ENHANCEMENT OF *DEGREE OF DEACETYLATION* OF CHITIN IN CHITOSAN PRODUCTION

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I declare that this thesis entitled "*Enhancement of Degree of Deacetylation of Chitin in Chitosan Production"* is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature : …….

Name : Stephenie A/K Kalut

Date : May 2008

To my beloved mother, Silia ak Mandung, and father, Kalut ak Legan
and also my siblings.
For *Rome was not built in a day*,
you were there to help me make it through.

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ABSTRACT

Chitosan is made from crustacean shells by a chemical process involving demineralization, deproteinization, decolorization, and deacetylation. The process of deacetylation involves the removal of acetyl groups from the molecular chain of chitin, leaving behind a complete amino group ($-NH_2$) and chitosan versatility depends mainly on this high degree chemical reactive amino groups. The purpose of this research is to observe the parameters that can enhance the degree of deacetylation of chitosan production to the highest percentage. The observed parameters are the temperature of heating, concentration of sodium hydroxide, and the time of heating. The obtained chitin was converted into the more useful soluble chitosan by reaction with sodium hydroxide (NaOH) solution of various concentrations, then the alkaline chitin was heated in an autoclave with different time and temperature of heating which dramatically reduced the time of deacetylation. The method used to determine the degree of deacetylation of chitosan is the linear potentiometric titration. From the result, the highest degree of deacetylation can be achieved at the temperature of 134^{o}C, and 70% concentration of sodium hydroxide with DDA% of 98.38% and 98.79% respectively. It took only 10 minutes to also achieve highest degree of deacetylation, 89.05%. In conclusion, the increasing of temperature and concentration of sodium hydroxide will increase the degree of deacetylation of chitin. The increasing of time of heating will decrease the degree of deacetylation.

ABSTRAK

Chitosan terhasil daripada kulit udang melalui proses kimia yang melibatkan proses “demineralization” iaitu penyingkiran bahan mineral, “deproteinization” iaitu penyingkiran bahan berprotin, “decolorization” iaitu penyingkiran bahan berwarna, dan “deacetylation” iaitu penyingkiran kumpulan ikatan polimer N-acetyl. Tujuan utama kajian ini dijalankan adalah untuk mengkaji factor-faktor yang meningkatkan peratusan proses penyingkiran ikatan polimer N-acetyl iaitu suhu pemanasan, kepekatan alkali iaitu natrium hidroksida, dan masa pemanasan. Chitin yang diperoleh akan ditukar kepada chitosan yang mempunyai lebih banyak kegunaan dan boleh melarut dalam air dengan baik. Ini dilakukan dengan mencampurkan chitin dengan larutan natrium hidroksida dengan kepekatan yang berbeza dalam masa dan suhu pemanasan yang berbeza dan membiarkan ia bertindakbalas. Selepas itu, chitin beralkali itu dipanaskan di dalam alat pemanas “autoclave” di mana proses pemanasan ini akan mempercepatkan masa untuk proses penyingkiran ikatan polimer N-acetyl. Kaedah yang digunakan untuk mengira peratusan penyingkiran ikatan polimer N-acetyl itu adalah pentitratan potensiometrik. Daripada hasil eksperimen yang dijalankan, didapati peratusan yang paling tinggi diperoleh pada suhu 134^{o}C, dengan peratusan sebanyak 98.38% dan pada kepekatan natrium hidroksida sebanyak 70% dengan peratusan sebanyak 98.79%. Masa pemanasan dalam 10 minit pula menghasilkan peratusan penyingkiran yang paling tinggi iaitu sebanyak 89.05%. Kesimpulannya, semakin tinggi suhu pemanasan dan kepekatan alkali, semakin meningkat peratusan proses penyingkiran ikatan polimer N-acetyl di dalam chitosan. Semakin meningkat masa pemanasan pula, semakin rendah peratusan proses penyingkiran ikatan polimer N-acetyl tersebut.

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LIST OF SYMBOLS

>	-	Bigger than
≈	-	Nearly equal to
DDA	-	Degree of Deacetylation
w/w	-	weight per weight
w/v	-	weight per volume
v/v	-	volume per weight
WBC	-	Water Binding Capacity
FBC	-	Fat Binding Capacity
KOH	-	Potassium hydroxide
HCl	-	Hydrochloric acid
NaOCl	-	Sodium hypochloride
NaOH	-	Sodium hydroxide

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Chitosan is a natural polysaccharide comprising copolymers of glucosamine and *N*-acetylglucosamine, and can be obtained by the partial deacetylation of chitin, from crustacean shells, the second most abundant natural polymer after cellulose. Chitin can be converted into chitosan by enzymatic means or alkali deacetylation, this being the most utilized method. During the course of deacetylation, part of polymer N-acetyl links are broken with the formation of D-glucosamine units, which contain a free amine group, increasing the polymer's solubility in aqueous means (Chen & Tsaih, 1998).

Chitosan has been widely used in vastly diverse fields, ranging from waste management to food processing, medicine and biotechnology. It becomes an interesting material in pharmaceutical applications due to its biodegradability and biocompatibility, and low toxicity. Chitosan has found wide applicability in conventional pharmaceutical devices as a potential formulation excipient. The use of chitosan in novel drug delivery as mucoadhesive, peptide and gene delivery, as well as oral enhancer have been reported

in the literature. Chitosan exhibits myriad biological actions such as hypocholesterolemic, antimicrobial, and wound healing properties. Since chitosan is a new substance, it is important to carry out precise standardization for its pharmaceutical and biomedical applications like other auxiliary substances.

