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OPTIMUM CONDITIONS FOR PRODUCING CHITOSAN FROM SHELL OF GREEN SHRIMP *PENAEUS SEMISULCATUS*

Abstract. The paper studies the conditions for obtaining chitosan from the shells (wastes from cutting the shell) of green shrimp *Penaeus semisulcatus*, caught in Egypt. Demineralization of raw materials was carried out using 1 M HCl at 45°C for 2 hours, and deproteinization was made with the use of 1 M HCl at 75°C for 4 hours. As a technology of obtaining chitosan there was used deacetylation of chitin (50 g dried shrimp shells), produced in accordance with the previously developed technology using NaOH treatment solution of different concentrations (2.5, 5, 7.5, 10 and 12.5 mol) and by heating (30, 45, 60, 75 and 90°C), which dramatically reduced the time of deacetylation. The dependence of the properties of the chitosan on the concentration of NaOH, temperature and duration of the experiment was examined. The optimal mode of chitosan obtaining – treatment with a solution NaOH with concentration of 10 M at 90°C for 2 hours was set. Chitosan obtained in these conditions, had a degree of deacetylation of 83.53%. The water binding capacity of the product was 521.65%, fat binding capacity was 405.65%, the solubility – 55.65%. The resulting chitosan is able to accumulate and transfer the valuable biologically active substances that have a high biodegradability, perform anti-microbial and anti-fungal activity, allowing to use it in food production.

Key words: Egypt, green shrimp, chitin, chitosan, degree of deacetylation, concentration.

Introduction

Approximately 70% of the landed value of shellfish is rejected as offal. This abundant waste material has either to be discarded or converted to value added products, and this has led to the production of several useful biochemical and nutrients, such as chitin, pigments and seafood peptones from these by products [1]. It is frequently present as a cell wall material in plants, and in the cuticle of animals. In addition, chitin in animal tissues are frequently calcified, such as in the case of shellfish. Some fungi contain chitosan; however, it is commercially produced by the deacetylation of chitin [2].

Chitosan is a natural biopolymer derived by deacetylation of chitin, a major component of the shells of crustacean such as crab, shrimp, and crayfish. During the past several decades, chitosan has been receiving increased attention for its commercial applications in biomedical, food, and chemical industries [3]. Chitosan is now widely produced commercially from crab and shrimp shells wastes.

Several techniques to extract chitin from different sources have been reported. The most common method is referred to as the chemical procedure. The chemical method for isolation of chitin from crustacean shell biomass involves various major steps: elimination of inorganic matter (calcium carbonate) in dilute acidic medium (demineralization), and usually demineralization are accomplished by using HCl. Followed by extraction of protein matter in alkaline medium (deproteinization), and it is traditionally done by treating shells wastes with aqueous solutions of NaOH or KOH. The effectiveness of alkali deproteinization depends on the process temperature, the alkali concentration, and the ratio of its solution to the shells [4–6].

Therefore, this study aimed to evaluate define the optimum parameters (acid /alkaline concentration, temperature and time) to extract chitosan from shrimp shells. The chemical compositions, functional properties of shrimp shell chitosan were studied.

I. Material and methods

Shells of green shrimp *Penaeus semisulcatus* were purchased from the *Abou Ghalli* Company for trading and exporting *Alabour* market, Egypt. The shells were manually scraped (free of loose tissue), collected and brought to the laboratory in the same day. Whenever, the shells were brought to the laboratory they were frozen immediately (at –18°C) and stored for further analysis. The shells were first washed several times with tap water and rinsed several times with distilled water. The rinsed shells were dehydrated in an electric draft oven at 45°C tell drying. The dried shells were grounded in a

grinder (Braun Biotech International GMBH. D.34212 Melsungen, Germany) to pass through a 1.6 mm sieves and stored at 4ºC in tight dark glasses till they were subjected to demineralization and deproteinization process. Generally, the raw material is crushed, washed with water or detergent and cut into small pieces. The mineral content of the exoskeleton of the different crustaceous is not the same and consequently different treatments may be used. The present work is the first systematic trial to investigate the extraction of chitosan from different indigenous sources in Egypt. A preliminary experiment to define the optimum demineralization condition of shrimp shells was carried out using 50 g dried shrimp shells.

