CHAPTER 3

MATERIALS AND METHODS

3.1 Crawfish Chitosan Production

3.1.1 Raw Material

Cooked undersized crawfish shell waste was obtained from a commercial crawfish processor (Bayou Land Seafood, Beaux Bridge, Louisiana). Upon receipt, shells of tail and the head were separated, and placed separately into double black polyethylene bags and kept in labeled (parts of shell and date) carton boxes. These were then stored at -20°C until utilized.

Preceding preparation of crawfish chitosan, the frozen tail shells were thawed at ambient temperature, washed under running warm tap water to remove soluble organics, adherent proteins and other impurities. The tail shells were then dried in the oven (Model # E32-Bakbar Turbofan oven-Moffat Limited, Christchurch, New Zealand) at 70°C for a period of 24 hrs or longer until completely dried shells were obtained. To obtain a uniform size product, the dried shell was ground through a centrifugal grinding mill (Model # DR64857-Retsch/Brinkmann ZM-1, Westbury, NY) and shifted with 20-(0.841-mm) and 40-mesh (0.425 mm) sieves. Dried ground shell was placed in opaque plastic bottles and stored at ambient temperature until used.

3.1.2 Isolation of Chitosan

1. DP (Deproteinization)

Depending upon the production sequence, the crawfish shells or demineralized shells was deproteinized with 3.5% (w/w) NaOH solution for 2 hr at 65ºC with constant stirring at a solid to solvent ratio of 1:10 (w/v) (No et al., 1989). Samples were then filtered under vacuum, and the filtrate was washed with tap water for 30 minutes and oven-dried.

2) DM (Demineralization)

Depending upon the production sequence, the crawfish shells or deproteinized shells were demineralized with 1N HCl for 30 min at ambient temperature with a solid to solvent ratio of 1:15 (w/v) (No et al., 1989), then filtered under vacuum. The filtrate was washed for 30 min with tap water and oven-dried.

3) DC (Decoloration)

Crawfish shells (also referred to as demineralized, deproteinized or crawfish chitin) were decolorized with acetone for 10 min and dried for 2 hr at ambient temperature, followed by bleaching with 0.315 % (v/v) sodium hypochloride (NaOCl) solution (containing 5.25% available chlorine) for 5 min at ambient temperature with a solid to solvent ratio of 1:10 (w/v), based on dry shell (No et al., 1989). Samples were then washed with tap water and dried under vacuum for 2-3 hrs until the powder was crispy.

4) DA (Deacetylation)

Removal of acetyl groups from chitin was achieved by autoclaving at a pressure of 15 psi for 30 min at 121°C using 50% concentrated sodium hydroxide solution (NaOH) with a solid to solvent ratio of 1:10 (w/v) according to No et al.(1989). The resulting chitosans were washed to neutrality in running tap water, rinsed with distilled water, filtered, and dried at 60°C for 24 hr in the oven.

Six crawfish chitosans were prepared. The abbreviation (D**C**MPA, DM**C**PA, DMP**C**A, DMPA**C**, D**PM**CA, and **DA**MPC) denotes the sequential processes used to prepare crawfish chitosans: D**C**MPA = decolorized + demineralized + deproteinized + deacetylated; DM**C**PA = demineralized + decolorized + deproteinized + deacetylated; DMP**C**A = demineralized + deproteinized + decolorized + deacetylated; DMPA**C** = demineralized + deproteinized +

deacetylated + decolorized; D**PM**CA = deproteinized + demineralized + decolorized + deacetylated; **DA**MPC = deacetylated + demineralized + deproteinized + decolorized.

Commercial crab chitosans Sigma91 and Vanson75 were purchased from Sigma Chemical Co. (St. Louise, MO) and Vanson Inc. (Redmond, WA), respectively. They were used as controls to compare with the physicochemical and functional properties of the crawfish chitosans developed in this study.

