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Determination of the pattern of acetylation of low-molecular-weight chitosan used in biomedical applications

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ABSTRACT

The microstructure of chitosan, a linear copolymer of glucosamine and *N*-acetylglucosamine units widely used in biomedical applications, is closely dependent on the conditions of its preparation. Knowledge of the structural differences between chitosan preparations is very important in determining the properties of chitosan and essential for structure-activity analysis where biological systems are concerned. Determination of the pattern of acetylation of chitosan samples (P_A parameter) by ^{13}C NMR spectroscopy hitherto required depolymerization of the native chitosans. The present study has demonstrated that this step is not necessary for determining P_A of low-molecular-weight chitosan samples.
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1. Introduction

Chitin, a natural product consisting of *N*-acetylglucosamine units linked by β-(1 → 4) bonds, is one of the most abundant natural polysaccharides in the world. Synthesized by an enormous number of living organisms, this biopolymer is as a structural component of the exoskeletons of crustaceans, the cuticles of insects, and the cell walls of fungi and yeast [1]. Industrially, chitin is used mainly as a raw material for the production of chitosan, which can be regarded as a deacetylated derivative of chitin. However, as the *N*-deacetylation reaction rarely goes to completion, most commercial and laboratory products tend to be copolymers of *N*-acetylglucosamine (GlcNAc) and glucosamine (GlcN) units (Fig. 1). The term chitosan includes not only the polymers derived from chitin by *N*-deacetylation [2], but also those derived from chitosan by *N*-acetylation [3]. The ratio of the two repeating units (GlcNAc and GlcN), and their distribution along the polysaccharide chain may depend greatly on the source and preparation conditions of chitosan [4]. It has been suggested that the chitosan preparations obtained by *N*-deacetylation under heterogeneous conditions adopt a blockwise pattern and they are insoluble in water [5]. The specific biological features of chitosan – biodegradability, biocompatibility, antibacterial activity, lack of toxicity and allergenicity, the ability to reduce cholesterol levels (LDL) – make it a very attractive biomaterial for diverse applications in pharmaceutical and medical fields [6–9]. It not only improves the solubility of poorly soluble drugs but also substantially enhances fat metabolism in the body [10]. It has been shown that conjugates of some kinds of anticancer agents with chitin and chitosan derivatives display good anticancer activity, with a concomitant reduction in the adverse effects of the original drug [11]. For medical applications, various forms of chitosan-based products are available: finely divided powders, films, membranes, gels, coatings, suspensions and hydrogels. The potential implementation of chitosan in medicine can only be explored, however, if its usable forms are properly developed and prepared. Most of the above applications depend strongly on the degree of acetylation (F_A) and probably also on the pattern of acetylation (P_A) [12]. Structural analysis of chitosan provides an insight into structure–property relationships and helps to predict its effect on the natural environment and/or in synthetic products.

As mentioned above, the distribution of GlcNAc and GlcN units along the chitosan chain can be characterized by the parameter P_A ($P_A = P_\Sigma$) [13,14]. If the statistics are consistent with the Bernouillian model for polymers, the values $P_A = 0$, 1 and 2 respectively indicate the perfect block, the random distribution, and the alternating distribution of *N*-acetyl groups along the chitosan chain (Fig. 2). The pattern of acetylation can be determined by ^{13}C NMR spectroscopy [15], but its analysis requires a sufficiently good resolution of the

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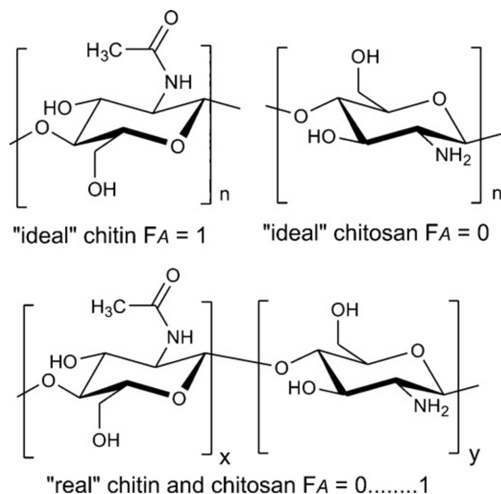

Fig. 1. The theoretical and natural structure of chitin and chitosan (F_A—degree of acetylation).

signals. So far, limited depolymerization of chitosan with nitrous acid has been recommended to enhance the resolution of the NMR spectra [15,16]. Depolymerization of chitosan with nitrous acid is a homogeneous reaction: the number of broken glycosidic bonds correlates roughly with the stoichiometric amount of added nitrous acid. The mechanism of this reaction involves the deamination of D-glucosamine units to form 2,5-anhydro-D-mannose [17]; artifact signals may appear in the spectrum as a result (Fig. 3). The aim of the present study was to show that the depolymerization of low-molecular-weight chitosan samples is not a necessary step in P_A analysis using ^{13}C NMR.