Demineralization was carried out using 1 M HCl at 45°C for 2 h with a solution/solid ratio of 1/15 v/w. The HCl, which showed the highest ash reduction rate (2 M) was applied during determination of the optimum temperature (45°C). The temperature (45°C) resulted in the highest ash reduction rate was applied during determination of the optimum treatment time (2 h). The optimum conditions for demineralization treatment were 2M HCl at 45°C for 2 h. A preliminary experiment to define the optimum conditions for deproteinization of demineralized shrimp shells and deproteinized was carried out using 50 g demineralized dried shrimp shells. Deproteinization was carried out using NaOH concentrations (1 M NaOH) at a temperature of 75°C for 4 h with a solution/solid ratio of 1/15 w/v. The NaOH which showed the highest protein reduction rate (1 M) was applied during determination of the optimum temperature (75°C). The temperature (75°C) showed the highest ash reduction rate was applied during determination of the optimum treatment time (4 h). The optimum conditions for deproteinization treatment of chitin were 1 M NaOH at 75°C for 4 h. The raw materials were obtained in solid form from the different sources, washed with water, desiccated at room.

Preliminary experiments were carried out by refluxing chitin in strong NaOH solution at normal atmosphere. The experiments took more than 2h producing low deacetylation content and the reaction was accompanied by drastic degradation of the final chitosan. To avoid long heating times, the refluxing in alkaline solution was tried in an autoclave under two atmospheres pressure. The heating lasted for several hours (2–3 h) and still the resulting chitosan was partially soluble in acetic acid indicating the low deacetylation extent. A preliminary experiment to define the optimum deacetylation condition of shrimp shell chitin was carried out using 50 g dried shrimp shell chitin (demineralized and deproteinized). Deacetylation was carried out using different NaOH concentrations (10% NaOH) at a temperature of 90°C for 4 h with a solution/solid ratio of 1/15 v/w. The NaOH which showed the highest degree of deacetylation (DD%) by using 10% NaOH, was applied during determination of the optimum temperature (90°C). The temperature (90°C) resulted in the highest of deacetylation (DD%) was applied during determination of the optimum treatment time (2 h). Finally, the optimum conditions 10% NaOH, 90°C and 4 h which recorded the highest degree of deacetylation (DD%) were applied to produce shrimp shell chitosan. It has indicated that [7, 8] deacetylation of chitin can be highly facilitated by steeping in strong sodium hydroxide solution at room temperature before heating. This approach was then adapted and the effect of steeping time on the feasibility of deacetylation was investigated.

Proximate composition. The procedure [9] was followed in the determination of moisture (method No. 32.1.03), crude fat (method No. 32.1.13), crude fiber (method No. 32.1.15), crude protein (method No. 32.1.22), and total ash (method No. 32.1.05). Total carbohydrate content was calculated by difference.

Measurement of Degree of Deacetylation (DD%). The acid–base titration method was used to determine the DD from the amino group content in chitosan. Chitosan (0.3 g) was dissolved in 30 ml of HCl standard solution (0.1 mol/l). Methyl orange and aniline blue mixing indicators were added [10]. A standard solution of 0.1 M NaOH was used for titration until the solution became blue green. The following formulas were used to calculate the DD:

$$(-NH_2)\% = \frac{0.016(C_1V_1 - C_2V_2)}{W}100;$$

$$DD\% = \frac{203(-NH_2\%)}{16 + 42(-NH_2\%)}100.$$

Where C1, V1, C2, and V2 are the concentrations and volumes for the HCl standard solution and NaOH standard solution, respectively, and W is the weight of the sample.

Solubility, Water Binding Capacity (WBC) and Fat Binding Capacity (FBC) of chitosan

Solubility. Chitosan (0.1 g) was placed into a centrifuge tube (known weight) then dissolved in 10 ml of 1% acetic acid for 30 min using an incubator shaker operating at 240 rpm and 25ºC. 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 residue particles were washed with distilled water (25 ml) then centrifuged at 10.000 rpm [4]. The supernatant was removed and the residue dried at 60°C for 24 h. Finally, weighed the dried residue and the percentage of solubility was calculated as followed:

$$\text{Solubility of chitosan } (\%) = \frac{(\text{Initial weight of tube + chitosan}) - (\text{Final weight of tube + chitosan})}{(\text{Initial weight of tube + chitosan}) - (\text{Initial weight of tube})} 100 .$$

Water and fat binding capacity. Water binding capacity (WBC) and fat binding capacity (FBC) of chitosan were measured using the method of [4, 11]. Briefly, the procedure was carried out by weighing a centrifuge tube containing 0.5 g sample, adding 10 ml of water or corn 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 5s every 10 min and centrifuged at 3200 rpm for 25 min. The supernatant was decanted and the tube was weighed again. WBC and FBC were calculated using the following formula:

WBC (%) = [water bound (g)/sample weight (g)] 100.

