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Semi-synthesis of Chitosan with High Molecular Weight and Enhanced Deacetylation Degree

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Abstract

Chitosan is a cationic polymer with different biomedical, biotechnological, environmental, industrial and agricultural applications. Various methods were suggested to prepare chitosan from its natural ancestor polymer, chitin, but the controlling of the molecular weight and degree of deacetylation of the resulting polymer was a problem where there is an inverse relationship between them. This study aimed at modifying the sequence of deacetylation process in combination with number of cooling/heating cycles for chitin to produce chitosans with high molecular weight as well as enhanced deacetylation. The produced chitosans were tested for their molecular weights, chemical structure, deacetylation degrees, antioxidant properties and purity. With the proposed modification of polymer deacetylation, different chitosans of different degrees of deacetylation but similar molecular weights were prepared. This may represent a more economical method for the production as well as applications of different types of chitosan.

Keywords: Chitin; Chitosan; Biopolymer; Degree of deacetylation; Heating/Cooling cycle

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Introduction

As the second most abundant natural polymer after cellulose [1], chitin is a principal component in the exoskeletons of silkworms, many insects and as supporting material in many marine organisms (e.g., Shrimp shells, lobster, krill, crab and bone plates of squid). From economic and technological points of view, these byproducts represent abundant, cheap, ecologically-friendly, renewable resources for the extraction of chitin and its derivatives where several million tons of chitin are harvested annually in the world [2]. During the past 20 years, a substantial amount of work has been reported on the synthesis of chitosan, as a polysaccharide derivative of chitin [3] with different physicochemical characteristics, and deacetylation degrees [4], and found various potential biomedical applications [5,6]. The major chemical structure of chitin is composed of the D-Glucosamine monomers and it becomes chitosan when the C-2s of these monomers substitute total or partial acetyl amines with amine groups [7]. This gives the unbranched cationic copolymer chitosan with a structure consisting of 2 main repeated units linked by β (1→4) glycosidic bonds; these are: (2-amino-2-deoxy-β-glucopyranose) and (2-acetamido-2-deoxy-β-D-glucopyranose) with the energetically favorable ($^4C_1$ chair) form [2] available in different grades depending upon the degree of acetylated moieties [8], as shown in (**Figure 1**). These changes are accompanied by changing in the average molecular weights as well as the chemical and biological properties.

Chitosan (Ch) is a pseudonatural, renewable cationic polysaccharide of superior biodegradability [9], biocompatibility [10], low-toxicity [11] with bioabsorbable and easy chelating power. It is an interesting glycosaminoglycan possessing rare bioactivity [12] with gel-forming capability either of the polymer itself [11] or with other compounds [12]. It has the ability to be injected into the body as a liquid below the Lower Critical Solution Temperature (LCST) to form a gel in situ at body temperature [13]. Many methods have been adopted for the extraction of chitosan from chitins with the aid of some enzymes [14], by fermentation with microorganisms [15] or with direct isolation using a refluxing method [16]. The chemical method of extraction is widely used to obtain chitin with a high quality

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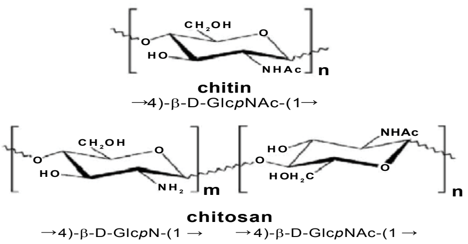

Figure 1 The chemical structures of Chitin and Chitosan. (GLcN refers to glucosamine and GlcNAc refers to N-acetylglucosamine).

by removing protein, pigments, lipids and inorganic materials (mainly CaCO3). In many chitin producing industries, the deproteination step is achieved by treatment with NaOH, followed by the demineralization (decalcification) step using HCl [2].

Degree of Deacetylation (DD) refers to the percentage of the average number of D-Glucosamine units present in the polymer chains when the distribution of the 2 constitutive residues is random. The term Chitin usually refers to a copolymer of (DD less than 70%), while Chitosan represents a series of copolymers of (DD <70%) [10] with lactic- average M.W in the range (10-10^3 KDa) [2]. Chitin is generally insoluble in standard polar and non-polar solvents, while chitosan dissolves in diluted acidic solutions [17] below pH 6.0, suitable for quaternisation of the amine groups with macro intrinsic pKa value of 6.3 [18] giving it a high positive charge density to make water-soluble cationic polyelectrolyte able to form salts with inorganic and organic acids [19]. Different deacetylation conditions can be influenced by changing both inter and intra-molecular repulsion forces [20]. Biodegradability in living organisms is DD-dependent where it increases as (DD) decreases [21,22], and, therefore, lower DD-chitosans induce acute inflammatory responses, but those with high DDs produce a minimal response due to their lower degradation rate.

