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Production and characterization of chitosan from shrimp waste

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Abstract

Chitosan has been produced from shrimp waste by chemical method involving demineralization, deproteinization and deacetylation. The quality of chitosan depends on the conditions of the chemical extraction process. The results showed that 3% HCl and 4% NaOH were suitable concentration for demineralization and deproteinization, respectively at ambient temperature ($28\pm 2^\circ\text{C}$). Chitosan with a high degree of deacetylation (81.24%) and high solubility (97.65%) was obtained by deacetylation with 60% NaOH for 24 hours at 60°C . Purified chitosan was characterized for intrinsic viscosity (13.2dl/g), molecular weight (1.05×10^6 Dalton), FBC (427.98%), WBC (537.29%) as well as yield (15.4%).

Keywords: Shrimp waste, Chitin, Chitosan, Characterization

Introduction

Shrimp is one of the important fisheries products worldwide including Bangladesh. This product is mostly exported in frozen condition that has undergone a process of separation of the head and skin (Budiyanto, 1993). The crude shrimp head and skin materials have only a low economic value and are treated as bio-waste or sold to animal feed manufacturers (Suchiva *et al.*, 2002). Shrimp industries generate large amounts of shrimp bio-waste during processing, approximately 45-55% of the weight of raw shrimp (Lertsutthiwong *et al.*, 2002). However, this bio-waste can be used to produce value-added products such as chitosan.

Chitosan is a modified natural carbohydrate polymer derived from chitin which has been found in a wide range of natural sources such as crustaceans, fungi, insects and some algae (Tolamite *et al.*, 2000). Generally, the shell of selected crustacean consists of 30-40% protein, 30-50% calcium carbonate and calcium phosphate, and 20-30% chitin (Knorr, 1984). After cellulose, chitin is the second most abundant natural biopolymer found in nature (No and Meyers, 1989).

Chitosan is a fiber-like substance and a homopolymer of β -(1 \rightarrow 4)-linked N-acetyl-D-glucosamine. Chitin is made up of a linear chain of acetylglucosamine groups while chitosan is obtained by removing enough acetyl groups ($\text{CH}_3\text{-CO}$) for the molecule to be soluble in most diluted acids. 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).

Chitosan is a non-toxic, biodegradable polymer of high molecular weight and is very much similar to cellulose, a plant fiber. The only difference between chitosan and cellulose is the amine ($-\text{NH}_2$) group in the position C-2 of chitosan instead of the hydroxyl ($-\text{OH}$) 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).

Chitosan is useful in a wide application in various industries such as pharmaceuticals, biochemistry, biotechnology, cosmetic, biomedical, paper industry, food and textile industries and others (Muzzarelli, 1985). These biopolymers offer a wide range of unique applications including bioconversion for the production of value-added food products, preservation of foods from microbial deterioration, formation of biodegradable films, recovery of waste material from food processing discards, purification of water and clarification and de-acidification of fruit juices (Shahidi *et al.*, 1999).

The extraction process of chitin from shrimp shell waste and its conversion to chitosan still needs more investigation especially on the process condition to obtain high quality chitosan. The objectives of this research are to determine optimal condition of chitosan production from shrimp processing waste and to investigate the characteristics properties of chitosan.

Materials and Methods

Fresh shrimp was collected from local market of Mymensingh. Shrimp head and skin was separated from shrimp using sharp knife. The collected shrimp wastes were then washed with tap water and crushed with mortar and pestle. Crushed shrimp waste was kept in a polyethylene bag at ambient temperature ($28\pm 2^\circ\text{C}$) for 24 hours for partial autolysis to facilitate chemical extraction of chitosan and to improve the quality of chitosan (Toan, 2009).

Isolation of chitosan

The following 3 (three) steps, namely Demineralization, Deproteinization and Deacetylation are followed for the isolation of chitosan. The details of the above three steps are discussed below and shown in Fig.1.

Demineralization

Demineralization of shrimp shell has been carried out with three different concentrations of HCl (4%, 3%, 2%) at ambient temperature ($28\pm 2^\circ\text{C}$) with a solid to solvent ratio 1:5 (w/v) for 16 hours (Toan, 2009). The residue was washed and soaked in tap water until neutral pH.