3.2 Physicochemical and Functional Properties Measurements

3.2.1 Moisture Content

Moisture content of the crawfish chitosan was determined by the gravimetric method (Black, 1965). The water mass was determined by drying the sample to constant weight and measuring the sample after and before drying. The water mass (or weight) was the difference between the weights of the wet and oven dry samples. Procedures were as follows: weighed and recorded weight of dish, placed 1.0g of chitosan sample in duplicates in the metal aluminum dish, recorded weight of dish with sample, then placed the sample with the lid (filter paper to prevent or minimize contamination) in the oven. Adjusted the oven temperature to 60°C, and dried the samples for 24 hrs or overnight. Took the sample from the oven and placed it in a desiccator until it cools to room temperature. Weighed the sample, and recorded this weight as weight of dry sample. Calculated moisture content as:

$$\frac{(\text{wet weight, g - dry weight, g})}{(\text{wet weight, g})} \times 100 = \%\ \text{of moisture content}$$

3.2.2 Nitrogen

The nitrogen of the crawfish chitosan was determined using a microprocessor-based, software-controlled instrument Model-TruSpec CN (Model # FP-428 Leco Corporation St. Joseph, MI. USA). There were three phases during an analysis cycle, i.e., purge, burn and

analyze. The encapsulated sample was purged of any atmospheric gases that had entered during sample loading. During the burn phase the sample was dropped into a hot furnace (850°C) and flushed with pure oxygen for a very rapid combustion. Finally, in the analyze phase, the remaining combustion product (nitrogen) was measured by the thermal conductivity cell. The final result was displayed as percent nitrogen (Theory of Operation Manual).

3.2.3 Ash

Ash of the crawfish chitosan was calculated according to the standard method # 923.03 (AOAC, 1990). Placed 2.0g of chitosan (triplicate) into previously ignited, cooled, and tarred crucible. The samples were heated in a muffle furnace preheated to 600°C for 6 hr. The crucibles were allowed to cool in the furnace to less than 200ºC and then placed into desiccators with a vented top. Allowed them to cool and weighed crucible and ash.

Calculation: $$\frac{\text{(Weight of residue, g)}}{\text{(Sample weight, g)}} \times 100 = \%\ \text{Ash}$$

3.2.4 Degree of Deacetylation

Chitosan samples prepared in the form of film were studied for the degree of deacetylation (DD). The chitosan films were prepared by casting 1.0% w/v chitosan in 1% acetic acid solution, followed by drying in a vacuum air for 12 hr. The chitosan films were deprotonated by washing 2-3 times with methanol. The chitosan films were kept in desiccators for 12hr and then placed in sealed plates before scanning. The DD of chitosan was established using a FTIR (Fourier Transform Infrared Spectroscopy) instrument (Model # M2000, Midac Corp, Irvine, CA. USA) with frequency of 4000-400 cm^{-1}. The degree of deacetylation (DD) of the chitosan was calculated using the baseline by Domszy and Roberts (1985). The computation equation for the baseline is given below:

$$DD = 100 - [(A_{1655} / A_{3450}) \; X \; 100 / 1.33]$$

where A_{1655} and A_{3450} were the absorbance at 1655 cm^{-1} of the amide-I band as a measure of the N-acetyl group content and 3450 cm^{-1} of the hydroxyl band as an internal standard to correct for film thickness. The factor '1.33' denoted the value of the ratio of A_{1655} / A_{3450} for fully N-acetylated chitosan.

3.2.5 Molecular Weight

For the determination of viscosity-average molecular weight (Dalton), the chitosan was dissolved in a mixture of 0.1 M acetic acid with 0.2 M NaCl, then the automated solution viscometer (Relative Viscometer Model Cat #9721-R56, Cannon instrument Corp., State College, PA. USA) was used to measure the intrinsic viscosity (η). The Mark-Houwink equation relating to intrinsic viscosity with empirical viscometric constants $K=1.81 \times 10^{-3}$ cm^3/g and $a=0.93$ (No et al., 2003) for chitosan was used to calculate the molecular weight using this equation: $[\eta]=KM^a$. Six or eight different dilute solutions were used to do this experiment (see Appendix B).

