Chitosan Preparation from Persian Gulf Shrimp Shells and Investigating the Effect of Time on the Degree of Deacetylation

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ABSTRACT

Chitosan is an amino polysaccharide which can be prepared from shrimp shells with several applications in medicine and tissue engineering. The most important parameter, that characterizes chitosan and its applications, is its degree of deacetylation. In this research the influence of deacetylation time on the quality of the produced chitosan was investigated by measuring the amount of glucosamine and acetyl glucosamine. The amount of glucosamine in the sample was measured using HPLC analysis based on derivation method. By deacetylation of the extracted chitin in 90 and 180 minutes, chitosan with deacetylation degree of 69.75% and 77.63% was obtained, respectively. Therefore, by increasing the deacetylation period in a constant temperature condition and NaOH concentration, the deacetylation degree is increased.

Keywords: Chitosan; Tissue Engineering; Chitin; Deacetylation; Shrimp

INTRODUCTION

Chitin is a natural polymer which is found in exoskeleton animals like shrimps. After extraction processes in food industries, these shells are usually useless. There are several methods which are being suggested for extraction of this natural polymer [1].

Chitin is a polysaccharide with the structure of "Poly N-Acetyl Glucosamine"(Figure 1).

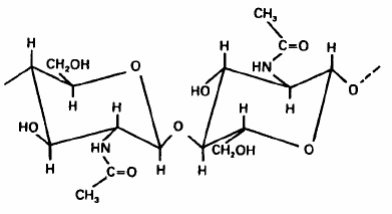

Figure1. Chitin Structure

Chitosan is taken from chitin. This cationic polymer can be prepared by deacetylation of the chitin (Figure 2) [2]. Actually, the main difference between chitin and chitosan is the percentage of the acetyl groups in their chemical structure.

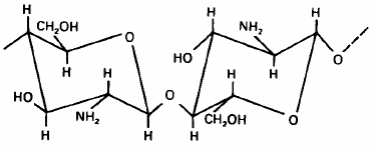

Figure 2. Chitosan Structure

If the percentage of acetyl glucosamine is more than 50%, we have chitin and if this percentage is less than half, the material is called chitosan. Chitosan is soluble in low concentrations of acetic acid; but chitin is insoluble [3&4]. First type amine groups present in chitosan cause this polymer to be soluble in acetic acid by absorbing protons when the pH of the solution is less than 6. Chitosan is biocompatible and is not toxic for live cells. Also, it is biodegradable. Lysozyme is the enzyme which degrades this biopolymer and hydrolyzes the remaining acetyl glucosamine. The hydrolysis process is slow, if the degree of deacetylation is high; this property of chitosan makes it so attractive in tissue engineering for designing and developing scaffolds for controlling the degradation period [4]. Chitosan has antibacterial activity [5] which can be prepared in the form of film or hydro-gel [6 & 7 & 8] to be used in burn and wound dressing and also for fabricating suturing threads [9]. Chitosan has several properties, such as biocompatibility, biodegradability, homeostasis and antibacterial activity, due to which it can be used in the treatment of wound [10].

By electro spinning, nanofibers of chitosan and its derivatives can be created with diameter of a few nanometers which is comparable with collagen fibers in extracellular matrix in range of 30 nm to 130

nm in diameter. Its biocompatibility and biodegradability enables researchers to produce in vitro biomimetic tissues. Application of chitosan as natural cationic polymer in gene transfer is under investigation which seems more useful than the virus method. Some chitosan derivatives have anti-coagulant properties, while others are useful for drug delivery such as anti cancer drugs [11].

Most proteins, fats and pigments of shrimp shells are separated by hydrolysis in the basic solution. Shrimp shells contain minerals; i.e., calcium carbonates which can be removed using acidic solution (Reaction 1) [12].

$$CaCO_3 + 2\,HCl \rightarrow CaCl_2 + CO_2 + H_2O$$

Reaction 1

In this step, chitin is obtained and for converting it to chitosan, acetyl groups must be dissociated from amine groups by hydrolysis process. This process is done using hot solution of concentrated basic solution, such as sodium hydroxide [13].

In this study, proteins and minerals were removed from shrimp shells, and after the second deproteinization, the influence of the time of deacetylation was investigated.