Chitosan can be characterized in terms of its quality, intrinsic properties (purity, molecular weight, viscosity, and degree of deacetylation) and physical forms. Furthermore, the quality and properties of chitosan product may vary widely because many factors in the manufacturing process can influence the characteristics of the final product. Chitosan is commercially available from a number of suppliers in various grades of purity, molecular weight, and degree of deacetylation. The variations in preparation methods of chitosan result in differences in its deacetylation degree, the distribution of acetyl groups, the viscosity and its molecular weight (Berger et al., 2005). These variations influence the solubility, antimicrobial activity among other properties, being that commercial chitosan usually has a deacetylation degree varying from 70% to 95%, and a molecular weight ranging from 50 to 2000 kDa (Rege et al., 2003).

The deacetylation degree is the proportion of glucosamine monomer residues in chitin. It has a striking effect on the solubility and solution properties of chitin. By convention, chitin and chitosan are distinguished by their solubility in dilute aqueous acids such as acetic acid (Muzzarelli, 1977). Chitin does not dissolve in dilute acetic acid. When chitin is deacetylated to a certain degree (~ 60% deacetylation) where it becomes soluble in the acid, it is referred to as chitosan. A typical deacetylation process of chitin involves the reaction of chitin powder or flake in an aqueous 40-50% sodium hydroxide solution at 100-120°C for several hours to hydrolyze N-acetyl linkages (Roberts, 1992). Repetition of the process can give deacetylation values up to 98% but the complete deacetylation can never be achieved by this heterogeneous deacetylation process without modification. Fully deacetylated (nearly 100%) chitosan can be

prepared by the alkaline treatment of a gel form instead of the powder form of chitosan (Mima et al., 1983).

1.2 Problem statement

The degree of deacetylation could influence the performance of chitosan in many of its applications. It determines the content of free amino groups in the polysaccharides and can be employed to differentiate between chitin and chitosan. The process of deacetylation involves the removal of acetyl groups from the molecular chain of chitin, leaving behind a complete amino group ($-NH_2$) and chitosan versatility depends mainly on this high degree chemical reactive amino groups. There are methods available to increase or decrease the degree of deacetylation. For example, increase either in temperature or strength of sodium hydroxide solution could enhance the removal of acetyl groups from chitin, resulting in a range of chitosan molecules with different properties and hence its applications. Preliminary experiments were carried out by refluxing chitin in strong NaOH solution at normal atmosphere. The experiments took more than 20 hours producing low deacetylation content and the reaction was accompanied by drastic degradation of the final chitosan.

1.3 Objective

The objective of this research is to enhance the degree of deacetylation of chitin in chitosan production.

1.4 Scope of study

The scope of this study covers the effect of temperature, concentration of NaOH solution, and time of heating in autoclave on the degree of deacetylation.

CHAPTER 2

LITERATURE REVIEW

2.1 Definition and Composition of Chitosan

Chitosan is a fiber-like substance derived from chitin. Chitin is the fiber in shellfish shell such as crab, lobster and shrimp. It is also found in common foods we eat such as grain, yeast, bananas, and mushrooms. Chitin, a naturally abundant polymer consists of 2-acetamido 2-deoxy-β-D-glucose through a β(1 → 4) linkage. In spite of the presence of nitrogen, it may be regarded as cellulose with hydroxyl at position C-2 replaced by an acetamido group. Like cellulose, it functions as structural polysaccharides. Its natural production is inexhaustible; arthropods, by themselves, count more than 10^6 species from the 1.2 X 10^6 of total species compiled for animal kingdom, constitute permanent and large biomass source. The chitin is deproteinized, demineralized and de-acetylated. It is a dietary fiber, meaning that it cannot be digested by the digestive enzymes of a person (Razdan A., and Petterson D., 1994). Chitin is a white, hard, inelastic, nitrogenous polysaccharide and the major source of surface pollution in coastal areas.

Chitin is made up of a linear chain of acetylglucosamine groups while chitosan is obtained by removing enough acetyl groups (CH_3-CO) for the molecule to be soluble in most diluted acids. This process is called deacetylation. The actual difference between chitin and chitosan is the acetyl content of the polymer. Chitosan having a free amino group is the most useful derivative of chitin (No and Meyers, 1992).