3.2.6 Viscosity

Viscosity of chitosan was determined with a Brookfield viscometer (Model DV-II + Brookfield Engineering Laboratories, Inc., Stonghton, MA.). Chitosan solution was prepared in 1% acetic acid at a 1% concentration on a dry basis. Measurement was made in duplicate using a No. 5 spindle at 50 rpm on solutions at 25°C with values reported in centipoises (cPs) units.

3.2.7 Solubility

Crawfish chitosan powder (0.1 g in triplicate) were placed into a centrifuge tube (known weight) then dissolved with 10 ml of 1% acetic acid for 30 min using an incubator shaker operating at 240 rpm and 25°C (C25KC, New Brunswick Scientific Co., Inc. NJ). The solution was then immersed in a boiling water bath for 10 minutes, cooled to room temperature (25°C)

and centrifuged at 10,000 rpm for 10 min. The supernatant was decanted. The undissolved particles were washed in distilled water (25ml) then centrifuged a 10,000 rpm. The supernatant was removed and undissolved pellets dried at 60°C for 24hr. Finally, weighed the particles and determined the percentage solubility. Calculation:

$$\frac{\text{(Initial weight of tube + chitosan) — (Final weight of tube + chitosan)}}{\text{(Initial weight of tube + chitosan) – (Initial weight of tube)}} \text{ X } 100 = \text{\% solubility}$$

3.2.8 Bulk Density

The bulk density of crawfish chitosan was determined using the following procedure. Each of the chitosan samples (20-40 mesh particle size) were placed into a 25 ml graduated cylinder tube until reaching the marked line of 25 ml without tapping the tube and recorded the volume of the sample. The procedure was repeated five times for each sample. On the other hand, each of chitosan samples was placed in the same cylinder tube but this time tapping the tube and recorded the volume of the sample. The procedure was also repeated five times for each sample. The bulk density was computed as grams per milliliter of the sample.

3.2.9 Color

The color of chitosan powder, expressed in L*, a*, b*, c*, h*, and whiteness values, was measured (five readings) using a Minolta Spectrophotometer CM-508d (Minolta Co, Ltd. Japan). The whiteness was calculated using a formula from NFI (1991); whiteness $=100-[(100-L^*)^2+ a^{*2} + b^{*2}]^{1/2}$.

3.2.10 Water Binding Capacity (WBC)

WBC of chitosan was measured using a modified method of Wang and Kinsella (1976). WBC was initially carried out by weighing a centrifuge tube containing 0.5 g of sample, adding 10 ml of water, and mixing on a vortex mixer for 1 min to disperse the sample. The contents were left at ambient temperature for 30 min with intermittent shaking for 5 s every 10 min and

centrifuged (Model # Z383K, HERMLE-National Labnet Company, Woodbridge, NJ. USA) at 3,500 rpm (6,000 x *g*) for 25 min. After the supernatant was decanted, the tube was weighed again. WBC was calculated as follows: WBC (%) = [water bound (g)/ initial sample weight (g)] x 100. All experiments were triplicated.

3.2.11 Fat Binding Capacity (FBC)

FBC of chitosan was measured using a modified method of Wang and Kinsella (1976). FBC was initially carried out by weighing a centrifuge tube containing 0.5 g of sample, adding 10 ml of oil (five types of oil: soybean oil (Pure Wesson® Congra Foods, Irvine, CA. USA), canola (Pure Wesson®), corn (Pure Wesson®), sunflower (Pure Wesson®), and olive (San Marc' Can-America Inc. Tampa, FL. USA)) and mixing on a vortex mixer for 1 min to disperse the sample. The contents were left at ambient temperature for 30 min with shaking for 5 s every 10 min and centrifuged (Model # Z383K, HERMLE-National Labnet Company, Woodbridge, NJ. USA) at 3,500 rpm (6,000 x *g*) for 25 min. After the supernatant was decanted, the tube was weighed again. FBC was calculated as follows: FBC (%) = [fat bound (g)/ initial sample weight (g)] x 100. All experiments were triplicated.

