Physicochemical Characterization of Thermally Treated Chitosans and Chitosans Obtained by Alkaline Deacetylation

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The thermal depolymerization of chitosan and alkaline deacetylation of chitin were characterized by measurement of viscosity, gel permeation chromatography (GPC), potentiometric titration (PT), and proton nuclear magnetic resonance spectroscopy (1H NMR). The depolymerization rates (DR) measured by kinematic viscosity (KV), apparent viscosity (AV), and GPC (Mw) until 4 h of treatment were $\text{DR}_{\text{KV}} = 21.9\% \text{h}^{-1}$, $\text{DR}_{\text{AV}} = 25.5\% \text{h}^{-1}$, and $\text{DR}_{\text{Mw}} = 23.3\% \text{h}^{-1}$ and for 5 to 10 h of treatment they decreased slowly to produce $\text{DR}_{\text{KV}} = 0.545\% \text{h}^{-1}$, $\text{DR}_{\text{AV}} = 0.248\% \text{h}^{-1}$, and $\text{DR}_{\text{Mw}} = 1.11\% \text{h}^{-1}$. The mole fraction of N-acetylglucosamine residues ($F_A$) of chitosans was not modified after 10 h of thermal treatment at 100°C. The initial $F_A$ values of chitosan without any treatment were $F_A^{\text{APT}} = 0.21$ and $F_A^{1\text{H NMR}} = 0.22$ and of chitosan treated for 10 h were $F_A^{\text{APT}} = 0.27$ and $F_A^{1\text{H NMR}} = 0.22$. The variables used to characterize the depolymerization process showed a good correlation. Six hours of thermal treatment is sufficient to obtain chitosans with a molar mass 90% smaller than that of the control chitosan without treatment.

1. Introduction

Chitin, a natural biopolymer, is a structural polysaccharide found in the exoskeleton of marine crustaceans (crab and shrimp shells) and insects. It is also widely found in fungi, such as Basidiomycetes, Ascomycetes, and Pycomycetes, where it is a component of cell walls and structural membranes of mycelia, stalks, and spores. Chitin and chitosan are $\beta$-(1,4)-aminoglucopyranans composed of N-acetylglucosamine (GlcNAc = A) and glucosamine (GlcN = D) residues. Chitosan and chitin are polydisperse polymers and the number of their subunits varies. They are distinguished by their solubility in 1% aqueous acetic acid. Chitin, containing ca. >40% GlcNAc residues ($F_A > 0.4$), is insoluble, whereas soluble polymers are named chitosan (for a review, see [1, 2]).

Several characteristics of chitosan are fundamental in describing the particular molar batch and predicting its chemical and physical properties: the average molar mass of the sample, its average degree of acetylation (DA, given as a percentage) or the fraction of acetylation ($F_A$, given as the mole fraction), and the local and global distribution of the acetylated amide moieties along the chain as well as the polydispersity index, the viscosity, and the ash content [1]. Presently, a substantial amount of research is devoted to the application of chitosan and derivatives for antimicrobial purposes against a wide range of phytopathogenic fungi [3–5] and pathogenic bacteria [6].

Chitosan, like other polysaccharides, is susceptible to a variety of degradation mechanisms, including oxidative-reductive free radical depolymerization and acid-, alkaline-, and enzyme-catalyzed hydrolysis. Degradation of polysaccharides occurs via cleavage of the glycosidic bonds [7]. Several studies have been done to evaluate the acid hydrolysis of chitosan. The acid hydrolysis of chitin was studied for the first time in 1992 by Roberts, who used HNO₃ [8]. Different acids have been used in the hydrolysis of chitosan: hydrochloric acid [9–13], phosphoric acid [14, 15], sulfuric acid and acetic anhydride [16], nitrous acid [17], and hydrogen fluoride [18].

Unlike acid hydrolysis, enzymatic hydrolysis of chitin and chitosan by chitinases (EC 3.2.1.14) and chitosanases
(EC 3.2.1.132) permits the production of different oligomers. Hydrolysis of chitin and chitosan catalyzed by specific chitinase and chitosanase enzymes has been employed in several studies [19–23]. A few studies have been done to evaluate the degradation of chitosan in solid form by thermal treatment [7, 24–29]. Thermal treatment of chitosan is an alternative method to obtain chitosans with small degree of polymerization (DP) and the same $F_A$. The reaction mechanisms of thermal treatment of chitosan have been studied [7, 28, 29]; however they were not completely elucidated.