MATERALS AND METHODS

For separation of proteins, minerals and deacetylation, sodium hydroxide and 37% hydrochloric acid were used. For the hydrolysis of chitosan, 37% hydrochloric acid and sodium hydroxide from Merck Company were used. For determining deacetylation degree, boric acid, acetonitrile (for HPLC grade) and 9-fluorenylmethoxycarbonyl chloride from Merck Company and glucosamine from Sigma-Aldrich Company were used. Double distilled water was used for rinsing of prepared chitosan after deacetylation and deionized water was prepared from equipment of Millipore (Direct-Q, Millipore Corp., Bedford, MA).

HPLC system (Adept Series, Cecil Instruments Ltd, with LIRP8-104433 column) was used for determining deacetylation degree. The shrimp shells which were collected from Persian Gulf were rinsed with water and then suspended in sodium hydroxide (NaOH) solution at room temperature for 24 hours to remove their proteins. The shells had to be fully suspended in the solution and it would be better to be stirred. Then, the shells were rinsed with water to remove sodium hydroxide and proteins.

In this step, the shrimp shells were suspended in 4% hydrochloric acid solution at room temperature. Carbon dioxide bubbles appeared in the solution which shows conversion of calcium carbonate to calcium chloride. After 24 hours, the shells were quite squashy and were rinsed with water in order to remove the acid and calcium chloride from the medium.

After removing the proteins in the first step, some proteins and pigments may still remain in deep layers of the shells. The shells obtained after removing calcium minerals were suspended in 2% sodium hydroxide for 24 hours and then washed, dried, and grinded.

In the fourth step the chitin was deacetylated to form chitosan. For this purpose, 10% (w/v) of chitin in 60% sodium hydroxide was heated for 90 minutes at 130^{o}C. Then, the solution was cooled and the prepared chitosan was rinsed with distilled water until pH of the solution reached to 7. In the second sample the above method was repeated, but for 180 minutes.

To determine the deacetylation degree, firstly, an appropriate amount of the prepared chitosan was hydrolyzed and a mixture of glucosamine and acetyl glucosamine was prepared. By the HPLC method with LIRP9-10-4433 column and derivation of glucosamine with Fmoc-Cl and absorption wavelength of 254 nm, amount of glucosamine was determined in hydrolyzed sample. Fmoc-Cl reacts with first type amine group. The relation of amount of glucosamine to chitosan settles the deacetylation percent [14].

About 20 mg of dried chitosan were added in 20 ml solution of 37% hydrochloric acid with concentration of 8 molar and was heated at 100^{o}C for 4 hours. In order to verify whether chitosan had been hydrolyzed completely, the solution was stored in a closed container at room temperature for 24 hours.

Then, the pH of the solution was adjusted to 7.0 by adding 2 molar concentration sodium hydroxide solution.

Borate buffer solution was prepared by the following procedure: 10 ml of boric acid solution (0.4 molar) was prepared and its pH

was set at 7.0 by adding sodium hydroxide (0.2 molar). About 100 micro-liter of this solution was mixed with 100 microgram per milliliter of Fmoc-Cl in acetonitrile and 10 micro-liter of the sample (prepared chitosan) and was reacted at least for 30 minutes. Then, the solution was prepared for injection to HPLC system.

Simultaneously, the same process was performed in case of glucosamine (1000 ug/ml) of Sigma-Aldrich Company as a pure standard control. Then, by comparing the result values (prepared chitosan) to the standard control, amount of glucosamine in hydrolyzed sample was determined.

RESULTS

Results of this study are presented in figures 3-6. According to the method described in the methodology section, the chitosan was prepared from shrimp shells of Persian Gulf in the Nano-medicine and Tissue Engineering Research Center of Shahid Beheshti University of Medical Sciences, which is presented in the figure3.

Figure 3. Prepared chitosan from shrimp shells in Nanomedicine and Tissue Engineering Research Center

The most common method for determining the deacetylation degree is HPLC analysis by cation exchange column. In this technique glucosamine which together with acetyl glucosamine is the product of hydrolysis of chitosan is measured. The result is proportional with the deacetylation percentage [12].