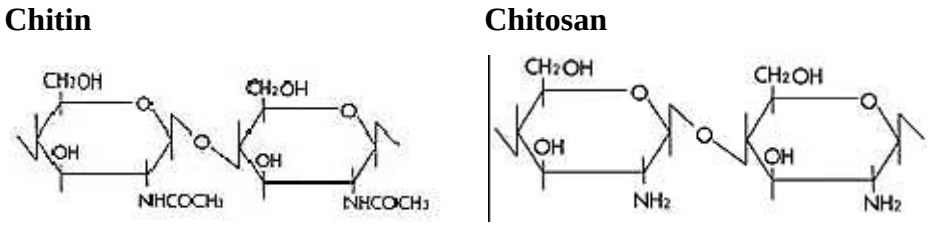


Figure 2.1 The structural formula of chitin and chitosan

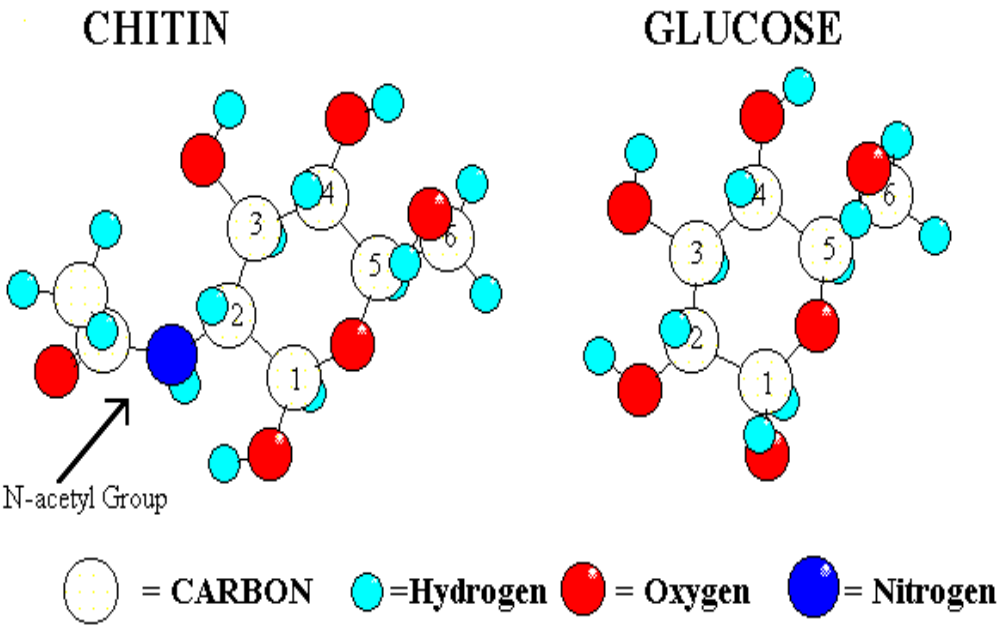
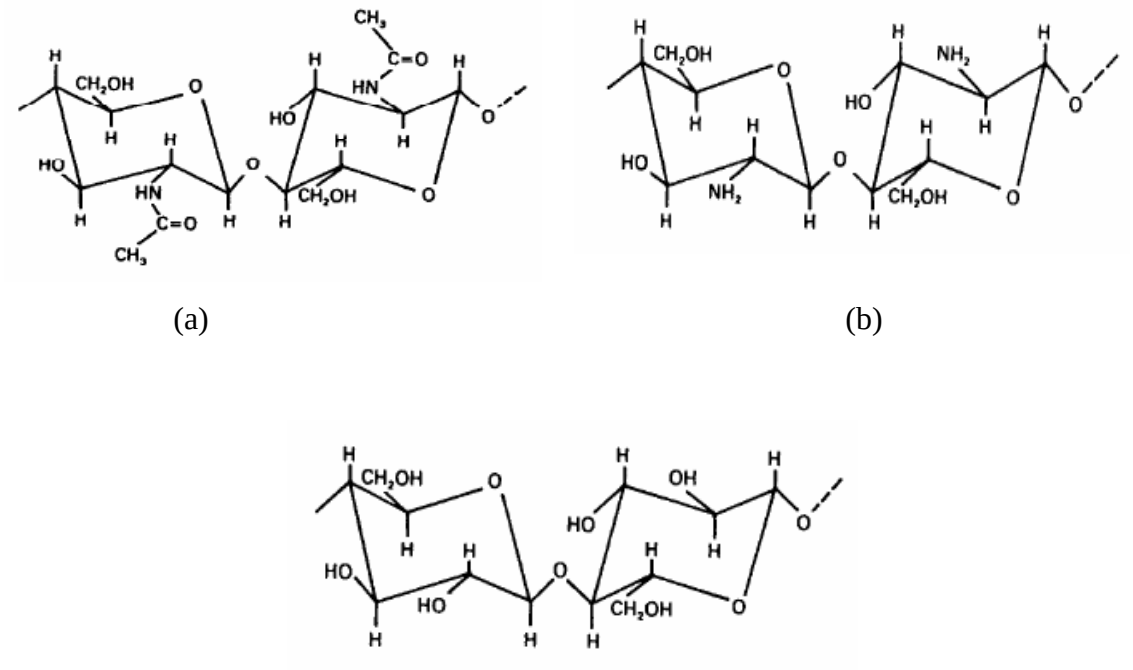


Figure 2.2 The structural formula of chitin and glucose.

2.2 Characteristics of Chitosan

Chitosan is a non toxic, biodegradable polymer of high molecular weight, and is very much similar to cellulose, a plant fiber.



(a) (b)

(c)

Figure 2.3 Structure of a) chitin, b) chitosan, and c) cellulose.

As seen in Figure 2.3, the only difference between chitosan and cellulose is the amine ($-NH_2$) group in the position C-2 of chitosan instead of the hydroxyl (-OH) group found in cellulose. However, unlike plant fiber, chitosan possesses positive ionic charges, which give it the ability to chemically bind with negatively charged fats, lipids, cholesterol, metal ions, proteins, and macromolecules (Li et al., 1992). In this respect, chitin and chitosan have attained increasing commercial interest as suitable resource

materials due to their excellent properties including biocompatibility, biodegradability, adsorption, and ability to form films, and to chelate metal ions (Rout, 2001).