The 1st step deproteination of chitin, followed by 2nd step demineralization were proved to have hydrolyzing effects on the resulting polymer [23]. In spite of that, such degrading effects can be controlled by controlling the demineralization period to get the required chitosan molecular weight for the subsequent applications. NaOH concentration, reaction temperature, period and the interaction between them play dominant roles in influencing the DD [15,24,25]. Chinadit et al. [26] reported that a multi-step process is required to obtain high degrees of deacetylation at low temperatures, however, high DD at high temperature can be reached in one step as well. The DD is an important characteristic influencing the polymer performance in many of its applications. The interactions between chitosan and the cells increase as the DD rises due to the increase in the free amino groups-content. Other biological properties (e.g., Analgesic, antitumor, hypocholesterolemic, hemostatic, antimicrobial, and antioxidant properties) are also affected by the chemical and physical properties of chitosan [27,28]. The objective of this research was to evaluate effects of the cooling process on the deacetylation step; as a modification step, aiming to obtain chitosan with high degree of deacetylation, but with avoiding chain degradation.

Experimental

Extraction of chitin

These steps were carried out according to Gopalakannan et al. [29] with some modifications. freeze dried shrimp shell waste was cut into pieces, washed with tap water and deproteinized by boiling in 1 N NaOH (1:5 w/v) for 30 min. The alkali was drained, replaced with another amount and the process was repeated to remove the residual proteins. The colors of the solutions were noticed till becoming clear and the pieces were washed with tap water. The shells were demineralised by soaking in 1 N HCl (1:5 w/v) at 40°C for 30 min. The acid was then drained off and the pieces were washed thoroughly with tap water till reaching the neutrality followed with distilled water washing. The chitin product was dried at R.T and stored under dry conditions till the further deacetylation procedures.

Preparation of chitosan

The deacetylation steps were performed according to Roberts [2]. The chitin was soaked in 50% aqueous NaOH (1:20 w/v) and divided into two different patches which were boiled at 100°C for 2 hours divided into 4 equal times with the changing of NaOH. The first patch was then washed with water and nominated as (Ch-1). The second patch, denoted as (Ch-2) was stored in solution at 4-9°C till cooling, then reboiled in 50% aqueous NaOH for additional 2 hours, re-cooled and reboiled for additional 1 hour. Finally, the chitosan from the (2) patches was drained off, washed with tap water, then distilled water and dried at room temperature for several days. A control patch was prepared where a fraction of the (Ch-1) was heated with NaOH for additional continuous 3 hours without any cooling steps to activate the deacetylation process with measuring the corresponding average molecular weights and DD. A summary for the different deacetylation processes is shown in **Figure 2**.

Characterization of the prepared chitosan products

Solubility test: As for the solubility of each patch product as well as the parent compound, chitin was tested by formation

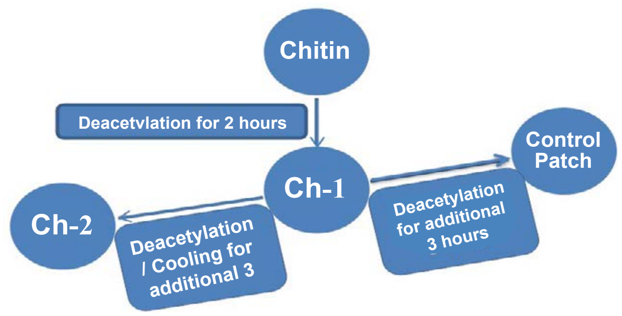

Figure 2 A summary for the deacetylation of the different patches.

of soluble polymer on preparation of (0.5% solution in 1% Acetic acid solution (HAc)) with continuous heating and shaking.