Unfortunately, this column is not a routine device in most medical research centers. So, another technique was set up which is known as derivation method. After hydrolysis of chitosan, Fmoc-Cl is added under certain conditions. This material has a chromophore part which could absorb a specific wavelength in UV spectrum. On the other hand, Fmoc-Cl could just react with glucosamine and acetyl glucosamine has no reaction with it. These two features make Fmoc-Cl useful for indicating the amount of glucosamine in sample solution by HPLC analysis. So, after that the percentage of deacetylation could be determined [14].

In the three chromatograms of the present work (Figures 4-6), the Y axis is absorbance (mA) and the X axis is time. As shown in Figure 4, two peaks occurred close to each other, marked as 3 and 4 which are related to absorption of Glucosamine in the standard chromatogram. In this finding, total areas under two curves are related to concentration of glucosamine (Figure 4). Peak 5 indicates absorption of Fmoc-Alcohol which is formed from reaction of extra Fmoc-Cl with water.

In the chromatogram of the first hydrolyzed chitosan sample (Figure 5), peaks 4 and 5 are correspondent to glucosamine and peak 9 is related to absorption of Fmoc-Alcohol. Peaks 6, 7 and 8 indicate impurities.

In the chromatogram of the second hydrolyzed chitosan sample (Figure 6), peaks 5 and 6 are correspondent to glucosamine and peak 10 occurred because of Fmoc-Alcohol absorption.

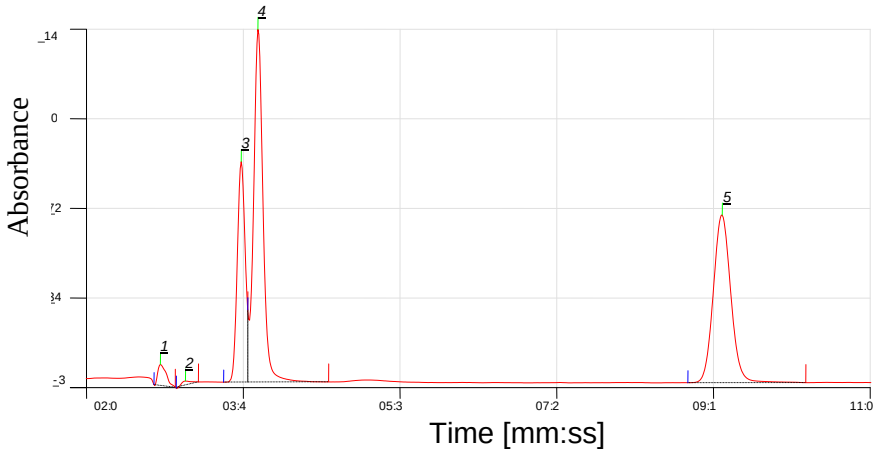

Figure 4. Standard chromatogram of glucosamine for 1 milligram per milliliter solution

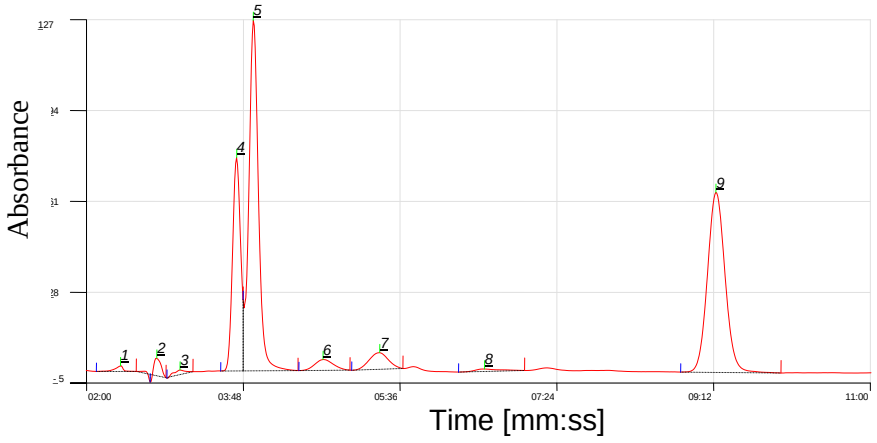

Figure 5. Chromatogram of hydrolyzed chitosan of sample 1 with concentration of 20 milligram of raw material in 15 ml of acidic solution