Deproteinization

Deproteinization of shrimp shell was done with 4% NaOH at ambient temperature ($28\pm 2^\circ\text{C}$) with a solid to solvent ratio 1:5 (w/v) for 20 hours (Toan, 2009). The residue was washed and soaked in tap water until neutral pH. Then purified chitin was dried until it became crispy. Chitin flakes were ground to small particles to facilitate deacetylation.

Deacetylation

Removal of acetyl groups from chitin was experimented using four different concentrations of NaOH (30%, 40%, 50%, 60%) at 65°C temperature with a solid to solvent ratio 1:10 (w/v) for 20 hours. (Toan, 2009). The residue was washed until neutral pH with tap water. The resulting chitosan was then dried at a cabinet dryer for 4 hours at $65\pm 5^\circ\text{C}$ and prepared for characterization.

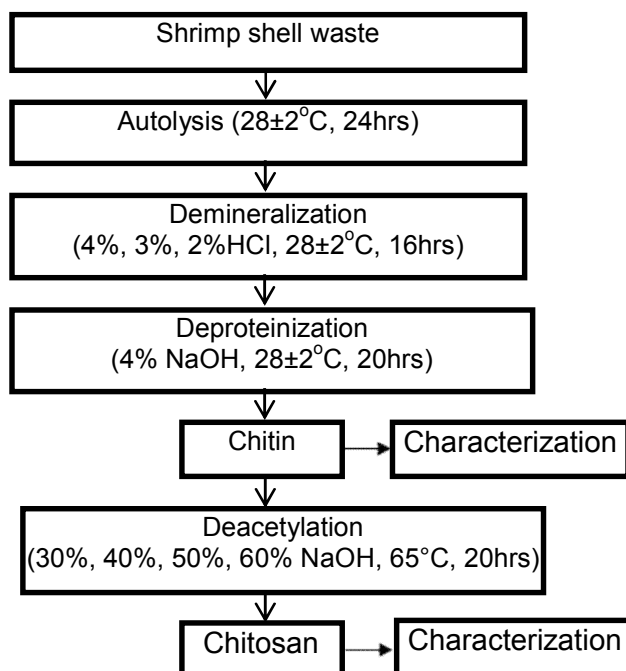


Fig. 1. Traditional Shrimp chitin and Chitosan Production Flow Scheme (Modified from Toan, 2009)

Physicochemical and functional properties measurements

Moisture and ash content: The different physicochemical and functional properties were measured as per the standard methods, e.g., Moisture content was determined by the gravimetric method (Black, 1965) and ash content by the standard method (AOAC, 2005). The procedures for other determinations are briefly discussed in the following sections:

Determination of degree of deacetylation: The degree of deacetylation (DD) was measured by the acid-base titration method (Domard & Rinaudo 1983) with modifications. In brief, chitosan (0.1 g) was dissolved in 30 ml HCl aqueous solution (0.1 mol/l) at room temperature with 5–6 drops of methyl orange added. The red chitosan solution was titrated with 0.1 mol/l NaOH solution until it turned orange. The DD was calculated by the formula:

$$DD (\%) = \frac{C_1 V_1 - C_2 V_2}{M \times 0.0994} \times 0.016$$

Where, C_1 = concentration of standard HCl aqueous solution (mol/l), C_2 = standard NaOH solution (mol/l), V_1 = volume of the standard HCl aqueous solution used to dissolve chitosan (ml), V_2 = volume of standard NaOH solution consumed during titration (ml), and M = weight of chitosan (g).

The number 0.016 (g) is the equivalent weight of NH_2 group in 1 ml of standard 1 mol/l HCl aqueous solution and 0.0994 is the proportion of NH_2 group by weight in chitosan.