Fourier transform-infrared (FTIR) spectroscopic characterization: Chitosans samples were prepared in the form of KBr pellets based on the method of Sabnis and Block [30] where the pellets (powdered chitosan: KBr=1: 3) were dried at 80°C in hot air oven for 24 hours before analysis, then characterized by (JASCO FT/IR-6100 type A spectrophotometer, Japan) in the range of (800-4000 cm^{-1}) using the transmission (T%) mode. The% N-deacetylation values were determined using the equation proposed by Terayama; Domszy and Roberts [31,32], but with using the baseline, proposed by Baxter et al. [33] to compare the intensity of the (amide I) band in chitosan with that of the (OH groups). The following equation was applied:

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

Where: A_{1655}: The absorbance at 1655 cm^{-1} for the amide (N-H) band as a measure of the N-acetyl group content.

A_{3450}: Absorbance at 3450 cm^{-1} for the (OH) band as (internal standard) to correct for film thickness or differences in chitosan powder concentration.

1.33: Correction factor in case of (A_{1655}/A_{3450}) for fully N-acetylated chitosan.

Measurement of the protein content: After complete drying, the protein content% of chitin as well as each patch product was measured according to the Bradford method using Coomassie Brilliant Blue (G-250) as the dye reagent [34] with triplicate measurements for each patch. The standard curve was built using the BSA concentrations (1, 2, 4, 6, 8 and 10 mg/ml).

Measurement of the ash content: For each chitosan patch, ash content was measured after heating the samples (in triplicates for each) at 100°C for 24 h.

Determination of the total antioxidant activity of different chitosans: Each sample (1.3 mg) of the different polymer powder was dissolved to 10 ml with 50 mM phosphate buffer (pH 7) and added to 10 ml absolute ethanol containing 130 µl linoleic acid with adjusting the total volume to 25 ml with distilled water. After incubation of the closed flasks at 37°C in the dark for 6 days, the degree of oxidation was evaluated by measuring the level of ferric thiocyanate according to Mitsuda et al. [35] as follows: 100 µl of the resulting oxidized linoleic acid solution were mixed with [4.7 ml, 75% ethanol, 100 µl, 30% ammonium thiocyanate and 100 µl (0.02 M $FeCl_2$ solution, prepared in 3.5% HCl)], stirred for 3 min with measuring the absorbance at 500 nm. Taking distilled water as a negative control and α-tocopherol as a standard substance, the capacity of each polymer to inhibit the peroxide formation in the linoleic acid system was calculated as follows:

(%) Inhibition (Anti-oxidative capacity)=[1 - ($A_{sample}/A_{control}$)] *100

Where: A_{sample} and $A_{control}$ are the absorbance of samples and control, respectively.

Determination of the Average Molecular Weights (M.W) of the Chitosans: Molecular Weights of chitosans from the two patches as well as the control were determined by the following methods:

The viscometric method: The dynamic viscosities were measured using (Synchro-Lectric rotational Viscometer Model DVIV+ Pro, Brookfield Engineering Labs Inc., Middleboro, MA, USA) operated at 25°C using the spindle (No. CPE 41) that rotated at 25 RPM. The spindle was immersed at first in the solvent (1% acetic acid), and then on the 2 chitosan solutions of the concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1%). The viscosity average molecular weight (Mv) value for each chitosan type was calculated on the basis of the (MHS) equation:

$[\eta_{int}]$ [ml/gm]=K. M^a

Where: η_{int}: the intrinsic viscosity, obtained by blotting both the inherent (η_{inh}) and reduced (η_{red}) viscosities for the solutions versus concentration and extrapolating to C=0, then determined by averaging the values of the intercept [36].

M (M_v): the polymer molecular weight.

The values of the equation constants for chitosan are (K=0.076 ml/gm and a=0.76) [37].

Gel Permeation Chromatography (GPC) method: The Number average Molecular Weight (Mn) and Weight average Molecular Weight (Mw) values of both chitosans were measured by GPC instrument (1100 Agilant) equipped with GPC-SEC start up kits with a flow rate of 2 ml/min, maximum pressure 150 bar, minimum pressure 5 bar, injection volume 50 µL and column temperature thermostat 25°C. The eluent was monitored by a refractive index detector of optical unit temperature 25°C and peak width 0.1 min. The molecular weights were determined from calibration curve with polyethylene oxide standards and the method used for the determination followed Liu et al. and Li et al. [38,39].

Statistical analysis: The SPSS Computer program (Version: 14) aided in the statistical analysis of the results. Data were analyzed using one way Analysis Of Variance (ANOVA) followed by post Hoc LSD test. The data were expressed as (Mean ± Standard Error). Differences were considered Statistically Significant at ($p \leq 0.05$) and Highly Significant at ($p \leq 0.01$).