The aims of this work were to investigate chitosan degradation in solid form by thermal treatment to obtain chitosan with small DP and alkaline deacetylation of chitin. In order to evaluate the extension of these processes, the capability of some analytical methods was further investigated. For molar mass determination two methods were used: viscometry and gel permeation chromatography (with a refractive index detector, GPC). For a determination of the degree of acetylation, potentiometric titration (PT) and high-field $^1$H NMR spectroscopy were used.

2. Materials and Methods

2.1. Raw Materials. Chitosan was supplied by Polymar (Fortaleza, Brazil). Chitin (Sigma Chemical Co. St. Louis, USA) was used in the deacetylation reaction to obtain chitosan with different degrees of acetylation. Dextran standards (American Polymers, Ohio, USA) were used for calibration of GPC columns (Mw: 11, 38, 72, 260, and 530 kDa). Sodium azide was from Sigma (St. Louis, USA), lactic acid was from Synth (Diadema, Brazil), acetic acid, sodium chloride, urea, hydrochloric acid, and deuterium chloride were from E. Merck (Darmstadt, Germany).

2.2. Thermal Depolymerization of Chitosan. Thermal depolymerization was performed by a procedure described in the literature [7], with slight modifications. Solid chitosan (8g) was placed in 9 cm glass Petri plates and thermally degraded in an oven at 100°C for up to 10 h. At 1 h intervals, during this treatment, 1.5 mL of distilled water was added to the chitosan.

2.3. Kinematic and Apparent Viscosities. The thermal depolymerization of all chitosan samples was analyzed by kinematic and apparent viscosities. Thermally treated chitosan samples were transferred to lactic acid solution (0.15 mol dm$^{-3}$), stirred in an orbital shaker for 3 h, and filtered through a glass sintered filter n°4. Kinematic viscosity (cSt) and apparent viscosity (Pa s) of the filtrate were determined using a Cannon-Fenske n°200 viscometer and a Brookfield Programmable DV-II rheometer at 25 ± 0.1°C, respectively, in accordance with the manufacturer's instructions.

2.4. Determination of Average Molar Mass by GPC. The extent of thermal depolymerization of chitosan was assessed by gel permeation chromatography with a refractive index detector (GPC). Heat treated chitosan samples were dissolved (1.0 mg dm$^{-3}$) in sodium acetate buffer (0.33 mol dm$^{-3}$ acetic acid, 0.1 mol dm$^{-3}$ NaOH, pH = 3.9 ± 0.2) and centrifuged

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Temperature (°C)</th>
<th>Time (h)</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermally treated chitosan$^*$</td>
<td>100</td>
<td>0 (control)</td>
<td>A</td>
</tr>
<tr>
<td>Chitosan obtained by alkaline deacetylation of chitin</td>
<td>100–110</td>
<td>1</td>
<td>D</td>
</tr>
</tbody>
</table>

Table 1. Chitosan samples used to determine the viscosity average molar masses (Mv).

<table>
<thead>
<tr>
<th>Code</th>
<th>Mv (g mol$^{-1}$)</th>
<th>Solvent</th>
<th>$K$ (dm$^{-3}$/g)</th>
<th>$a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>113,000–492,000</td>
<td>HOAc, 0.1 mol dm$^{-3}$ NaCl, 4 mol dm$^{-3}$</td>
<td>0.893 × 10$^{-4}$</td>
<td>0.71</td>
</tr>
<tr>
<td>2</td>
<td>90,000–1,140,000</td>
<td>HOAc, 0.2 mol dm$^{-3}$ NaCl</td>
<td>0.181 × 10$^{-5}$</td>
<td>0.93</td>
</tr>
<tr>
<td>3</td>
<td>13,000–135,000</td>
<td>HOAc, 0.3 mol dm$^{-3}$ NaCl</td>
<td>0.341 × 10$^{-5}$</td>
<td>1.02</td>
</tr>
</tbody>
</table>

Table 2: Viscosity parameters of chitosans (for references and discussion, see Roberts [8]).