Determination of intrinsic viscosity: Chitosan was prepared in acetate buffer (0.5M AcOH – 0.2M NaOAc). A calibrated, standard size A, borosilicate glass Ostwald U-tube viscometer clamped into a leveled water bath was used to determine the reduced viscosity of the solutions from the mean flow time of five determinations of the solutions and the solvent at $30 \pm 0.5^\circ C$. The mean flow times of the polymer solutions and the reference solvent were used to determine the kinematic viscosity (ν).

$$\nu = \eta / \rho$$

Where η is dynamic viscosity and ρ is density of fluid. Since the polymer solutions were sufficiently dilute, the dynamic viscosity was assumed to be equal to the kinematic viscosity. The viscosities of the solvent and the polymer solutions were used to calculate the relative viscosity, specific viscosity and reduced viscosity using the following relationships:

$$\text{Relative viscosity } (\eta_{rel}) = t/t_s,$$

$$\text{Specific viscosity } (\eta_{sp}) = (t/t_s) - 1,$$

$$\text{Reduced viscosity } (\eta_{red}) = \eta_{sp}/c,$$

$$\text{Intrinsic viscosity } ([\eta]) = (\eta_{red})_{c \rightarrow 0}$$

Where t is the mean flow time of polymer solution, t_s is mean flow time of solvent, and c is polymer concentration in (g/dl).

A plot of reduced viscosity against polymer concentration (Huggin's plot) on extrapolation to infinite dilution gave the intrinsic viscosity of the polymer. The intrinsic viscosity was determined from the Huggins equation (Wang *et al.*, 2004):

$$\eta_{sp}/c = [\eta] + K[\eta]^2 c$$

Molecular weight: For the determination of viscosity-average molecular weight (Dalton), the intrinsic viscosity (η) of the polymer was used. From the intrinsic viscosity, the molecular weight was determined employing the Mark-Houwink equation (Wang *et al.*, 1991):

$$[\eta] = KM^a$$

Where M is viscosity average molecular weight; K and a are constants, whose values depend on the polymer type and the chosen solvent. For chitosan and the solvent (0.5 M AcOH – 0.2 M NaOAc), these constants are 3.5×10^{-4} and 0.76, respectively and they do not depend on the deacetylation degree (Terbojevidh *et al.*, 1997). Five different dilute solutions were used to do this experiment.

Water binding capacity: Water binding capacity (WBC) of chitosan was measured using a modified method of Knorr, (1982). 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 (29°C) for 30 min with intermittent shaking for 5 s every 10 min and centrifuged at 3000 rpm for 25 min. After the supernatant was decanted, the tube was weighed again. WBC was calculated as follows:

$$\text{WBC (\%)} = \frac{\text{water bound (g)}}{\text{Initial sample weight (g)}} \times 100$$

Fat binding capacity: Fat binding capacity (FBC) of chitosan was measured using a modified method of Knorr, (1982). FBC was initially carried out by weighing a centrifuge tube containing 0.5 g of sample, adding 10 ml of oil (soybean oil) 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 at 3,000 rpm for 25 min. After the supernatant was decanted, the tube was weighed again. FBC was calculated as follows:

$$\text{FBC (\%)} = \frac{\text{Fat bound (g)}}{\text{Initial sample weight (g)}} \times 100$$

Statistical analysis

All the above determinations were carried out in triplicate and average values as well as standard deviations were reported. Mean separations were analyzed using the ANOVA and Fisher's least significant difference (LSD) procedure at $\alpha = 0.05$ using statistical software (StatGraphics, 1999).

Results and Discussion

Moisture content

Results of the moisture content of fresh shrimp shell, chitin and chitosan samples are presented in Table 1, 2, and 3. Ushakumari and Ramanujan (2012) investigated that moisture of shrimp shell waste is 71.6% which is closely related to the value (69.3%) obtained in this study. The lowering of moisture content in the present study due to the lack of homogeneity of the sample. Moisture content of chitin lies from 8.50% to 9.23% which is lower than the value (12.90% for mussel shell) reported by Abdulkarim *et al.* (2013). This may be due to the source of chitin and drying conditions. According to Li (1992), commercial chitosan products contain less than 10% moisture. The shrimp shell chitosan samples had a moisture content ranging from 7.69% to 8.25%. Chitosan is hygroscopic in nature (Khan *et al.*, 2002) hence it can be affected by moisture absorption during storage.