Results and Discussion

The objective of this research was the synthesis of chitosans with high degrees of deacetylation with conserving the molecular weights of the resulting polymer chains as a method for enhancing the properties of the chitosans employing a cooling process and testing its effects on properties of the resulting polymers. Chitin is insoluble in most solvents, but, on heating with (NaOH), it turns into chitosan that is soluble in diluted organic acids. Thus, the solubility refers to formation of chitosan and the deacetylation efficiency. This relates to the DD as its raising makes the solubility easier as shown in **Table 1** with a significant differences in solubility between the (2) chitosans after fast shaking ($p \leq 0.001$).

Figure 3 and Table 2 illustrate the I.R spectra for (Ch-1 and Ch-2) with each corresponding bands. Due to the overlapping between the bands in the spectral region (1100-1000 cm^{-1}) for both chitosans, it was difficult to identify the peaks corresponding to the stretching of (O-H) groups of carbon atoms (C3 and C6) as well as the C-O stretching vibration and bridge oxygen stretching bands.

Many methods were proposed to measure the D.D (e.g., Dye adsorption measurement, residual salicylaldehyde determination and hydrobromide salt titration [33], but the I.R-spectroscopy provides a rapid, accurate technique with a high level of precision to detect the different DDs. There was a significant difference in the degrees of deacetylation between the 2 types where the calculated (DD%) for (Ch-1) was (90% ± 0.577), but for (Ch-2) was about (98% ± 0.265) as shown in **Figure 4A and 4B** and **Table 1** values of the corresponding (A_{1655} and A_{3450}). A_{3450} (for Ch-2)=$\log_{10}$(xb/xa), A_{3450} (for Ch-1)=$\log_{10}$(xf/xe), A_{1655} (for Ch-2)=$\log_{10}$(yd/yc), A_{1655} (for Ch-1)=$\log_{10}$(yh/yg).

There were significant differences in protein and ash contents between the 2 prepared chitosans. (Ch-2) showed significant less protein as well as ash content than (Ch-1) ($p \le 0.001$) (**Table 1**). This shows that the prolonged deacetylation steps on heating with (NaOH) share in more deproteination as well as more removal for the retained CaCO3 within the chitosan products.

The absorbance (A) and% inhibition as a measure of the total antioxidant activity of the prepared chitosan samples are summarized in **Table 1**. Absorbance for control ($A_{control}$) was 1.263 and for standard (A_{st}) was 0.555 with inhibition% (56.1 ± 1.155). Versus (α-tocopherol), there was a significant difference in the %inhibition for both Ch-1 ($p=0.017$) and Ch-2 ($p \ge 0.001$). The increases in DD of chitosan seem to increase the inhibition ability and antioxidant activity of chitosan, so (Ch-2) as well as (α-tocopherol) had significant higher antioxidant activities than (Ch-1). In addition, the results of Park et al. [40] showed that the chitosans with high DDs exhibit also higher scavenging activity than those with lower DDs.

The current study shows the ability to prepare 2 chitosan polymers of different DDs with different periods of heating with NaOH, but having similar M.Ws, lower than those of many commercial chitosans. The Viscosity, Number and Weight Average Molecular Weights for (Ch-1, Ch-2 and the control chitosan patch) are summarized in **Table 1** with no significant differences among those of (Ch-1 and Ch-2). The **Figure 5a and**

Table 1 Properties of the prepared chitosans.

Parameter	Chitosan-1 (Ch-1)	Chitosan-2 (Ch-2)	Control chitosan
M_v (Da)	2.15×10^4	2.14×10^4	1.4×10^4
M_n (Da)	4.3×10^3	3.8×10^3	2×10^3
M_w (Da)	2.2×10^4	2.19×10^4	1×10^4
Solubility %	92 ± 0.577	98.5 ± 0.29	
Ash content % ± SEM	1.84 ± 0.012	0.78 ± 0.012	
Protein content % ± SEM	0.875 ± 0.009	0.589 ± 0.006	
DD % ± SEM	90 ± 0.577	98 ± 0.265	
Inhibition % ± SEM	51.5 ± 0.577	78.5 ± 1.155	