at 10 000 × g for 60 s. A calibration curve was established with dextrin standards (2.5 mg dm$^{-3}$) of Mw 11, 38, 72, 260, and 530 kDa (American Polymers, Mentor, OH, USA) dissolved in water containing 0.05% (m/v) sodium azide. The GPC system (Waters Corporation, Milford, Massachusetts, USA) consisted of a model 515 pump, a model 717 automatic injector, and a 410 differential refractometer, and the data were handled by Waters’ Millenium GPC software. Two columns of polymethyl methacrylate hydroxylate (Waters Ultradrygel 1000 and Ultradrygel 500), with respective exclusion volumes of 1.0 × 10$^6$ and 8.0 × 10$^4$ Da, were connected in series. The injected sample volume was 200 µL and the mobile phase was the same sodium acetate buffer as that used to dissolve the sample, flowing at 0.8 mL min$^{-1}$ with a temperature of 40°C. The accuracy of GPC method, used to determine the Mw of thermally treated chitosan samples, is 3-4%.

2.5. Determination of Viscosity Average Molar Mass (Mv) of Chitosan. The viscosity average molar mass of the five chitosans described in Table 1 was also determined by intrinsic viscosity [8].

Three different solvents were tested in order to find the most suitable (Table 2). $K$ and $a$ are constants that are independent of molar mass over a considerable range of molar masses and depend on the polymer, solvent, temperature, and, in the case of polyelectrolytes, the nature and concentration of the low-molar-mass electrolyte added. The Ubbelohde viscometer was kept at a temperature of 25.0 ± 0.1°C by means of a water bath. The solvent flow times were preferably longer than 100 s. Chitosan average molar mass was determined using a Ubbelohde viscometer, type 53101 from Schott GmbH.

In order to select the best solvent, Mv of sample A (raw material) was determined using solvents 1, 2, and 3, and for samples B, C, D, and E solvent 3 was used. A 0.5% stock
solution (w/v) was prepared for each chitosan and the range of concentrations used was 1.0 to 5.0 g dm$^{-3}$. When the dissolution was complete, the solution was filtered through a Schott glass sintered filter n° 4. The correct concentration of dissolved polysaccharide was calculated as the difference between the initial amount of polymer and the insoluble part, using

$$C = \frac{m_1 - (m_2 - m_0)}{\nu},$$

where $C$ is the concentration of chitosan solution, $m_0$ is the mass of dry filter, $m_1$ is the mass of chitosan sample, and $m_2$ is the mass of filter containing insoluble particles after drying.

The intrinsic viscosity was determined according to

$$[\eta_i] = \frac{(t_1 - t_0)/t_0}{c},$$

where $t_1$ is the flow time for the chitosan solution, $t_0$ is the flow time for the solvent system, and $\eta_i$ is the intrinsic viscosity.

The limiting viscosity number was found by the extrapolation of Mark-Houwink’s relationship between the intrinsic viscosity and the concentration of chitosan in the investigated solution to concentration of chitosan of zero.

The average molar mass was obtained according to

$$M_v = \left(\frac{\eta_i}{K}\right)^{1/a},$$

where $M_v$ is the viscosity average molar mass, $\eta_i$ is limiting viscosity number, and $K$ and $a$ are Mark-Houwink constants.

### 2.6. Alkaline Deacetylation of Chitin

The kinetics of homogeneous alkaline deacetylation of $\alpha$-chitin was reported to be a pseudo-first-order reaction at high temperature (80 to 120°C) [30] and also at low temperature (−5 to −35°C) [31]. Deacetylation of chitin was achieved in accordance with a modified procedure developed by Canella and Garcia [32]. Sixteen grams of chitin (Sigma Chemical, St. Louis, USA) were suspended in 200 mL of 50% NaOH solution (m/v) and stirred at 900 rpm in a batch reactor under reflux. In Table 3 the temperatures and times used to obtain samples D and E are described and a summary of the purification process is given (Table 3).

### 2.7. Determination of the Degree of N-Acetylation by Potentiometric Titration

The mole fraction of N-acetylglucosamine residues ($F_A$) was determined using potentiometric titration, as described by Raymond et al. [33]. The 1% chitosan sample (w/v) was added to HCl 0.1 mol dm$^{-3}$ and titrated with a solution of NaOH 0.1 mol dm$^{-3}$. The neutralization point was determined potentiometrically.