2. Experimental

2.1. Materials

Chitosan A and B were purchased from Chipro (Bremen, Germany), chitosan S, U, W and Y were received from Bioneer (Horsholm, Denmark). D_2O and DCl were obtained from Deutero GmbH (Kastellaun, Germany), and $NaNO_2$ from Fluka (Seelze, Germany). All chemicals used were of analytical grade.

2.2. NMR sample preparation

All chitosan samples were divided into two portions of 25 and 100 mg. The 25 mg portions were prepared directly for NMR analysis. Each was dissolved in a solution of 1 mL of 99.9% D_2O and 5 μL of DCl by means of sonification (15 min, 60 °C) and then lyophilized. These steps were repeated twice to enable labile hydrogen to be replaced by deuterium. Next, the deuterium-containing chitosan samples – 5 mg for ^{1}H NMR and 20 mg for ^{13}C NMR experiments – were dissolved in a solution containing 0.7 mL of 99.95% D_2O and

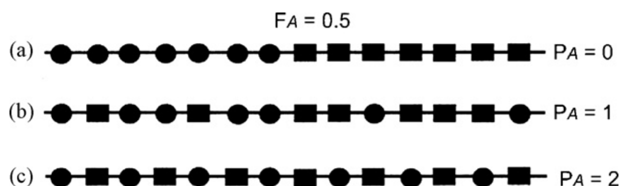

Fig. 2. The scheme of (a) the perfect block (P_A = 0), (b) the random distribution (P_A = 1) and (c) the alternating distribution (P_A = 2) of *N*-acetylglucosamine (■) and glucosamine (●) units along a chitosan chain (F_A = 0.5).

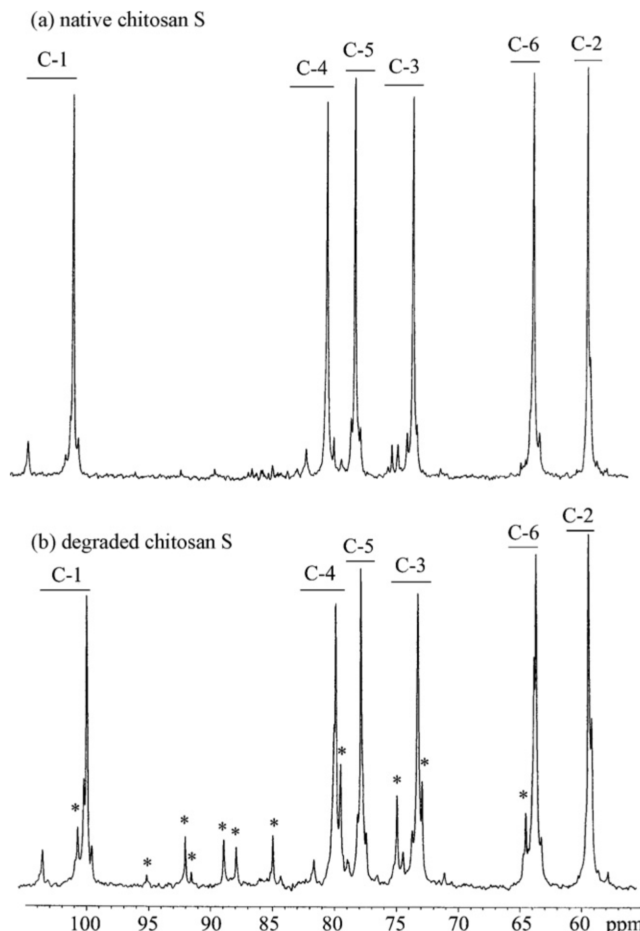

Fig. 3. The parts of the ^{13}C NMR spectra of (a) native and (b) degraded chitosan S samples obtained during the present study. As shown in (b), artifact signals (*) are visible in the spectrum of the degraded sample besides the signals typical of chitosan (signals C-1 to C-6 in a and b).