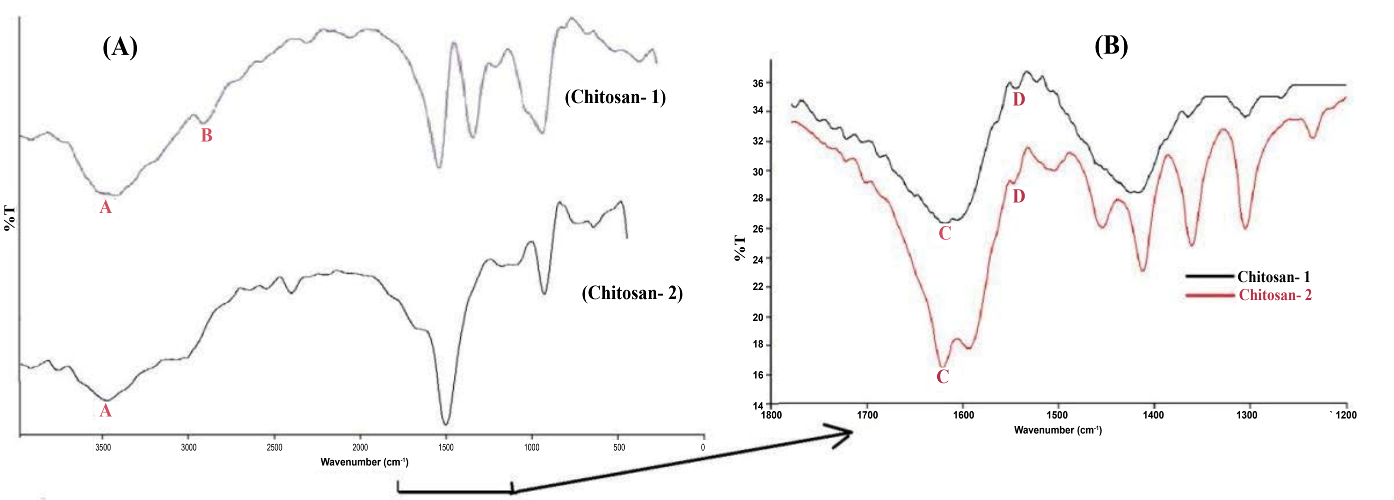

Figure 3 The FT-IR spectra for the Chitosan patches (1, 2). (A) I.R spectra within the range (500-4000 cm^{-1}). (B) I.R spectra within range (1200-1800 cm^{-1}).

Table 2 The different group frequencies wave-numbers (cm^{-1}) for the two prepared chitosans (Ch-1 and Ch-2).

Functional Groups Assignments	Absorption Position cm^{-1}
(O–H) stretching mode of (-OH) groups.	3450
Asymmetric C-H stretching vibration of ($-CH_2$ groups) and pyranose ring.	2900
Asymmetric stretching vibrations of the superimposed (C=O groups) of the (amide bond I), linked to (OH) groups by H-bonding.	1620
In-plane bending vibrations of (NH_2 groups) in non-acetylated 2-amino- glucose 1^{ry} amine.	1605-1590
(N–H) deformation band of (amide I) or (N-H) stretching of (amide II).	1545
(N-H) stretching of (amide I).	1430-1425
Stretching vibration of (amide III).	1365-1360
(CH_2) bonds wagging.	1330-1325
(C-OH) stretching vibration.	1250-1200
(C-O-H) out of plane bending and (CH_2) twisting	950-750