3.2.12 Emulsion Capacity at Various pHs

The effect of chitosan on the emulsifying capacity of soy protein (Isolated soy protein, 90% of protein, PRO FAM 892, ADM Protein Specialties Decatur, IL) was determined in triplicate at various pH values by modifying the method of Prinyawiwatkul et al.(1993). Initially, 0.5g of chitosan was dissolved in 100 ml of 1% acetic acid to prepare 0.5% chitosan solution (pH 3.14). An emulsion was prepared by blending 9 ml of 0.5% chitosan solution and 38 ml of 1% soy protein solution, later they were adjusted to pH 2, 4, 6, 8, and 10 using either 0.5N NaOH or 1N HCl. Soy bean oil (Wesson vegetable oil - Hunt-Wesson, Inc. Fullerton, CA) supplemented

with 0.03% Oil-Red-O biological stain (Aldrich Chemical Co., Inc., Milwaukee, WI) was dispended from a 50 ml burette through a 2 cm diameter hole at the bottom of an inverted blender jar which contained chitosan and protein solutions adjusted previously to a certain pH. To this was added soybean oil drop wise at a speed of 0.5 ml/s while the mixture was blended at low speed in an Osterizer blender (Model #6698, Oster Division of Sunbeam Products, Inc., Boca Raton, FL.) until the emulsion broke. The breakpoint or endpoint can be described when visible viscosity of emulsion disappeared and the mixture became oil-like in appearance. Phase inversion (coalescence) occurred was considered as the emulsion capacity of soy protein suspensions. Emulsifying capacity was expressed as milliliters of soybean oil emulsified per gram of soy protein.

3.2.13 Emulsion Capacity with Different Concentrations of Chitosan

The effect of various concentrations of chitosan on the emulsifying capacity of soy protein (Isolated soy protein, 90% of protein, PRO FAM 892, ADM Protein Specialties Decatur, IL) was determined in triplicate by modifying the method of Borton et al. (1968). Initially, chitosan was dissolved in acetic acid (1%, v/v) at concentrations of 0% (control), 0.1%, 0.5%, and 1.0%. Emulsion was prepared by blending 38ml protein solution (1%) and 9ml of chitosan (0.1%, 0.5%, 1.0%) solution, while soybean oil (Wesson vegetable oil - Hunt-Wesson, Inc. Fullerton, CA), which was supplemented with 0.03% Oil-Red-O biological stain (Aldrich Chemical Co., Inc., Milwaukee, WI) was dispended drop wise from a 50 ml buret through a 2 cm diameter hole at the bottom of an inverted blender jar at a speed of 0.5 ml/s while the mixture was blended at low speed in an Osterizer blender (Model #6698, Oster Division of Sunbeam Products, Inc., Boca Raton, FL.) until the emulsion broke. Emulsion capacity was expressed as milliliters of soybean oil emulsified per gram of soy protein.

3.2.14 Emulsion Viscosity

Emulsions containing 80% of the amount of oil needed to reach the breakpoint were used for emulsion viscosity measurements (Prinyawiwatkul et al., 1993). Emulsion viscosity (EV) was determined at 25°C using a Brookfield viscometer (Model DV-II+ (Brookfield Engineering Laboratories, Inc., Stonghton, MA.) and a Helipath Stand equipped with a T-B spindle operated at 2.5 rpm. The emulsion viscosity was computed as cps. Two readings were recorded on duplicate samples of emulsions.

3.3 Statistical Analysis

All experiments were carried out in triplicate, except for duplicate determinations of nitrogen content, moisture content, and emulsion viscosity. Average values (means) and standard deviations were reported. Mean separations were analyzed using the ANOVA (SAS) and Tukey's studentized range tests at $\alpha = 0.05$.