The values of $F_A$ were calculated according to

$$F_A = 1 - \frac{V_{NaOH} \times M_{NaOH}}{(m_{ch}/M_{ch})},$$

where $F_A$ is the mole fraction of N-acetylglucosamine residues, $m_{ch}$ is the chitosan sample mass, $M_{ch}$ is the molar mass of glucosamine unit, $V_{NaOH}$ is the volume of NaOH 0.1 mol dm$^{-3}$ solution used to neutralize the protonated free amino groups, and $M_{NaOH}$ is the molar concentration of NaOH solution.

### 2.8. Determination of the Degree of N-Acetylation by High-Field $^1$H NMR Spectroscopy

In accordance with a procedure adapted from a publication by Vårum et al. [34], a sample of roughly 100 mg of chitosan was suspended in 10 mL of 0.07 mol dm$^{-3}$ HCl at room temperature with stirring overnight. A small mass of NaNO$_2$ (9-10 mg) was added to the stirring solution and left to react for 4 h. The solution was lyophilized and then ion-exchanged with D$_2$O three times. The samples were dissolved in roughly 1.5 mL of D$_2$O and filtered through cotton to remove any insoluble mass. $^1$H NMR spectra of the samples were obtained in a Bruker 300 MHz NMR spectrometer at room temperature after 32 scans with a delay time of 3 to 4 seconds. The degree of acetylation is given by

$$F_A = \frac{7(I_B+F_E)}{[4(I_B+I_C+I_D)+I_B+I_E]},$$

where $I$ is the peak intensity, represented as an integral, and the subscripts A to E identify particular peaks indicated in Figures 4(a) and 4(b). Peaks A and B correspond to the anomeric protons GlcN and GlcNAc, respectively; C and D correspond to ring protons; E corresponds to the methyl protons.

### 2.9. Statistical Analysis

The result of each treatment was the average of two or three repetitions depending on the analysis realized. The results were analyzed by ANOVA and regression at $P \leq 0.05$. The models were selected analyzing the determination coefficients ($R^2$) and significance of regression coefficients tested by Student’s t-test.

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**Table 3: Conditions of alkaline deacetylation of chitin and purification of chitosan samples.**

<table>
<thead>
<tr>
<th>Batch</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>100–110</td>
<td>110–122</td>
</tr>
<tr>
<td>Time (minutes)</td>
<td>60</td>
<td>80</td>
</tr>
</tbody>
</table>

**Steps:**
- Neutralization of supernatant with NaOH 1 dm$^{-3}$/L until pH = 8.5 is reached
- Washing of suspension until low conductivity is achieved
- Freezing at −80°C and lyophilization

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**Notes:**
- The values of $R^2$ and significance of regression coefficients tested by Student’s t-test.

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**Formulas:**
- $C = \frac{m_1 - (m_2 - m_0)}{\nu}$
- $[\eta_i] = \frac{(t_1 - t_0)/t_0}{c}$
- $M_v = \left(\frac{\eta_i}{K}\right)^{1/a}$
- $F_A = 1 - \frac{V_{NaOH} \times M_{NaOH}}{(m_{ch}/M_{ch})}$
3. Results and Discussion

3.1. Evaluation of Thermal Depolymerization. The most commonly used methods for determination of Mv, Mw, and Mn are viscosity, light scattering (SLS: static light scattering and MALSS: multiple-angle laser light scattering), and GPC (gel permeation chromatography) or SEC (size exclusion chromatography) [35].

Insoluble materials were observed in the raw material from Polymar. Approximately, 1% of insoluble material was retained on sintered glass filter n° from Polymar. Approximately, 1% of insoluble material was retained on sintered glass filter n°4 from 1% chitosan solution (m/v) in lactic acid (0.15 mol dm$^{-3}$). The formation of insoluble material increased with increase in treatment time. Approximately, 2% and 6% of insoluble materials were retained on sintered glass filter n° 4% from 1% chitosan solutions of samples thermally treated for 3 and 10 h, respectively. Some insoluble materials were also observed by Holme et al. [7]. The formation of insoluble material can be explained by interchain cross-link formation involving free amino groups and reducing ends [36].

The thermal degradation of chitosan samples in solid state treated at 100°C during 10 h was analyzed by viscosity (kinematic, intrinsic, and apparent viscosities) and gel permeation chromatography with a refractive index detector (GPC). Values of kinematic and apparent viscosities and molar mass (Mw) are shown in Figures 1(a) and 1(b). The empirical models adjusted to the experimental data of kinematic viscosities (6), apparent viscosities (7), and average molar masses (8) were statistically significant at $P \leq 0.05$.