FBC (%) = [fat bound (g)/sample weight (g)] 100.

Statistical analysis. Statistical analysis was carried out using analysis of variance (ANOVA), and least significant difference (LSD) was obtained to compare the means of treatments, using Costat version 6.311 (Copyright 1998–2005, CoHort Software). Duncan's multiple range test was used to compare the treatment means [12].

II. Results and discussion

Proximate composition of crude shrimp shells (moisture, protein, fat, ash, carbohydrates, and fiber content), was presented in Table 1. The protein content was 36.63%, while ash content was 44.96%. Our results showed a low content of lipids (4.85%), while shrimp shells total fiber and total carbohydrates content were 6.18 and 7.38%, respectively. These results are in the same trend of [13–15] who stated that dry *Crangon crangon* shells consist of 10–38% proteins, 31–44% minerals. While ours are close to that reported by Hopkins [16]. The content of total fiber was 29.41% in chitin compared to 6.18% of shrimp shells waste.

Table 1

Chemical composition of crude shrimp shells (on dry weight basis)

Samples	Chemical composition, %					
	Moisture	Protein	Fat	Carbohydrates	Ash	Fiber
Crud shrimp	13.05	36.63	4.85	7.38	44.96	6.18

Deacetylation treatment (chitosan production)

Effect of NaOH concentration. Increasing the concentration of NaOH up to 10 M resulted in a significant ($p \leq 0.05$) increase in the degree of deacetylation Table 2. However, the degree of deacetylation was significantly ($p \leq 0.05$) decreased by NaOH concentration increasing than 10 M. Water binding capacity was significantly ($p \leq 0.05$) increased with increasing in NaOH concentration up to 10 M. While, it was significantly ($p \leq 0.05$) decreased when the concentration of NaOH increased up to 12.5 M. The same trend was observed in fat binding capacity. Solubility was significantly ($p \leq 0.05$) affected by concentration of NaOH. Increasing the concentration of NaOH resulted in a significant ($p \leq 0.05$) decreased in solubility. These results are in good agreement with those obtained [8, 17] who indicated that chitosan could be produced from chitin by deacetylation with highly concentrated (10–12.5 M) solutions of sodium hydroxide. The WBC values of chitosan were similar to that reported by [18, 19] ranging from 523 to 539%. According to [20] WBC and FBC five commercial

chitosan products ranged from 458 to 805 and 314 to 535%, respectively. Water binding capacity and fat binding capacity of six commercial chitosan products observed by [11] were in the range of 355–611 and 217–403%, respectively. The WBC (526.49%) and FBC (487.27%) of the obtained chitosans observed in this study were comparable to those of commercial chitosans reported by [11, 19, 20].

Table 2

Effect of different concentrations of NaOH on the degree of deacetylation and functional properties of shrimp shells chitosan

Concentration NaOH, M	Degree of deacetylation and functional properties, %			
	DD%	WBC	FBC	Solubility
2.5	49.87 [e]	501.20 [bc]	422.89 [c]	67.03 [a]
5	53.20 [d]	489.94 [c]	412.32 [d]	62.51 [b]
7.5	60.53 [c]	511.01 [b]	464.73 [b]	55.37 [c]
10	75.83 [a]	526.49 [a]	487.27 [a]	51.40 [d]
12.5	72.83 [b]	506.22 [b]	415.63 [d]	47.39 [e]
L.S.D	2.55	11.64	4.17	2.60

[*] Means in the same column with different letters are significantly different at ($p \leq 0.05$).

Effect of temperature. Effect of temperature on the degree of deacetylation and functional properties of shrimp shells chitosan were presented in Table 3. The degree of deacetylation was not significantly ($p > 0.05$) affected by the temperature of extraction up to 60°C. However, more than 60ºC resulted in a significant ($p \leq 0.05$) increase in the degree of deacetylation. The highest ($p \leq 0.05$) water binding capacity was noticed when the chitin extracted at 90°C (528.53%) while the lowest ($p \geq 0.05$) value was observed when the chitin treated at 45°C (450.60%). The fat binding capacity had the similar trend of water binding capacity. Solubility was significantly ($p \leq 0.05$) affected by temperature. Solubility was significantly ($p \leq 0.05$) increased with temperature up to 60°C. The temperature higher than 60°C resulted in a significant ($p \leq 0.05$) decrease in the solubility. Conversion of chitin to chitosan involves a treatment with concentrated NaOH at high temperature for the removal of acetyl moieties from the N-acetylglucosamine units of chitin. Thus, the contact time of chitin with e. g. 50% NaOH for deacetylation plays a crucial role for the viscosity of chitosan [13, 15]. Degree of deacetylation is an important parameter affecting solubility, chemical reactivity and biodegradability. Depending on the source and preparation procedure, DD may range from 30 to 90% [21]. Water binding (WBC) and fat binding capacities (FBC) of commercial chitosan are lower than the extracted chitosan. Water and fat binding capacities of different commercial chitosan were reported as 458–805 and 314–535%, respectively, by [22]. WBC and FBC of six commercial chitosan products observed by [4, 11] were in the range of 355–611 and 217–477%, respectively. The WBC (492.67%) and FBC (383.04%) of commercial chitosan in the present study were compatible to those reported by [22, 23]. Water solubility of chitosan has been found to rely on degree of deacetylation (DD) and randomly 50% deacetylated chitosan is soluble in neutral water or even under alkaline conditions. On the other hand, water-soluble chitosan with about 50% DD can be prepared from chitosan by N-acetylating with acetic anhydride [23–25].