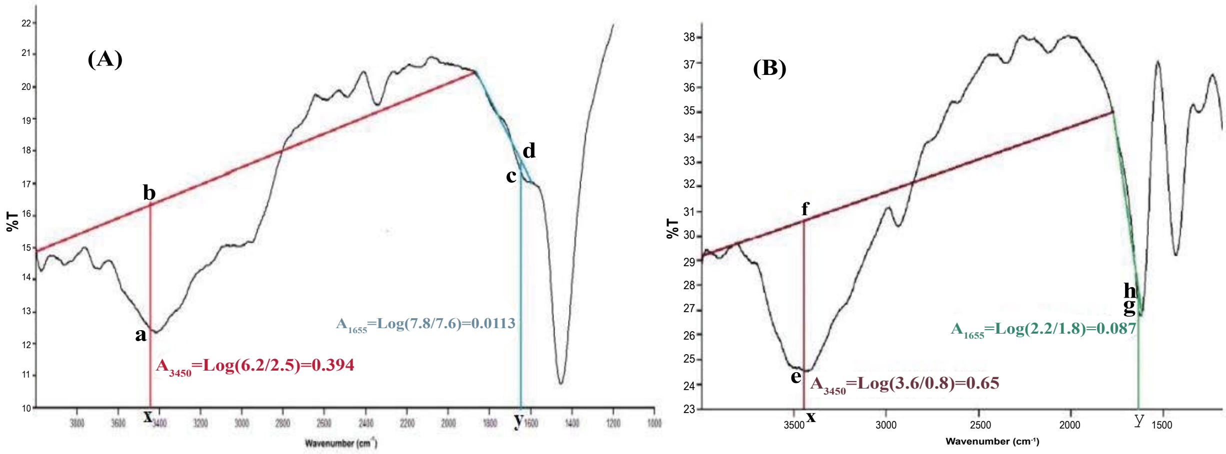

Figure 4 The FTIR spectra for Chitosan-2 (A) and Chitosan-1 (B) showing the base lines for calculating the DD.

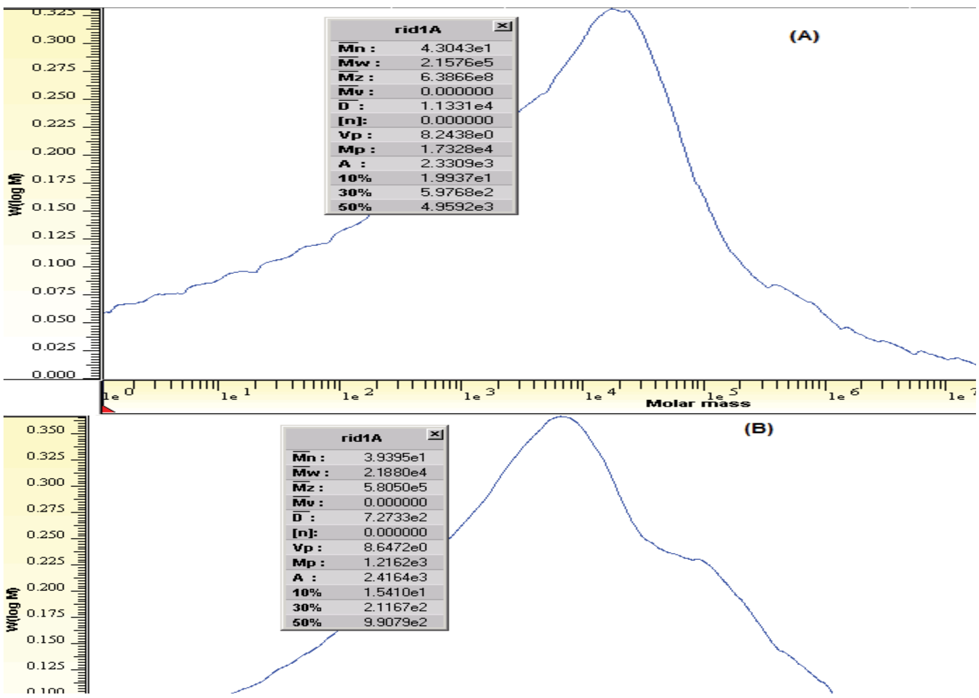

Figure 5 Molecular weight curves produced by the GPC for Chitosan-1 (A) and Chitosan-2 (B).

5b show the molecular weight curves produced by the GPC for the Ch-1 and Ch-2. It seems that the continuous heating of the polymer during the deacetylation process with NaOH causes a decrease in the molecular weight with the rising in the DD (The control patch: Data are not shown), but the fractionation of the chains can be prevented with cooling the polymer before reheating. This is a very important result as preparing chitosans of high deacetylation degrees is known to associate with their degradation and decrease in bulk density (Sehol et al. Trung et al.) [16,41].

The cooling step may become a standard step between each repetition for the heating of the polymer for controlling the molecular weights of the resulting polymers for the subsequent applications requiring high DD with high molecular weights such as the formation of polyelectrolyte complexes with the negatively charged polymers.

Conclusion

Dividing the deacetylation stage during the preparation of chitosan into a number of stages with a cooling step between each two steps was suggested to be an efficient method for the semi-synthesis of chitosans of different degrees of deacetylation but similar weights. This method represents a more economical method for the production of such polymers comparing to the traditional methods used in industry and opens the way for more applications.

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