4 μL of DCl (pD 3–5). The samples were transferred into 5 mm NMR tubes. In the case of the ^{13}C NMR analyses acetone was added to the prepared samples as the internal standard. The 100 mg portions of chitosan were depolymerized. Each was dissolved in 10 mL of 0.07 M HCl with stirring at room temperature for 24 h. Then, following the addition of 5 mg $NaNO_2$, the solution was stored for 4 h and thereafter lyophilized. The depolymerized samples were prepared for NMR analysis according to the procedure described above. 50 mg of these samples were used for each ^{13}C NMR experiment.

2.3. NMR spectroscopy

The one-dimensional ^{1}H and ^{13}C NMR spectra were recorded with a Varian Mercury 400 MHz spectrometer (Palo Alto, USA) in D_2O solutions. ^{1}H NMR spectra were recorded at 80 °C, the ^{13}C NMR spectra at 60 or 70 °C. At these temperatures the solvent peak (HOD) did not interfere with any of the signals due to chitosan. Normal carbon spectra are not quantitative due to NOE effect and different relaxation times of different carbons (carbons with long T1's may be saturated, which results in reduced intensity). Accordingly, all ^{13}C NMR spectra were recorded with inverse-gated proton decoupling by using the WALTZ-16 sequence, which suppressed the NOE effect. Moreover, to achieve full relaxation of all the carbon atoms, an acquisition time of 3 s, a delay time of 5 s, and a pulse width (60°) of 7.1 μs were applied. 32 and 30 000 scans were acquired for the proton and carbon spectra, respectively. Chemical shifts were

reported relative to internal acetone (δ_H 2.225, δ_C 31.45). The ^{1}H NMR analysis of each sample was performed in two ways: without and with the addition of acetone.

2.4. Determination of the degree of acetylation F_A

F_A was determined by ^{1}H NMR spectroscopy using the method described by Hirai et al. [18]. In order to obtain more accurate experimental data, a linear drift correlation between 1 and 6 ppm was applied prior to signal integration in ^{1}H NMR spectra. This procedure subtracts a linear function from the baseline and improves baselines that are offset from zero or have a linear slope [19]. Integration boundaries were set manually from an inspection of the spectrum.

2.5. Determination of the pattern of acetylation P_A

The distribution of *N*-acetyl groups along the chitosan chain was determined by ^{13}C NMR spectroscopy according to the method presented by Vårum et al. [15]. MestRec-4.9.9.9 software for PC was applied for processing the spectra. A linear drift correlation between 0 and 110 ppm was used. A line-fitting procedure was additionally applied. This uses the Levenberg–Marquardt non-linear least squares and Downhill Simplex algorithms for estimating the following peak parameters: position, intensity, line width and line shape function. Moreover, line fitting also includes options for locking any of the parameters during optimization, as well as the ability to compare the resulting calculated spectrum as a sum of the individual components with the experimental spectrum [19]. ^{13}C NMR data was compared with the Bernouillian statistics for polymers [13,14].

2.6. Determination of the molecular weight

The molecular weight was determined by triple detection size-exclusion chromatography (SEC^3). Weight-averaged molecular weights (M_W) were determined as described previously [20]. Both native and $NaNO_2$ degraded samples were analyzed to monitor the change in molecular weight during the reaction.

3. Results and discussion

3.1. M_W and F_A parameters of the original and degraded chitosan samples

In the present work, six chitosan samples with different initial molecular weights (M_W) from 9.7 to 122 kg/mol were used. All native and depolymerized samples were subjected to ^{13}C NMR in order to perform P_A analysis and to compare the resolution in the NMR spectra. The degrees of acetylation (F_A) of native and degraded samples were measured (see Table 1). The P_A values were calculated with the aid of the experimental sequences F_{AA}, F_{DD}, and F_{AD} and compared with the random Bernoullian statistics for polymers [13,14].

3.2. P_A parameter of the original and degraded chitosan samples

In the ^{13}C NMR spectrum of chitosan, the carbon signals have different chemical shifts depending upon the nature of the neighboring units (GlcNAc A or GlcN D). The intensities of the C-5 carbon signals were used for the determination of the experimental sequences AD, DD, AA and DA (Fig. 4). The relative experimental intensities of ^{13}C resonances in the analyzed chitosan samples

Table 1
Physicochemical data (M_W—molecular weight, F_A—degree of acetylation, P_A—pattern of acetylation) of native and degraded chitosan samples. Typical standard deviations may be as high as 2% for M_W, 3% for F_A and 10% for P_A owing to uncertainties during line fitting and depending on spectral quality. P_A of native samples A and B could not be determined.