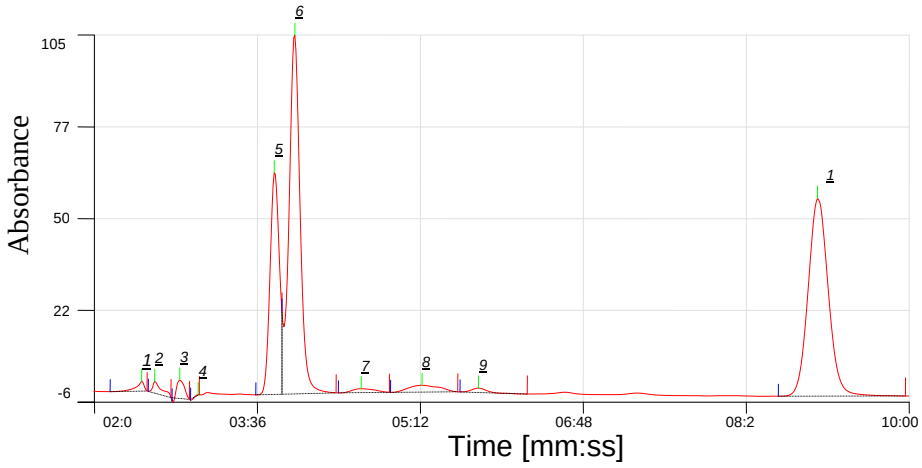

Figure 6. Chromatogram of hydrolyzed material in sample 2 with concentration of 20 milligram of raw material in 20 ml of acidic solution

DISCUSSION

The Degree of Deacetylation (D.D.A.) can be calculated by the following equation [16]:

$$DDA = 100 - \left[\frac{mole\ of\ NAG}{mole\ of\ NAG + mole\ of\ NG} \times 100\right] \quad (1)$$

in which NAG is N-Acetyl Glucosamine and NG is N-Glucosamine. With simplification of the above formula, equation 2 is derived from equation1:

$$DDA = \frac{\frac{m_G}{M_G}}{\frac{m_G}{M_G} + \frac{m_{AG}}{M_{AG}}} \times 100 \quad (2)$$

in which mG is the mass of obtained Glucosamine from hydrolyzed sample and mAG is the mass of Acetyl Glucosamine of hydrolyzed sample and MG = 179.17 and MAG = 221.21 are the molecular mass of the Glucosamine and Acetyl Glucosamine, respectively. The mass of unhydrolyzed chitosan is:

$$m_{\text{Chitosan}} \approx m_G + m_{AG} \quad (3)$$

Based on equations 2 and 3, equation 4 is proposed which is as follows:

$$D.D.A. = \frac{m_G \times 100}{m_G + (m_{Chit} - m_G) \times 0.80995} \quad (4)$$

For determining of D.D.A., 20 mg of prepared chitosan was hydrolyzed. In the solution of the first sample, 13.025 mg glucosamine was measured. By equation 4, D.D.A was computed as being 69.75%. In the second solution 20 mg of chitosan was hydrolyzed and 14.751 mg glucosamine was obtained; thus, the measured D.D.A was equal to 77.63%.

Deacetylation of obtained chitin from shrimp shells for 24 hours with 50% caustic soda solution at 40oC improved the D.D.A. value up to 75% [15]. In the same research, it has been shown that the deacetylation could be increased to 87% with the same concentration of NaOH at 65oC for 20 hours.

Pranee Lertsutthiwong, et al [12] in a study on Effect of Chemical Treatment on the Characteristics of Shrimp Chitosan applied 50% sodium hydroxide solution for deacetylation at 90oC for 5 hours, found an increase in the D.D.A. value to 88%. The observation of the above author suggests that higher temperature results in higher D.D.A, which is similar to our findings.

Considering the fact that the temperature is an influencing factor to increase or decrease DDA-value, and deacetylation period, respectively, this research has shown the influence of temperature as a good candidate for manipulating the D.D.A. value and improving the deacetylation period.

The present observations indicate that the prepared chitosan in this study is soluble in 1% acetic acid solution and increasing the time of deacetylation (from 90 to 180 minutes) could result into an increase (7.9%) in degree of deacetylation.

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