Consider

$$Y_{KV} = -0.03373X^3 + 0.85342X^2 - 7.05007X + 20.19318,$$

$$R^2 = 0.99193,$$  \hspace{1cm} (6)

$$Y_{AV} = -0.05609X^3 + 1.62181X^2 - 1.10938X + 4.17165,$$

$$R^2 = 0.96158,$$  \hspace{1cm} (7)

$$Y_{Mw} = 4.29172X^2 - 74.75343X + 323.88758,$$

$$R^2 = 0.95506.$$  \hspace{1cm} (8)

Values of Mw determined by GPC were calculated using chromatograms and calibration curve for the dextran standard. Both chromatograms and calculation of the calibration curve for the dextran standard were obtained with the software Millenium (for chromatograms and calibration curve, see Figures 1Sa and b in Supplementary Material available online at http://dx.doi.org/10.1155/2014/853572). From the chromatographic profiles for control and chitosans treated for up to 10 h (Figure 2) and calibration curve were obtained the molar mass distribution of chitosan samples and their Mw.

The kinematic and apparent viscosities and molar masses of chitosan samples treated for 6 h at 100°C decreased more than 90% (Table 1S). After 10h of treatment, decreases in kinematic and apparent viscosities and Mw of the chitosans of 92.0%, 96.5%, and 96.7%, respectively, were observed.

Thermal depolymerization of chitosan chloride with different $F_A$ was studied by Holme et al. [7] at different temperatures. The decrease in apparent viscosity obtained for chitosan $F_A = 0.35$ treated at 105°C for 10 h was about 90% [7]. In our study for chitosan $F_A = 0.22$% treated at 100°C for 10 h, a decrease of 96.5% in apparent viscosity was observed (Table 1S).

The thermal depolymerization of chitosan occurs in two phases. In the first stage (0–4 h of treatment) an increase in chitosan depolymerization, predicted by the linear model, was observed (Figure 3) and in the second stage (5–10 h of treatment) a tendency towards process stabilization (Figures 1(a) and 1(b)).

Similar results were observed by viscosity and GPC measurements. In Figure 3 it can be seen that the depolymerization rate increased with time. The depolymerization rates measured by kinematic viscosity, apparent viscosity, and molar mass for 4 h were 21.9, 25.5, and 23.3% h$^{-1}$, respectively.

It was observed that the depolymerization rates in the period of 5 to 10 h of treatment decreased slowly, and the values observed were $DR_{KV} = 0.545$, $DR_{AV} = 0.248$, and $DR_{Mw} = 1.11%$ h$^{-1}$ (for linear regression, see Supplementary Figure 2S).

Viscometry and GPC gave similar results in the analysis of chitosans with a range of molar masses from 235,000 to 43,000 g mol$^{-1}$ and polydispersity indices from 7 to 3.5; however, in the analysis of chitosans with a range of molar masses from 22,000 to 8,000 g mol$^{-1}$ and polydispersity indices from 2.3 to 1.3 the methods gave different results. This difference may be related to the hydrodynamic volume of the macromolecules, which is a function of molar mass, conformational properties, and polymer-solvent interactions, as described by Terbojevich and Cosani [35].

Table 1S shows the decrease in the values of kinematic and apparent viscosities and molar mass as measured by viscometry and GPC. Each parameter had significantly different values for the increase up to $t_{KV} = 7$ h, $t_{AV} = 4$ h, and $t_{Mw} = 6$ h. Chitosans thermally treated at 100°C for 8, 9, and 10 h did not show a significant decrease in AV and Mw. The degradation of chitosan in the first 4 h of thermal treatment, measured by KV, AV, and GPC, was significant ($P \leq 0.05$).