2.2.1 Degree of Deacetylation in Chitosan

The process of deacetylation involves the removal of acetyl groups from the molecular chain of chitin, leaving behind a compound (chitosan) with a high degree chemical reactive amino group ($-NH_2$). This makes the degree of deacetylation an important property in chitosan production as it affects the physicochemical properties, hence determines its appropriate applications (Rout, 2001). Deacetylation also affects the biodegradability and immunological activity (Tolaimate et al., 2000).

A sharp nomenclature border has not been defined between chitin and chitosan based on the degree of N-deacetylation (Rout, 2001). In an earlier study by Rudall (1963), he reviewed evidences suggesting that approximately one in every six to seven residues in the chain has a proportion of free amino groups that manifests some histochemical properties. In any case, the degree of deacetylation can be employed to differentiate between chitin and chitosan because it determines the content of free amino groups in the polysaccharides. There are two advantages of chitosan over chitin. The first one is, in order to dissolve chitin, highly toxic solvents such as lithium chloride and dimethylacetamide are used whereas chitosan is readily dissolved in diluted acetic acid. The second advantage is that chitosan possesses free amine groups which are an active site in many chemical reactions (Knaul et al., 1999).

The degree of deacetylation of chitosan ranges from 56% to 99% with an average of 80%, depending on the crustacean species and the preparation methods (No and Meyers, 1995). Chitin with a degree of deacetylation of 75% or above is known as chitosan (Knaul et al., 1999). Various methods have been reported for the determination of the degree of deacetylation of chitosan. These included ninhydrin test, linear potentiometric titration, near-infrared spectroscopy, nuclear magnetic resonance spectroscopy, hydrogen bromide titrimetry, infrared spectroscopy, and first derivative UV-spectrophotometry (Khan et al., 2002).

The infrared spectroscopy method, which was first proposed by Moore and Roberts (1980), is commonly used for the estimation of chitosan degree of deacetylation values. This method has a number of advantages and disadvantages. First, it is relatively fast and unlike other spectroscopic methods, does not require purity of the sample to be tested nor require dissolution of the chitosan sample in an aqueous solvent (Baxter et al., 1992). However, the infrared method utilizing baseline for degree of deacetylation calculation, and as such there may be possible argument for employment of different baseline which would inevitably contribute to variation in the degree of deacetylation values. Secondly, sample preparation, type of instrument used and conditions may influence the sample analysis. Since chitosan is hygroscopic in nature and samples with lower degree of deacetylation may absorb more moisture than those with higher degree of deacetylation, it is essential that the samples under analysis be completely dry (Khan et al., 2001; Blair et al., 1987).

2.2.2 Molecular Weight

Chitosan is a biopolymer of high molecular weight. Like its composition, the molecular weight of chitosan varies with the raw material sources and the method of preparation. Molecular weight of native chitin is usually larger than one million Daltons while commercial chitosan products have the molecular weight range of 100,000 – 1,200,000 Daltons, depending on the process and grades of the product (Li et al., 1992). In general, high temperature, dissolved oxygen, and shear stress can cause degradation of chitosan. For instance at a temperature over 280˚C, thermal degradation of chitosan occurs and polymer chains rapidly break down, thereby lowering molecular weight (Rout, 2001). Also, maximal depolymerization caused by utilization of high temperature or concentrated acids, such as hydrochloric acid followed by acetic acid and sulfurous acid, results in molecular weight changes with minimal degradation with the use of EDTA (Rout, 2001). The molecular weight of chitosan can be determined by methods such as chromatography (Bough et al., 1978), light scattering (Muzzarelli, 1977), and viscometry (Maghami and Roberts, 1988)

2.2.3 Viscosity

Just as with other food matrices, viscosity is an important factor in the conventional determination of molecular weight of chitosan and in determining its commercial applications in complex biological environments such as in the food system. Higher molecular weight chitosans often render highly viscous solutions, which may not be desirable for industrial handling. But, a lower viscosity chitosan obtained from crawfish waste as shown in this thesis research may facilitate easy handling.

Some factors during processing such as the degree of deacetylation, molecular weight, concentration of solution, ionic strength, pH, and temperature affect the production of chitosan and its properties. For instance, chitosan viscosity decreases with an increased time of demineralization (Moorjani et al., 1975). Viscosity of chitosan in acetic acid tends to increase with decreasing pH but decrease with decreasing pH in HCl, giving rise to the definition of 'Intrinsic Viscosity' of chitosan which is a function of the degree of ionization as well as ion strength. Bough et al. (1978) found that deproteinization with 3% NaOH and elimination of the demineralization step in the chitin preparation decrease the viscosity of the final chitosan products. Moorjani et al. (1975) also stated that it is not desirable to bleach the material (i.e., bleaching with acetone or sodium hypochlorite) at any stage since bleaching considerably reduces the viscosity of the final chitosan product.

Similarly, No et al. (1999) demonstrated that chitosan viscosity is considerably affected by physical (grinding, heating, autoclaving, ultrasonication) and chemical (ozone) treatments, except for freezing, and decreases with an increase in treatment time and temperature. Chitosan solution stored at 4˚C is found to be relatively stable from a viscosity point of view (No et al., 1999). The effect of particle size on the quality of chitosan products was investigated by Bough et al. (1978), who reported that smaller particle size (1mm) results in chitosan products of both higher viscosity and molecular weight than those of either 2 or 6.4 mm particle size. They further enumerated that a larger particle size requires longer swelling time, resulting in a slower deacetylation rate. But, in contrast, Lusena and Rose (1953) reported that the size of chitin particle within the 20-80 mesh (0.841-0.177 mm) range had no effect on the viscosity of the chitosan solutions.