Table 1. Analysis of shrimp waste

Parameter	Value
Moisture content (%)	69.30
Ash content (%)	32.27
Yield (%)	45.00

Ash

Ash measurement is an indicator of the effectiveness of the demineralization step for removal of calcium carbonate. Elimination of the demineralization resulted in products having 31-36% ash (Bough *et al.*, 1978). The fresh shrimp shell had 32.27% ash content. The ash content of chitin obtained in this study was ranged from 0.36 % to 4.25% with significant difference. From the experimental data it shows that

4% and 3% HCl can effectively reduce the ash content of chitin up to 0.36% and 0.48%, respectively. A high quality grade of chitosan should have less than 1% of ash content. Some residual ash of chitosan may affect their solubility, consequently contributing to lower viscosity, or can affect other more important characteristics of the final product (No *et al.*, 1995). Table 3 shows shrimp chitosan contained less than 1% ash with a range of 0.26% to 0.3% with no significance difference.

Yield

Yield has been calculated for shrimp waste, chitin and chitosan. Waste from the fresh shrimp was found to be 45%. The yield obtained here found to be within the range reported by Lertsutthiwong *et al.* (2002) who showed that waste content varied approximately from 45 to 55% of the weight of raw shrimp. Yield of chitin extracted in this study from shrimp waste varied from 13.12% to 17.36% depending on concentration of HCl used during different treatments. This is due to lower concentration of HCl could not remove minerals from shrimp shell and subsequently increased the yield. Chitosan yielded in this study was 15.40% (Table 4), which was higher than that reported by Brzeski (1982) (14% yield of chitosan from krill). On the other hand, the yield was lower than those reported by Alimuniar and Zainuddin (1992) who reported 18.6% yield from prawn waste and No and Meyers (1989) who obtained approximately 23%. Chitosan yield in this research is relatively lower. This might be due to depolymerization of the chitosan polymer, loss of sample mass/weight from excessive removal of acetyl groups from the polymer during deacetylation and loss of chitosan particles during washing.

Table 2. Characteristics of chitin produced by different chemical treatments

Treatments	Parameters		
	Moisture content (%)	Ash content (%)	Yield (%)
2% HCl, 16hr & 4% NaOH, 20hr	8.50(0.12) ^a	4.25(.02) ^a	17.36
3% HCl, 16hr & 4% NaOH, 20hr	9.23(0.14) ^b	0.48(.04) ^b	14.02
4% HCl, 16hr & 4% NaOH, 20hr	9.02(0.37) ^b	0.36(.04) ^c	13.12

*Numbers in parentheses are standard deviations (\pm). Means with different letters in each column are significantly different ($p < 0.05$).

Degree of deacetylation

Based on the Table 3, it is indicated that the degree of deacetylation (DD) is influenced by NaOH concentration. Acetyl groups bounded in chitin is difficult to be removed. So, it needs high concentration of NaOH and temperature (Hargono *et al.*, 2003). In this case, the increase of NaOH concentration addressed to enhance the deacetylation grade where the highest deacetylation grade (81.24%) could be reached at NaOH concentration of 60%. The percentage of NaOH more than 60% was not observed since the process become inefficient especially in final washing to obtain chitosan product. In this case, more NaOH will be disposed and more purified water has to be required to get chitosan product. The degree of deacetylation of shrimp chitosan samples ranged from 45.5% to 81.24% based on different concentration of NaOH treatment. According to No and Meyers (1995), DD of chitosan ranges from 56% to 99%.

Solubility

The solubility of chitosan is one of important parameters for quality of chitosan, where higher solubility will produce a better chitosan. There are several critical factors affecting chitosan solubility including temperature and time of deacetylation, alkali concentration, 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 solubility of chitosan obtained in this study was ranged from 48.3 % to 97.65% with significant difference with respect to NaOH concentration. Deacetylated with 40% and 50% NaOH showed lower solubility which is due to lower DD value. Proportionally increase in solubility

was observed with increasing deacetylation degree. Shrimp shell chitosan samples treated with 50% and 60% NaOH were found to have excellent solubility ranging from 96.01% to 97.2% with no significant difference (Table 3). Brine and Austin (1981) noted that lower solubility values suggested incomplete removal of protein and acetyl group. Since solubility of chitosan depends on the removal of acetyl group from chitin, therefore the lower DD value could adversely interfere with the results.