Table 3

Effect of different temperatures on the degree of deacetylation and functional properties of shrimp shell chitosan

Temperature, °C	Degreeof deacetylation and functional properties, %			
	DD%	WBC	FBC	Solubility
30	74.97 [c]	498.43 [d]	414.17 [b]	54.31 [c]
45	75.16 [c]	450.60 [e]	391.36 [d]	60.02 [b]
60	76.67 [c]	502.56 [c]	385.52 [e]	64.55 [a]
75	79.28 [b]	510.51 [b]	407.33 [c]	57.70 [b]
90	82.76 [a]	528.53 [a]	512.91 [a]	50.41 [d]
L.S.D	2.55	2.40	5.07	2.70

[*] Means in the same column with different letters are significantly different at ($p \leq 0.05$).

Effect of time. No significant ($p > 0.05$) differences were detected in the DD% between the chitin treated for 4 h (76.83%) and that treated for 5 h Table 4.

The highest value of WBC was obtained when the shrimp shell chitin treated for 2 h (537.71%), while the lower water binding capacity was detected when shrimp chitin treated for 5 h (491.27%). Fat binding capacity showed the highest value when shrimp shell chitin treated for 4 h (424.48%), however it showed low values by using 1 and 2 h (311.17 and 345.89%) respectively. The effect of heating time on the degree of deacetylation and of solubility of shrimp shell chitosan showed clear decrease in both parameters due to the increase of time.

The results (Tables 2–4) indicated that the optimum condition for producing shrimp shell chitosan were extraction with 10 M NaOH at 90°C for 2 h. Shrimp shell chitosan produced under the optimum condition had 83.53% degree of deacetylation, 521.65% water binding capacity and 405.65% fat binding capacity. It seems that the purity for the product to be considered as chitosan was 80.5%. Accordingly, all crab chitosans were nearly pure chitosans. For the purity of crab chitosan products, the reaction time of 60 min was sufficient [26]. Water binding (WBC) and fat binding capacities (FBC) of commercial chitosan are lower than the extracted chitosan. Water and fat binding capacities of different commercial chitosan were reported as 458–805 and 314–535%, respectively, by [22]. Water Binding capacity and FBC of six commercial chitosan products observed by [11] were in the range of 355–611 and 217–477%, respectively. The WBC (492.67%) and FBC (383.04%) of commercial chitosan in the present study were compatible to those reported by [22, 27].

Table 4

Effect of different times on the degree of deacetylation and functional properties of shrimp shell chitosan

Time, h	Degree of deacetylation and functional properties, %			
	DD%	WBC	FBC	Solubility
1	85.35 [ab]	519.75 [c]	311.17 [e]	53.79 [b]
2	86.67 [a]	537.71 [a]	345.89 [d]	59.79 [a]
3	81.94 [b]	517.36 [c]	418.46 [b]	48.55 [c]
4	76.83 [c]	531.69 [b]	424.48 [a]	43.71 [d]
5	78.01 [c]	491.27 [d]	383.76 [c]	40.99 [e]
L.S.D	3.67	2.40	2.08	1.34

[*] Means in the same column with different letters are significantly different at ($p \leq 0.05$).

Effect of optimum condition for producing shrimp shell chitosan on the degree of deacetylation and some functional properties. The effect of optimum conditions for producing shrimp shell chitosan (10 M NaOH at 90°C for 2 h), on the degree of deacetylation and some function properties. Shrimp shell chitosan produced under the optimum condition had 83.53% degree of deacetylation, 521.65% water binding capacity and 405.65% fat binding capacity. The solubility of the produced chitosan reached to 55.65%. The values obtaining for the produced shrimp shell chitosan are too close to those stated by several investigators. It is stated that the degree of deacetylation [28] is an important parameter affecting solubility, chemical reactivity and biodegradability. Depending on the source and preparation procedure, DD may range from 30 to 90%.