2.2.4 Solubility

While chitin is insoluble in most organic solvents, chitosan is readily soluble in dilute acidic solutions below pH 6.0. Organic acids such as acetic, formic, and lactic acids are used for dissolving chitosan. The most commonly used is 1% acetic acid solution at about pH 4.0 as a reference. Chitosan is also soluble in 1% hydrochloric acid but insoluble in sulfuric and phosphoric acids. Solubility of chitosan in inorganic acids is quite limited. Concentrated acetic acid solutions at high temperature can cause depolymerization of chitosan (Roberts and Domszy, 1982). Above pH 7.0 chitosan solubility's stability is poor. At higher pH, precipitation or gelation tends to occur and the chitosan solution forms poly-ion complex with anionic hydrocolloid resulting in the gel formation (Kurita, 1998).

The concentration ratio between chitosan and acid is of great importance to impart desired functionality (Mima, 1983). At concentrations as high as 50 percent organic solvent, chitosan still works as a viscosifier causing the solution to remain smooth. There are several critical factors affecting chitosan solubility including temperature and time of deacetylation, alkali concentration, and prior treatments applied to chitin isolation, ratio of chitin to alkali solution, and particle size.

The solubility, however, is controlled by the degree of deacetylation and it is estimated that deacetylation must be at least 85% complete in order to achieve the desired solubility (No et al., 1995). The acid-soluble chitosans with >95% solubility in 1% acetic acid at a 0.5% concentration could be obtained by treatment of the original chitin with 45-50% NaOH for 10-30 min. Chitosans treated with 45% NaOH for only 5 min, and/or with 40% NaOH for 30 min, were not deacetylated sufficiently to be soluble in 1% acetic acid. Insoluble particles were found in both solutions. According to Bough et al. (1978), a reaction time of 5 min with 45% NaOH may not be enough for chitin

particles to be sufficiently swollen. A decrease of the NaOH concentration to 40% required increased time of >30 min to obtain a soluble chitosan (No et al., 2000).

2.2.5 Bulk Density

The bulk density of chitin from shrimp and crab is normally between 0.06 and 0.17 g/ml, respectively (Shahidi and Synowiecki, 1991), indicating that shrimp chitin is more porous than crab chitin. Krill chitin was found to be 2.6 times more porous than crab chitin (Anderson et al., 1978). In a study conducted by Rout (2001), the bulk density of chitin and chitosan from crawfish shell, is very high (0.39 g/cm^3). This perhaps could be due to the porosity of the material before treatment. But once crawfish shell had been demineralized or deproteinized or both there seem to be very minor variations unpacked in bulk density between chitin and chitosan produced. A comparison of the bulk densities of crawfish and commercial chitin and chitosan indicated some variations, which can be attributed to crustacean species or sources of chitosan and the methods of preparation (Rout, 2001), as also stated earlier by Brine and Austin (1981). Rout (2001) reported that increased degree of deacetylation decreased bulk density.

2.2.6 Color

The pigment in the crustacean shells forms complexes with chitin (4-keto and three 4, 4'-diketo-ß-carotene derivatives) (Rout, 2001). Chitosan powder is quite flabby

in nature and its color varies from pale yellow to white whereas starch and cellulose powder have smooth texture and white color.

2.2.7 Water Binding Capacity (WBC) and Fat Binding Capacity (FBC)

Water uptake of chitosan was significantly greater than that of cellulose and even chitin (Knorr, 1982). Basically, WBC for chitosan ranges between 581 to 1150% with an average of 702%, according to Rout (2001). In his report, Rout (2001) also noted that reversing the sequence of steps such as demineralization and deproteinization had a pronounced effect on WBC and FBC. Deproteinization of demineralized shell also gives higher WBC compared to the process when demineralization of the deproteinized shell is conducted. Besides, the process of decoloration also causes a decrease in WBC of chitosan than those of unbleached crawfish chitosan.

The fat uptake of chitin and chitosan ranges from 315 to 170% with chitosan having the lowest and chitin the highest fat uptake (Knorr, 1982). In a study by Rout (2001) on this aspect, he reported that the average FBC of crawfish chitosans and commercial crab chitosans for soybean oil was 706% and 587%, respectively. The inclusion of decoloration step during the production of chitosan was found to decrease the fat binding capacity of crawfish chitosans, affect the viscosity of chitosan (Moorjani, 1975). The decreased viscosity as evidenced may be a cause for decrease in fat binding capacity among unbleached and bleached crawfish chitosan samples.

Rout (2001) also reported that changing the sequence of steps, i.e., when demineralization is conducted prior to deproteinization, followed by deacetylation,

caused an increase in FBC compared with when deproteinization is performed prior to demineralization, followed by deacetylation.

2.2.8 Emulsification

Even though chitosan alone does not produce emulsions, Cho et al. (1998) reported that emulsifying capacity of egg yolk (protein) increased with the addition of chitosan compared with the control. At 0.5% chitosan concentration, better emulsifying capacity was observed compared with at 0.1 or 0.3% chitosan. In general, chitosan emulsions tend to be very stable under temperature changes and aging. With viscosity, the degree of deacetylation is reported to be a determining factor in the emulsification properties of chitosan. The protein solution containing chitosan with intermediate degree of deacetylation produces less effective emulsion compared with that containing chitosan with higher DDA.

