.05$) than chitosan-glucosamine one (2.35) treated under the same temperature for 2 days. Although chitosan type did not show effects on Maillard reaction extent, the interactions between chitosan type and heat treatment demonstrated that one exception occurred in fructose solutions treated with LTLT, i.e., α- vs. β-chitosan-fructose solution applied LTLT. Since chitosan-fructose solutions were heated at 65 °Cfor a longer time of 6 days, it may be possible that more sugar molecules were attached to the β-chitosan chain which was more reactive than α-form, resulting in a higher branch chitosan that scattered more light and gave a higher Maillard reaction extent.

Yield and solubility of α- and β-chitosan sugar derivatives

Yield (%)

A soft and cotton-like chitosan with mesopores was acquired after freeze-drying the Maillard reacted chitosan-sugar solution.Chitosan type affected the yield of the chitosan-derivatives ($P<0.01$) (Table 4.2), and an interaction existed between chitosan type and heat treatment ($P<0.001$).Overall, the yields of β-chitosan-derivatives (8.48%) were 1.21 times higher than that of α-chitosan products (7.00%) ($P<0.01$) (Table 4.4), sameas the yields of β-chitosan-glucose derivatives (51%) than that of α-chitosan analog (46%) when treated at 65°C for 3 days(Chung and others 2005).Although the results from Chung and others (2005) might not be directly comparable to our data, the difference in the yield may be explained by the different sugar types used and heat treatments applied.Especially the HTST heat treatment at 121°C might depolymerize chitosan linear chain in a higher degree and more chitosan fractions might lose during the dialysis process.The interaction

indicated an exception, in which the yield of α-chitosan-fructose derivative with LTLT (6.95%) was higher ($P<0.05$) than that of the β-chitosan product (8.39%).

Generally, heat treatment did not show effect on the yield statistically ($P>0.05$) (Table 4.2), buta distinct trend associated with heat treatment within each chitosan type was observed (Table 4.4).The α-chitosan-glucosamine with HTST (5.88%) had a lower yield ($P<0.05$) than that treated with LTLT (9.03%), where β-chitosan-glucosamine with HTST (9.75%), in contrast, possess a greater production yield ($P<0.05$) than the one with LTLT (6.82%).

Also, sugar type did not show any impact on the yield of the chitosan-derivative products in general. Since fructose and glucosamine are both monosaccharide, Chung and others (2005) indicated that both sugars performed in a similar matter that high concentration of monosaccharide results in high yields. The only exception was that the yield of β-fructose derivative with LTLT (9.56%) was higher ($P<0.05$) than that of β-chitosan-glucosamine one (5.19%).

Solubility

The solubility was affected by sugar type ($P<0.01$) and heat treatment ($P<0.001$),but not chitosan type (Table 4.2), and there were interactions between chitosan type and sugar type ($P<0.01$) and heat treatment ($P<0.001$), respectively.LTLT treatment had given all chitosan-derivatives a higher solubility (8.44 g/L) than HTST (3.83 g/L) ($P<0.001$).Relatively low temperatures led to a slower Maillard reaction, while high temperatures resulted in formation of insoluble variant (Cabodevila and others 1994).The

interactions between chitosan type andsugar type demonstrated that the effect of sugar type on solubility only occurred in the β-chitosan products prepared with LTLT ($P<0.05$), where β-chitosan-fructose derivatives (9.56 g/L) had higher solubility than the glucosamine (5.19 g/L). Chung and others (2005) also reported that the solubility of α-chitosan-fructose derivatives (17.1 g/L) was higher than α-chitosan-glucosamine derivative (16.2 g/L). It is worth to mention that after reacting chitosan and sugar solutions under dramatic reaction conditions or longer reaction time (i.e. > 6 days),microcrystals formed in chitosan derivatives were observed during the freeze-drying process leading to decreased solubility (Cabodevila and others 1994). In the case of β-chitosan-sugar derivatives with LTLT, it is possible that the relatively high rate of chitosan-glucosamine Maillard reaction was attributed to the present of extra amino groups from glucosamine. Relatively high solubility of chitosan-fructose derivatives was observed, although the rate of the Maillard reaction for the chitosan and fructose was lower comparing to glucosamine. Since fructose is a ketose, the products generated from Heyn's rearrangement and isomerization were resistant to crystal block formation in the molecules (Whistler and BeMiller, 1996). Hence, it is readily to product high solubility of chitosan-sugar derivative from fructose. On the other hand, glucosamine is aldose and crystals may form during the process of freeze-drying owing to the products were created from the Amadoris rearrangement and isomerization (Whistler and BeMiller, 1996). Thus, relatively low solubility was observed from chitosan-glucosamine derivatives. Moreover, the interaction between chitosan type and heat treatment showed that chitosan type only had effect on products derived from glucosamine solutions treated with LTLT

($P<0.05$).No difference was found by using different chitosan type on the solubility ($P>0.05$).