Among many characteristics, the degree of deacetylation is one of the most important chemical characteristics, which influences the performance of chitosan in many of its applications [29–31]. In addition, DD, which reveals the content of free amino groups in the polysaccharide [31] can be used to differentiate between chitin and chitosan. It is reported [10, 26] DD value is 89.7%.

Fat binding capacity signifies how the chitosan can easily bind or absorb fat especially when used in the manufacturing of dietary supplements. The trend recorded for water binding capacity was similarly observed for fat binding capacity. Values for un-irradiated and irradiated shrimp chitosan were 560.55 and 431%, respectively, for local frytol, while that for the commercial chitosan samples (un-irradiated and irradiated) were 490.10 and 529.05%, respectively. Reported that the average fat binding capacities of craw fish chitosan and commercial crab chitosan for soybean oil were 706 and 587%, respectively. The values obtained in this research were lower than the values reported by [32]. Water binding (WBC) and fat binding capacities (FBC) of commercial chitosan are lower than the extracted

chitosan. Water and fat binding capacities of different commercial chitosan were reported as 458–805 and 314–535%, respectively, by [20]. WBC and FBC of six commercial chitosan products observed by [23] were in the range of 355–611 and 217–477%, respectively. The WBC (492.67%) and FBC (383.04%) of commercial chitosan in the present study were compatible to those reported by [31, 32].

Chitin was subjected for deacetylation using alkali where the acetamide group was converted into amino group and nitrogen content showed obvious increase which further increased when the deacetylation process was repeated. The degree of deacetylation of the chitosan after second deacetylation was higher than that of chitosan after first deacetylation as reflected in the nitrogen content values [33].

Conclusion

Chitin has been extracted from different sources indigenous for Egypt. It was found that constitute a pollution hazard in Egypt could be an economic source of chitosan. The obtained chitin was hydrolyzed using steeped in a strong sodium hydroxide solution for different periods of time, which reduced the deacetylation time dramatically to produce chitosan with reasonably high molecular weight, and high deacetylation percent. The best condition to production of shrimp shell chitosan was 10 M NaOH at 90ºC for 2 h. The optimum conditions for producing shrimp shell chitosan (10 M NaOH at 90°C for 2 h), on the degree of deacetylation and some functional properties. Shrimp shell chitosan produced under the optimum condition had 83.53% degree of deacetylation, 521.65% water binding capacity and 405.65% fat binding capacity. The solubility of the produced chitosan reached to 55.65%. The values obtaining for the produced shrimp shell chitosan are too close to those stated by several investigators.

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ОПТИМАЛЬНЫЕ УСЛОВИЯ ПОЛУЧЕНИЯ ХИТОЗАНА ИЗ ПАНЦИРЯ ЗЕЛЕНОЙ КРЕВЕТКИ *PENAEUS SEMISULCATUS*

Исследовались условия получения хитозана из панциря (отходов от разделки панциря) зеленой креветки *Penaeus semisulcatus,* отловленной в Египте. Деминерализацию сырья проводили используя 1 М HCl при температуре 45 °С в течение 2 часов, депротеинизация осуществлялась с использованием 1 М HCl при температуре 75 °С в течение 4 часов. В качестве процедуры получения хитозана использовалось деацетилирование хитина (50 г сушеных панцирей креветок), полученного по разработанной ранее технологии, обработкой раствором NaOH различных концентраций (2,5; 5; 7,5; 10 и 12,5 М) и при нагревании (30, 45, 60, 75 и 90 °С), что резко сократило время деацетилирования. Была исследована зависимость свойств хитозана от концентрации раствора NaOH, температуры и продолжительности эксперимента. Установлен оптимальный режим получения хитозана – обработка раствором NaOH в концентрации 10 М при температуре 90 °С в течение 2 часов. Хитозан, полученный в этих условиях, имел степень деацетилирования 83,53 %. Водоудерживающая способность продукта составляла 521,65 %, жироудерживающая способность – 405,65 %, растворимость – 55,65 %. Полученный хитозан способен накапливать и переносить ценные биологически активные вещества, имеет высокую биоразлагаемость, проявляет противомикробную и противогрибковую активность, что позволяет использовать его при производстве продуктов питания.

Ключевые слова: Египет, зеленая креветка, хитин, хитозан, степень деацетилирования, концентрация.

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