2.2.9 Antimicrobial Properties

Recent studies in antibacterial activity of chitosan have revealed that chitosan is effective in inhibiting growth of bacteria. The antimicrobial properties of chitosan depend on its molecular weight and the type of bacterium. Gram-positive bacteria retain crystal violet dye after iodine fixation and alcohol decolorization, whereas gram-negative bacteria do not. Gram-negative bacteria have an additional outer membrane containing lipopolysaccharide (endotoxin). For gram-positive bacteria, chitosan with

470 KDa was the most effective, except for *Lactbacillus sp.,* whereas for gram-negative bacteria, chitosan with 1,106 KDa was effective. Chitosan generally showed stronger bactericidal effects for grampositive bacteria (*Listeria monocytogenes, Bacillus megaterium, B. cereus, Staphylococcus aureus, Lactobacillus plantarum, L. brevis, and L. bulgaris*) than for gram-negative bacteria (*E.coli, Pseudomonas fluorescens, Salmonella typhymurium, and Vibrio parahaemolyticus*) in the presence of 0.1% chitosan (No et al., 2002).

Koide (1998) reported that chitin and chitosan in vitro show antibacterial and anti-yeast activities. One of chitosan derivatives, N-carboxybutyl chitosan, was tested against 298 cultures of different pathogenic microorganisms that showed bacteriostatic and bactericidal activities, and there were marked morphological alterations in treated microorganisms when examined by electron microscopy (Muzzarelli, 1990). Conversely, growth inhibition and inactivation of mould and yeasts seem to depend on chitosan concentration, pH, and temperature (Rout, 2001). According to Cuero (1999), the antimicrobial action of chitosan is influenced by intrinsic and extrinsic factors such as the type of chitosan (e.g., plain or derivative), degree of chitosan polymerization, host nutrient constituency, substrate chemical and/ or nutrient composition, and environmental conditions such as substrate water activity.

In an extensive research by Tsai and Su (1999) on the antimicrobial activity of chitosan prepared from shrimp against Ecoli, they found that higher temperature and acidic pH of foods increased the bactericidal effect of chitosan. They also explained the mechanism of chitosan antibacterial action involving a cross-linkage between polycations of chitosan and the anions on the bacterial surface that changes membrane permeability. Chitosan has been approved as a food additive in Korea and Japan since 1995 and 1983, respectively (KFDA, 1995). Higher antibacterial activity of chitosan at lower pH suggests that addition of chitosan to acidic foods will enhance its effectiveness as a natural preservative (No et al., 2002).

2.2.10 Formation of Film

Chitosan coating have been shown to significantly delay fruit spoilage or decaying of fruits and vegetables such as tomatoes, strawberries, etc., at different temperatures. Chitosan coated fruits were not only firmer and higher in titratable acidity, but were slow to decay and exhibited less pigmentation than control samples at the end of storage (El Ghaouth et al., 1992). The low molecular weight chitosan has a greater inhibitory effect against phytopathogens than the high molecular weight chitosan (Hirano et al., 1989).

Chitosan has an ability to form film which makes it suitable for use as food preservation for control of psychotropic pathogen in fresh or processed meat and fish products packaged under modified atmosphere (Smith et al., 1994). According to Charles et al. (1994), the most potential application of chitosan is as a coating agent in the area of fruit preservation. The biodegradability of chitosan is one of the most advantageous features for concern of the environmental damage occurring by improper disposal of petrochemical based plastics (Knorr, 1991).

N, O-carboxymethyl chitosan can form a strong film that is selectively permeable to such gases as oxygen and carbon dioxide. Apples coated with this material remain fresh for up to six months. The chitosan coating has been shown to delay ripening of banana for up to 30 days where as chitosan film manifests a slightly yellow appearance, with the color darkening as thickness increased (Setha et al., 2000).

2.3 Production of Chitin and Chitosan

Chitosan is easily obtained from crab especially Dungeness crab (*Cancer magister*), shrimp particularly the Pacific shrimp (*Pandalus borealis*), lobster, or crawfish shells. These are the richest source of chitin and the major sources of crustaceans that are processed into chitin and chitosan (Knorr, 1991). While much research has been done with chitosan extraction from crab shell, limited information is available on the extraction possibilities with crawfish shell waste.

Previous studies demonstrated that crawfish and crustacean wastes, as well as organically-rich shellfish processing streams in general, can no longer be considered as disposable "waste" products with minimal economic value, but should be considered as profitable alternatives leading to valuable products of commerce (No et al., 1992). Similar research studies by Lee (1989) demonstrated that the astaxanthin-rich shell from crawfish waste is a valuable natural resource for commercially feasible pigment which is marketed as a fish food additive in aquaculture, especially for Salmon.

Apart from the recoverable pigment, it has been shown that crawfish shell waste possesses a significant and renewable major resource for the biopolymer chitin (23.5% on a dry basis compared to 14-27% and 13-15% of the dry weight of shrimp and crab processing waste, respectively) and chitosan (No and Meyers, 1989,1992). Therefore, the applications of crawfish shell wastes as a source of astaxanthin, chitin and chitosan represent a total byproduct utilization concept with realistic implications in other crustacean waste recovery industries (No and Meyers, 1989). Further significance can be seen in the utilization of astaxanthin pigment, chitin, and protein from crawfish shell as mentioned earlier in a variety of fields with different applications.