Chitosan is recognized as intractable polysaccharide because of its rigid crystalline structure and the primary amino group or acetamide residues which play a critical role in the formation of conformational features via intra- and/or intermolecular hydrogen bonding (Nishimura and others 1991; Heras and others 2001). By replacing the two hydrogen atoms of amino groups in chitosan and then introducing a hydrophilic compound like monosaccharaides can improve the chitosan solubility.

Conclusion

Maillard reaction was demonstrated to be a feasible method to improve the chitosan solubility with the present of fructose or glucosamine as reducing sugar. Sugar type and heat treatment are critical factors affecting the solubility of water-soluble chitosan derivatives prepared from Maillard reaction, while no difference was observed between α- and β-chitosan in the yield when subjected to same Maillard reaction. Chitosan-fructose derivatives had higher solubility due to the fact that fructose is a ketose and products generated from Heyn's rearrangement and isomerization were resistant to the formation of crystal blocks in water soluble chitosan. On the other hand, glucosamine is aldose and crystals might form during the freeze-drying process. Moreover, relatively low temperature resulted in a slower Maillard reaction, while high temperature treatment caused the formation of insoluble variant. The yield of β-chitosan-derivatives was 1.21 times higher than that of α-chitosan analog. Relatively high Maillard reaction rate of chitosan-glucosamine may attribute to the extra amino group from glucosamine in

addition of those amines from chitosan, especially under high temperature conditions. Study demonstrated that α-chitosan-glucosamine and β-chitosan-fructose solution heated by LTLT produced the highest yield and solubility of water soluble chitosan. For further studies, the improvement of the yield of water soluble chitosan modified via Maillard reaction and its antimicrobial activity and antioxidant capacity should be evaluated.

Table 4.1- Selected results from analysis of variance (ANOVA) tables (P = 0.05) for statistical significance and interactions among different treatment factors for chitosan-sugar solutions treated after Maillard reactions.

Source of variation	df[a]	*F*-value		
		pH	TSS[b]	Maillard Reaction extent
Linear terms				
Chitosan type (C)	1	355.27***[c]	7.36*	3.38
Sugar type (S)	1	1601.67***	15.36**	3917.10***
Heat treatment (H)	1	35.27***	7.36*	62.29***
Interaction terms				
C × S	1	56.07***	2.27	0.97
C × H	1	135.00***	2.27	27.00***
S × H	1	160.07***	7.36*	1010.80***
C × S × H	1	29.40***	0.82	3.68

[a] df = degree of freedom.

[b] TSS = total soluble solids.

[c] The *F* value followed by *** represents the corresponding *P* value <0.001, by ** means *P*<0.01, and by * means *P* <0.05.

Table 4.2- Selected results from analysis of variance (ANOVA) tables (P = 0.05) for statistical significance and interactions among different treatment factors for water-soluble chitosan derived from various chitosan-sugar solutions after Maillard reactions.

		***F*-value**	
Source of variation	**df**[a]	**Yield (%)**	**Solubility (g/L)**
Linear terms			
Chitosan type (C)	1	19.14** [b]	0.68
Sugar type (S)	1	0.61	49.70**
Heat treatment (H)	1	0.13	348.73***
Interaction terms			
C × S	1	3.62	19.47**
C × H	1	30.31***	58.65***
S × H	1	0.00	8.12*
C × S × H	1	12.34**	12.53**

[a] df = degree of freedom.

[b] The *F* value followed by *** represents the corresponding *P* value <0.001, by ** means *P*<0.01, and by * means *P* <0.05.

Table 4.3-pH, total soluble solids content, and Maillard reaction extent of chitosan-sugar solutions after Maillard reactions.[a]

	pH			
	α-chitosan		**β-chitosan**	
Formulation	**HTST[b]**	**LTLT[c]**	**HTST**	**LTLT**
Fructose	4.39±0.01$_{A(y)}$	4.31±0.01$_{A(z)}$	4.42±0.01$_{A(x)}$	4.45±0.01$_{A(w)}$
Glucosamine	4.23±0.01$_{B(y)}$	4.27±0.01$_{B(x)}$	4.24±0.01$_{B(y)}$	4.32±0.01$_{B(w)}$
	Total Soluble Solids (%)			
	α-chitosan		**β-chitosan**	
Formulation	**HTST**	**LTLT**	**HTST**	**LTLT**
Fructose	2.57±0.06$_{A(xy)}$	2.40±0.00$_{B(y)}$	2.67±0.12$_{A(x)}$	2.53±0.06$_{B(xy)}$
Glucosamine	2.67±0.12$_{A(x)}$	2.60±0.00$_{A(x)}$	2.63±0.06$_{A(x)}$	2.70±0.00$_{A(x)}$
	Maillard Reaction Extent [d]			
	α-chitosan		**β-chitosan**	
Formulation	**HTST**	**LTLT**	**HTST**	**LTLT**
Fructose	0.42±0.01 $_{B(z)}$	1.27±0.04 $_{B(y)}$	0.41±0.01 $_{B(z)}$	1.42±0.05 $_{B(x)}$
Glucosamine	2.70±0.10 $_{A(x)}$	1.97±0.01 $_{A(y)}$	2.55±0.02 $_{A(x)}$	2.16±0.09 $_{A(y)}$

[a] The results are the mean of 3 replicates ± SD.