In our work, two depolymerization rates were found, the first one for up to 4 h of heating ($DR_{KV} = 21.9$, $DR_{AV} = 25.5$, and $DR_{Mw} = 23.3%$ h$^{-1}$) and the lower rate ($DR_{KV} = 0.545$, $DR_{AV} = 0.248$, and $DR_{Mw} = 1.11%$ h$^{-1}$) for the treatment time interval from 5 to 10 h, when a decrease in molar mass of more than 90% had already been achieved. Three techniques used to evaluate the thermal depolymerization process had linear relationships ($P \leq 0.05$) in the treatment time interval up to 4 h; that is, the slope of the linear regression line predicts the depolymerization rate (DR) of chitosan treated thermally in a specific time interval. Analysis of thermal depolymerization kinetics of chitosans based on the KV, AV, and Mw data showed good agreement with DR values that did not vary statistically at $P \leq 0.05$. The viscosities and the average molar masses had a statistical tendency to become stable from 5 h on, and further heating did not seem to cause further chitosan depolymerization.
The mechanism of thermal degradation of chitosan was studied by Holme et al. [7]. The oxidative-reductive degradation mechanism was discarded after it was confirmed that thermal degradation with and without oxygen (nitrogen atmosphere) did not affect the degradation rate. Chitosan chloride in solid state \( F_A = 0.16 \) with pH 4, 5, and 6 was thermally treated at 105°C and an increase in thermal degradation with the increase in H⁺ concentration was observed. It was confirmed that acid hydrolysis is the primary mechanism of the thermal degradation of chitosan chloride. Acid hydrolysis of the glycosidic linkages involves both protonation of the glycosidic oxygen and addition of water to yield the reducing sugar end group [37]. Zawadzki and Kaczmarek [28] studied the changes of chitosan structure during storage in vacuum or in oxygen atmosphere at room and elevated temperatures (up to 600°C) using FTIR spectroscopy and thermogravimetry. They observed that gradual increase of temperature (20–150°C) and regulation of heating time allow for the complete dehydration without destruction of the chitosan chemical structure in vacuum and \( F_A \) remained constant on this range of temperature.

In our study chitosan in solid state was used with the addition of water at time intervals of 1 hour to maintain the H⁺ concentration during the heating treatment in oxygen atmosphere at 100°C and to hydrolyze the glycosidic linkages. The thermal treatment was carried out at 100°C to produce chitosans with different DP and the same \( F_A \).

3.2. Determination of Viscosity Average Molar Mass of Chitosan. The \( M_v \) of control chitosan (A) was determined using solvent 1 \((0.2 \text{ mol dm}^{-3} \text{ of acetic acid, } 0.1 \text{ mol dm}^{-3} \text{ of sodium chloride, and } 4 \text{ mol dm}^{-3} \text{ of urea})\), solvent 2 \((0.1 \text{ mol dm}^{-3} \text{ of acetic acid and } 0.2 \text{ mol dm}^{-3} \text{ of sodium chloride})\), and solvent
3 (0.33 mol dm$^{-3}$ of acetic acid and 0.3 mol dm$^{-3}$ of sodium chloride) as described by Roberts [8]. From the linear regressions of control chitosan (A) using solvents (1), (2), and (3) were calculated intrinsic viscosities and Mv values using the Mark-Houwink-Kuhn-Sakurada equation, $1,254,259$ g mol$^{-1}$, $121,048$ g mol$^{-1}$, and $193,400$ g mol$^{-1}$, respectively. (For linear regressions, see Supplementary Figure 3S.)

The strong effects of the three different solvents were shown to be significant ($P \leq 0.05$) when used for determination of viscosity average molar mass. The Mv value of chitosan (A) determined in solvent 1 (0.2 mol dm$^{-3}$ of acetic acid, 0.1 mol dm$^{-3}$ of sodium chloride, and 4 mol dm$^{-3}$ of urea) was $1,254,259$ g mol$^{-1}$, in solvent 2 (0.1 mol dm$^{-3}$ of acetic acid and 0.2 mol dm$^{-3}$ of sodium chloride) was $121,048$ g mol$^{-1}$, and in solvent 3 (0.33 mol dm$^{-3}$ of acetic acid and 0.3 mol dm$^{-3}$ of sodium chloride) was $193,400$ g mol$^{-1}$. Several set values for $K$ and $\alpha$ of chitosan have been proposed in the literature [8]. The $K$-values depend on the average molar mass used and on the molar mass distribution of the samples [38]. The polydispersity index of sample A

Figure 4.: $^1$H NMR spectra of control chitosan sample A (a) and chitosan sample C (b), thermally treated at 100°C for 10 h.
Table 4: Average molar mass (Mw), degree of polymerization, kinematic and apparent viscosities and viscosity average molar mass (Mv) of chitosans obtained by thermal treatment and alkaline deacetylation.