Chitin was obtained in pilot scale, according to the procedure of Soares, Moura, Vasconcelos, Rizzi, and Pinto (2003), through the stages of demineralization, that consists of the reduction of raw material's ashes; deproteinization, where there is a reduction of shrimp wastes' protein nitrogen; and deodorization, for the reduction of shrimp's characteristic odor. Chitin was dried in a tray drier until reaching commercial moisture content (5.0–6.0%, wet basis).

2.3.1 Isolation of Chitin

Isolation of chitin from crawfish shell wastes involves four traditional steps: demineralization, deproteinization, decolorization, and deacetylation. However, the isolation of chitin specifically consists of only two steps: demineralization and deproteinization, which involves the dissolution of calcium carbonate with 1.0 N HCl and the removal of proteins with 3% NaOH, respectively.

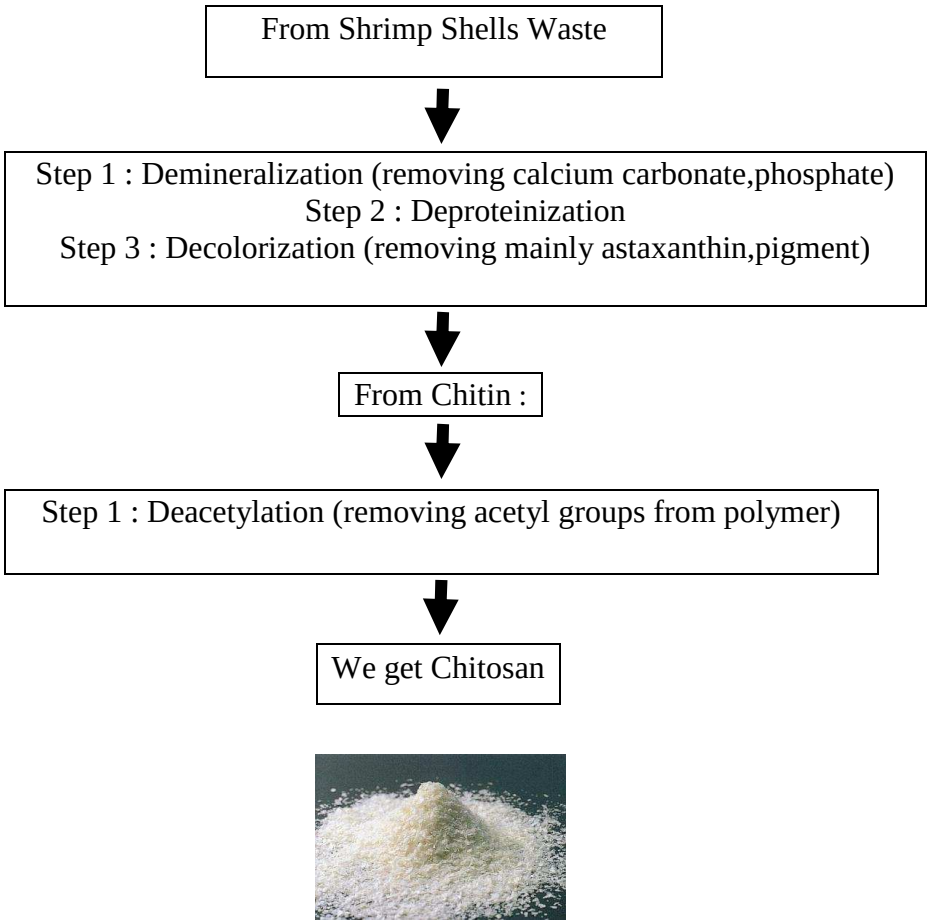


Figure 2.4 Isolation of chitin**.**

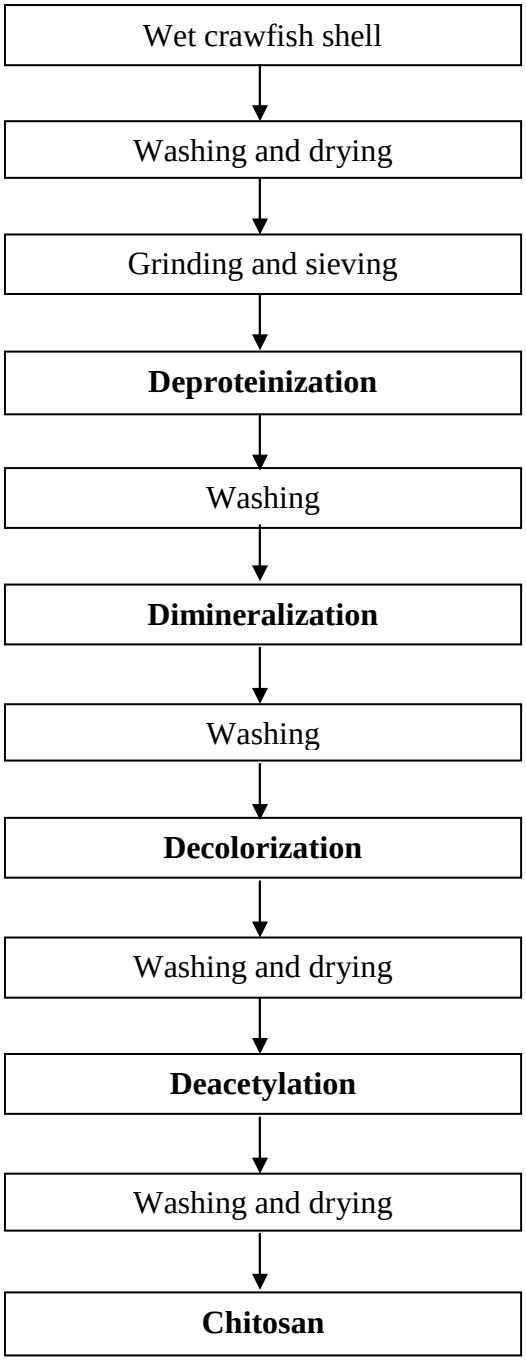


Figure 2.5 Traditional Crawfish Chitosan Production Flow Scheme (Modified from No and Meyers, 1995)

2.3.2 Deproteinization

Chitin occurs naturally in association with protein (chitinoprotein). Some of this protein can be extracted by mild methods, but other portion is not readily extracted, suggesting strong covalent bonding to chitin (Attwood and Zola, 1967). With regards to chemical structure, protein is bound by covalent bonds to the chitin through aspartyl or histidyl residues, or both, thus forming stable complexes such as glycoproteins. Crustacean shell waste is usually grounded and treated with dilute sodium hydroxide solution (1-10%) at elevated temperature (65-100ºC) to dissolve the proteins present. Reaction time usually ranges from 0.5 to 12 hr depending on preparation methods. Prolonged alkaline treatment under severe conditions causes depolymerization and deacetylation. To obtain uniformity in reaction, it is recommended to use relatively high ratios of solid to alkali solution of 1:10 or 1:15-20 with proper agitation because a minimum ratio of 1:4 (w/v) of shell weight to potassium hydroxide (KOH) solution, had only a minor effect on the deproteinization efficiency of shells (No and Meyers, 1995).

2.3.3 Demineralization

The conventional demineralization process of crustacean waste is costly and causes environmental problems. Hydrochloric acid is the most commonly used chemical in the demineralization of crustacean waste. The use of this strong acid are to harm the physiochemical properties of chitin, results in a harmful effluent wastewater, and increases the cost of chitin purification process. Percot et al. reported that using hydrochloric acid (HCl) for the demineralization of chitin results in detrimental effects on the molecular weight and the degree of acetylation that negatively affects the intrinsic properties of the purified chitin. The authors elaborated on the importance of the

optimization of the extraction process parameters (pH, time, temperature and solids to acid ratio) in order to minimize chitin degradation and bring the impurity levels down to the satisfactory level for specific applications. Therefore, a less harmful cheaper demineralization process is needed.