Table 3. Effect of NaOH concentration at deacetylation step on the characteristics of chitosan

NaOH concentration (%)	Parameters			
	Moisture (%)	Ash (%)	Solubility (%)	DD (%)
30	8.25(0.29) ^a	0.30(0.06) ^a	48.30(2.01) ^a	45.50
40	7.69(0.32) ^{ab}	0.29(0.02) ^a	71.27(1.25) ^b	61.24
50	8.32(0.36) ^b	0.26(0.01) ^a	97.02(0.56) ^c	79.57
60	7.96(0.06) ^b	0.27(0.02) ^a	97.65(0.29) ^c	81.24

*Numbers in parentheses are standard deviations (\pm). Means with different letters in each column are significantly different ($p < 0.05$).

Intrinsic viscosity

Viscosity is an important factor in the conventional determination of molecular weight of chitosan. Higher molecular weight chitosan often provide highly viscous solutions, which may not be desirable for industrial handling. 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'. Intrinsic viscosity is an important rheological parameter which is used to characterize the hydrodynamic properties of polymers and also to determine the weight average molecular weight of polymers by use of the Mark-Houwink's equation (Wang *et al.*, 1991). 'Intrinsic Viscosity' of chitosan is the function of the degree of ionization as well as ion Strength (Moorjani *et al.*, 1975). Intrinsic viscosity of chitosan obtained in this study was 13.20 dl/g.

Molecular weight

Chitosan is a biopolymer of high molecular weight (MW) and varies with the sources and also the methods of preparation (Fernandez-Kim, 1991). The MW of native chitin is usually larger than one million Daltons while commercial chitosan products fall between 100,000 to 1,200,000 Daltons (Li *et al.*, 1992). No and Meyers (1995) reported an average MW of $0.12-1.5 \times 10^6$ Da. The molecular weight of shrimp chitosan obtained in this study was 1.05×10^6 Da. Several factors during production, including high temperature, concentration of alkali, reaction time, previous treatment of the chitin, particle size, chitin concentration, dissolved oxygen concentration and shear stress may influence the MW of chitosan (Li *et al.*, 1992).

Table 4. Intrinsic viscosity, molecular weight, FBC, WBC and Yield of shrimp shell chitosan

Intrinsic Viscosity (dl/g)	Molecular weight (Dalton)	FBC (%)	WBC (%)	Yield (%)
13.20	1.05×10^6	427.98	537.29	15.40

Water binding capacity

Water binding capacity of shrimp chitosan was found 537.29% (Table 4). Cho *et al.*, (1998) reported the WBC for five commercial chitosan from shrimp and crab shell range of 458% to 805%. On the other hand, Rout (2001) found that WBC for chitosan ranges between 581 to 1150% with an average of 702%. However, Rout (2001) also commented that reversing the sequence of steps such as demineralization and deproteinization had a pronounced effect on WBC.

Fat binding capacity

Fat binding capacity of shrimp chitosan was measured using soybean oil. Shrimp chitosan sample showed 427.98% FBC which is in agreement with those (314 to 535% with an average of 417%) reported by No *et al.* (1998). Rout (2001) reported that changing the sequence of steps (such as an increased in FBC is observed when demineralization is conducted prior to deproteinization, followed by deacetylation and decreased FBC is observed when deproteinization is performed prior to demineralization, followed by deacetylation).

Conclusion

Chitin and chitosan extracted from shrimp waste by chemical methods have been characterized in this investigation. Among the treatments used in the study 3% HCl and 4% NaOH found to be used successfully to extract chitin. Although 60% NaOH treatment yields highest deacetylated chitosan with maximum solubility, 50% NaOH treatment could be used to get high quality chitosan of 79.57% degree of deacetylation and 97.02% solubility with minimum chemical utilization.

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