[b] HTST = high temperature short time (121 °C for 15 min).

[c] LTLT = low temperature long time (65 °C for 6 days for fructose and 2 days for glucosamine).

[d]Maillard reaction extent was measuring absorbance of various chitosan-sugar solutions at 420 nm by UV spectrophotometer.

[e] Means followed by the same superscript (A-B) in the same column and (w-z) in the same row are not significantly different (P > 0.05) based on Tukey-Kramer multiple comparison test.

Table 4.4- Yield and solubility of water-soluble chitosan derived from various chitosan-sugar solutions after Maillard reactions.

	Yield (%)			
	α-chitosan		**β-chitosan**	
Formulation	**HTST**[b]	**LTLT**[c]	**HTST**	**LTLT**
Fructose	6.15±1.02 A(y)	6.95±1.38 A(xy)	8.94±0.85 A(x)	8.39±0.78 A(xy)
Glucosamine	5.88±0.17 A(y)	9.03±0.15 A(x)	9.75±0.57 A(x)	6.82±0.90 A(y)
	Solubility (g/L)			
	α-chitosan		**β-chitosan**	
Formulation	**HTST**	**LTLT**	**HTST**	**LTLT**
Fructose	3.06±0.62 A(y)	9.79±0.15 A(x)	5.23±0.57 A(y)	9.56±0.35 A(x)
Glucosamine	2.55±0.50 A(z)	9.45±0.58 A(x)	3.90±0.26 A(yz)	5.19±0.18 B(y)

[a] The results are the mean of 3 replicates ± SD.

[b] HTST = high temperature short time (121°C for 15 min).

[c] LTLT = low temperature long time (65°C for 6 days for fructose and 2 days for glucosamine).

[d] Means followed by the same superscript (A-B) in the same column and (x-z) in the same row are not significantly different (P > 0.05) based on Tukey-Kramer multiple comparison test.

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Chapter 5 : General Conclusion

This project was the first study to evaluate the antibacterial properties of β-chitosan based film and to investigate the effect of heat treatment on preparing water soluble α- and β- chitosan through Maillard reaction. Also, this study was the very few reported ones to investigate the physicochemical properties of β-chitosan film, yet the only work focused on chitosan derived from jumbo squid (*Dosidicus gigas*) pens.

The physicochemical properties and antibacterial activity of β-chitosan films are compatible to α-chitosan films, but with simple sample preparation. Generally, β-chitosan acetate film had the most ideal film properties with high density, tensile strength, and elongation, but low water vapor permeability. Also, low molecular weight (~300 kDa) β-chitosan films significantly ($P < 0.05$) inhibited the growth of *E. coli* and *L. Innocua* with similar antibacterial activity, where *E. coli* was more sensitive than *L. Innocua*. Moreover, the β- and α-chitosan acetate film with molecular weight of ~300 kDa showed similar antibacterial activity. As a result, β-chitosan based films at molecular weight of ~300 kDa were suggested to employ as an intelligent package with a control-released manner of releasing active chitosan fragment from the film matrix to food surface to control the growth of Gram-positive and Gram-negative bacterial. In addition, it may use as an antibacterial food wrap or coating that directly contact with the food surface to control moisture loss and prevent bacterial growth. For instant, β-chitosan film can be employed on food surface with a relatively high water activity such as cheese, meat, poultry, and cod.

Maillard reaction was shown in this study to be a practical procedure to improve chitosan solubility in a wider pH range for increasing the possible applications of chitosan. Developed chitosan samples were able to dissolve in distilled water at natural pH, yet with various solubility. Sugar type (fructose and glucosamine) and heat treatment with distinct temperature and time were identified as the critical factors influencing the solubility of water-soluble chitosan derivatives. Maillard reaction occurred at 65°C with a longer time of 2 to 6 days generated the highest water solubility of chitosan among all samples tested.

The current work contributed several major findings related to β-chitosan, however, several aspects were proposed for further studies. First, it's important to evaluate the β-chitosan wrap or coating on various food products. Secondly, the anti-fungi property of β-chitosan films should be investigated to compare with α-chitosan based films. Thirdly, further work were necessary to improve the yield of water soluble chitosan modified via Maillard reaction and to further evaluate its antimicrobial activity and antioxidant capacity to demonstrate the potential usage in food applications as an versatile food additive.

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