The current study proposes the use of a novel demineralization process in which organic acids (lactic and acetic) are used. Using organic acids such as lactic and/or acetic acids for the demineralization process is a Am. J. (2007) promising idea, since organic acids can be produced from low cost biomass such as cheese whey, are less harmful to the environment, can preserve the characteristics of the purified chitin, and the resulting organic salts from the demineralization process can be used as an environmentally friendly deicing/ anti-icing agents and/or as preservatives.

2.3.4 Decolorization

Acid and alkali treatments alone produce a colored chitin product. For commercial acceptability, the chitin produced from crustacean sources, needs to be decolorized which is a process to remove astaxanthins and pigments or bleached to yield cream white chitin powder (No et al., 1989). The pigment in the crustacean shells forms complexes with chitin. In earlier research studies, one 4-keto-and three 4, 4'-diketo-ßcarotene derivatives was firmly bound to the exoskeletal chitin of red kelp crab. The level of association of chitin and pigments varies from species to species among crustacean. Several workers have used reagents to eliminate pigments from crustacean exoskeleton, usually crab.

However, with crawfish shell the reagents alone do not seem as effective as the procedure developed currently. This suggests that carotenoids, are more strongly bound to the crawfish shell matrix than are those reported from other crustacea (No et al., 1989). Hence, the stronger the bond the more harsh treatment is required to prepare a white colored chitin. During the process of decoloration, it should be noted that the chemical used should not affect the physicochemical or functional properties of chitin and chitosan. No et al. (1989) was able to prepare a near white colored crawfish chitin by extraction with acetone and dried for 2 hr at ambient temperature, followed by bleaching with 0.315 % (v/v) sodium hypochloride solution (containing 5.25% available chlorine) for 5 min with a solid to solvent ratio of 1:10 (w/v), based on dry shell. But, the color of chitin products varied from cream white to intermediate pink color (No et al., 1989). Without prior acetone extraction, bleaching for more than 1 hr was needed to obtain a commercially acceptable white product.

2.3.5 Deacetylation

The major procedure for obtaining chitosan is based on the alkaline deacetylation of chitin with strong alkaline solution. Deacetylation is the process to convert chitin to chitosan by removal of acetyl group. It is generally achieved by treatment with concentrated sodium or potassium hydroxide solution (40-50%) usually at 100ºC or higher for 30 min or longer to remove some or all of the acetyl groups from the polymer (No and Meyers, 1989). The N-acetyl groups cannot be removed by acidic reagents without hydrolysis of the polysaccharide, thus, alkaline methods must be employed for N-deacetylation (Muzzarelli, 1977).