<table>
<thead>
<tr>
<th>Code</th>
<th>GPC(^1) Mw (g mol(^{-1}))</th>
<th>DP(^2)</th>
<th>Apparent viscosity(^7) (mPa s)</th>
<th>Kinematic viscosity(^4) (cSt)</th>
<th>Intrinsic viscosity(^5) Mv (g mol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>235,000</td>
<td>1,383</td>
<td>29.9</td>
<td>13.9</td>
<td>193,400</td>
</tr>
<tr>
<td>B</td>
<td>62,500</td>
<td>366</td>
<td>2.3</td>
<td>1.9</td>
<td>93,594</td>
</tr>
<tr>
<td>C</td>
<td>7,700</td>
<td>45</td>
<td>1.4</td>
<td>1.2</td>
<td>10,978</td>
</tr>
<tr>
<td>D</td>
<td>193,000</td>
<td>1,171</td>
<td>4.9</td>
<td>5.1</td>
<td>30,219</td>
</tr>
<tr>
<td>E</td>
<td>184,000</td>
<td>1,089</td>
<td>4.7</td>
<td>5.2</td>
<td>91,203</td>
</tr>
</tbody>
</table>

\(^1\)Gel permeation chromatography. \(^2\)Degree of polymerization [DP = Mw/(203 × F\(A\) + 163 × (1 − F\(A\)))]. \(^3\)Brookfield rheometer. \(^4\)Cannon-Fenske no. 200 viscometer. \(^5\)Ubbelohde type 5100/1 Schott GmbH viscometer. 

The average molar mass of chitosan treated by thermal treatment and alkaline deacetylation was determined by GPC. By GPC analysis, the average molar mass of chitosan A was 235,000 g mol\(^{-1}\), of the closest value to the Mv determined by viscosity (value 193,400 g mol\(^{-1}\)) in solvent 3. The same was observed for samples B, C, D, and E, indicating that 0.33 mol dm\(^{-3}\) of acetic acid and 0.3 mol dm\(^{-3}\) of sodium chloride were the most suitable solvents for this determination.

The relationship between Mv and intrinsic viscosity \(\eta_i\) is expressed by the Mark-Houwink-Kuhn-Sakurada equation, \(\eta_i = kM^a\), where the viscosity parameters \(K\) and \(\alpha\) depend on the polymer, the temperature, the solvent, and the salt concentrations [1]. Different values of Mv were obtained by intrinsic viscosity \(\eta_i\) for chitosan sample A using three different solvents by the high polydispersity index of raw material (6.97) calculated by GPC. Roberts [8] reports different ranges of Mv for different solvents (Table 2). Chitosan samples with a high polydispersity index (6.97), whose average molar masses were determined by viscometry, showed very large discrepancies; however, for chitosan with a low polydispersity index there was no discrepancy of average molar masses obtained by viscometry.

It was observed that methods used to characterize thermally treated chitosan samples and chitosan samples obtained by alkaline deacetylation showed a good correlation between parameter values analyzed to determine molar masses and viscosities. Of these three methods, GPC and apparent and kinematic viscosities cited and discussed as well as intrinsic viscosity used in the determination of Mv, intrinsic viscosity was shown to be reliable and efficient, since it can be closely correlated to the average Mw obtained by GPC (Figure 1(b)).

In Table 4 the average molar masses, degrees of polymerization, apparent and kinematic viscosities, and viscosity average molar masses of chitosans obtained by thermal treatment and alkaline deacetylation are described.

In Table 5 the observed decrease in Mw (GPC), Mv (intrinsic viscosity), and apparent and kinematic viscosities of chitosans thermally degraded at 100°C for 3 and 10 h is described.

<table>
<thead>
<tr>
<th>Code</th>
<th>PT(^1)</th>
<th>F(A)</th>
<th>NMR(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.21</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.23</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.27</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>0.09</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>0.19</td>
<td>0.16</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Potentiometric titration. \(^2\)Nuclear magnetic resonance. nd: not determined.

3.3. Determination of Mole Fraction of N-Acetylglucosamine Residues in Chitosan. Initially, it was also our goal to compare two analytical methods to determine mole fraction of N-acetylglucosamine residues in chitosan. The most commonly applied methods for determination of the \(F_A\) in chitin and chitosan are infrared (IR), crosspolarization/magic angle spinning nuclear magnetic resonance (CP/MAS \(^{13}\)C-NMR), first-derivative UV and CD spectroscopy, potentiometric and dye adsorption titration, pyrolysis-gas chromatography, quantification of acetic acid in hydrolysates by liquid chromatography, and thermal and elemental analysis [1]. The potentiometric titration method and the \(^1\)H NMR were chosen for the study of their ability to determine the acetyl group fraction of our group of chitosans (Table 5). Titration was chosen because it is a very simple and inexpensive analytical procedure and \(^1\)H NMR because it is one of the most cited tools currently used for this task.

Another goal of this present work was to verify the effect of the heating on the degree of acetylation of chitosans. The
same group of chitosan samples which had been previously analyzed for average Mw was also analyzed for $F_A$.

It was observed that the thermal treatment used to depolymerize chitosans did not seem to significantly affect the $F_A$ of the chitosan samples, when determined either by titration or by $^1$H NMR (Table 5). Figures 4(a) and 4(b) show $^1$H NMR spectra of chitosan samples A and C. $F_A$ of control chitosan (A) and chitosan thermally treated for 10 h (C) was 0.22 and 0.22, respectively, as determined by $^1$H NMR, and 0.21 and 0.27, respectively, as determined by PT. Thus, thermal treatment of chitosan at 100°C for 10 h has very little or even no ability to modify the glucosamine bonds.

The $F_A$ value obtained by the titration method is precise when some steps are taken to standardize the HCl and NaOH solutions and filtrate the chitosan hydrochloride solution to avoid mistakes when chitosan samples are partially soluble. Raymond et al. [33] observed that titration of chitosan is useful for analysis of low $F_A$ samples. In our study the range of $F_A$ Samples was 0.08 to 0.22 and the accuracy of the $^1$H NMR and PT methods in determining $F_A$ did not differ.

The chitosan samples characterized in this work were used to evaluate barrier properties of chitosan films, whose water permeability of chitosan films is 50% reduced when molar mass of the original chitosan is reduced from 235 kDa (DP 1,383) to approximately 13.7 kDa (DP 45), [39] and antifungal activities in filamentous fungi, whose chitosan samples with low molar masses were more effective [40].

4. Conclusions

This study showed that thermal treatment results in chitosans with different molar masses but the same degree of acetylation, as determined by $^1$H NMR spectroscopy and potentiometric titration. The process of thermal depolymerization under the conditions used in this study was shown to be appropriate for obtaining chitosans with smaller molar masses, given that in this process strong hydrolytic reagents, such as hydrochloric, sulfuric, and phosphoric acids, are not used. However, the lost of insoluble particles, 2% and 6% (m/m) in thermally treated chitosans for 3 and 10 h, respectively, were observed. The variables used to characterize the depolymerization process showed a good correlation. Six hours of thermal treatment was sufficient to obtain chitosans with a molar mass 90% smaller than that of the control chitosan without treatment.

Nomenclature

- AV: Apparent viscosity mPas
- $F_A$: Mole fraction of N-acetylg glucosamine residues (-)
- DR: Depolymerization rate % h$^{-1}$
- KV: Kinematic viscosity cSt
- Mv: Viscosity average molar mass g mol$^{-1}$
- Mw: Mass average of molar mass (determined by GPC) g mol$^{-1}$
- C: Concentration of chitosan solution g dm$^{-3}$
- $m_0$: Mass of dry filter g
- $m_1$: Mass of chitosan sample g
- $m_2$: Mass of filter containing insoluble particles after drying g
- $t_1$: Flow time for the chitosan solution s
- $t_0$: Flow time for the solvent system s
- $K$: Mark-Houwink constant dm$^3$ g$^{-1}$
- $A$: Mark-Houwink constant (-)
- $M_{ch}$: Molar mass of glucosamine unit
- $161$ g mol$^{-1}$
- $V_{NaOH}$: Volume of NaOH solution cm$^3$
- $M_{NaOH}$: Molar concentration of NaOH solution mol dm$^{-3}$
- PT: Potentiometric titration (-).

Greek Letters

- $\eta_i$: Intrinsic viscosity dm$^3$ g$^{-1}$
- $\eta_L$: Limiting viscosity number dm$^3$ g$^{-1}$.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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