Sample	Native chitosan			Degraded chitosan		
	M_W (kg/mol)	F_A	P_A	M_W (kg/mol)	F_A	P_A
Chitosan S	9.7	0.09	0.99	3.3	0.10	0.90
Chitosan U	16.8	0.09	0.87	4.0	0.10	0.87
Chitosan W	26.8	0.10	0.91	6.2	0.10	0.90
Chitosan Y	40.9	0.10	0.87	5.1	0.10	0.95
Chitosan B	98	0.02	–	6.0	0.02	0.46
Chitosan A	122	0.09	–	6.4	0.08	0.79

were normalized according to Bernoullian statistics [13,14] and presented as:

$$F_{AD} = \frac{I_{AD} + I_{DA}}{I_{AD} + I_{DA} + I_{DD} + I_{AA}} \quad (1)$$

$$F_{AA} = \frac{I_{AA}}{I_{AD} + I_{DA} + I_{DD} + I_{AA}} \quad (2)$$

$$F_{DD} = \frac{I_{DD}}{I_{AD} + I_{DA} + I_{DD} + I_{AA}} \quad (3)$$

where I_{AD}, I_{DD}, I_{AA} and I_{DA} are the experimental intensities of signals AA, DD, AA and DA in the analyzed samples, respectively. In Eqs. (1)–(3) F_{AA} (F_{DD}) is the probability that two A (D) groups are adjacent to each other and F_{AD} the probability that one group A has a D neighbor, and vice versa. The obtained data were transformed into one single P_A parameter (Eq. (4)) [14]

$$P_A = \frac{F_{AD}}{(2F_{AA}) + F_{AD}} + \frac{F_{AD}}{(2F_{DD}) + F_{AD}} \quad (4)$$

and listed in Table 1.

The P_A values obtained for original and degraded chitosan S, which had the lowest molecular weight, were 0.99 and 0.90, respectively (Table 1). This indicated a random-dominated pattern of *N*-acetyl groups along this chain. P_A of the native sample seemed to be more accurate because, as Fig. 3 shows, many additional signals appeared in the ^{13}C NMR spectrum of the depolymerized chitosan S. This means that depolymerization of such a small molecule (9.7 kg/mol) could have a bearing on the accuracy of the P_A estimate. The results for chitosan U (M_W 16.8 kg/mol) and chitosan W (M_W 26.8 kg/mol) were roughly the same: 0.87/0.87 and 0.91/0.90,

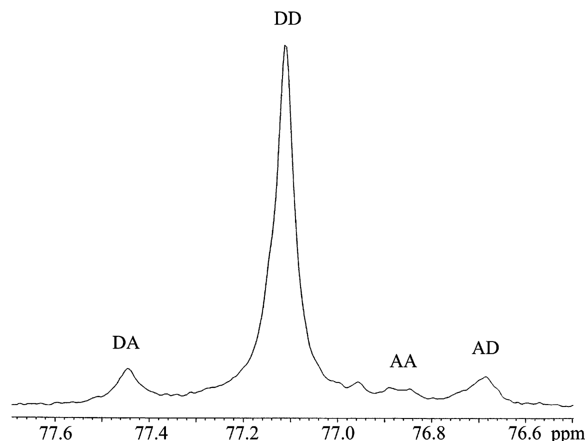

Fig. 4. The part of the ^{13}C NMR spectrum of native chitosan S containing the C-5 signals used for determining the experimental sequence of GlcNAc (A) and GlcN (D) units along the chain.

respectively. These values also indicated a random-dominated pattern (P_A from 0.50 to 1). The respective P_A values for native chitosan Y (M_W 40.9 kg/mol) and degraded chitosan Y (M_W 5.1 kg/mol) were 0.87 and 0.95. Even though the lines in the spectrum of the original chitosan Y exhibited broadening, extraction of the real signal intensity was possible and the results were comparable. To assess the limit of sensitivity for this method two samples with a higher molecular weight but different F_A were taken. Chitosan B (M_W 98 kg/mol) displayed the lowest degree of acetylation (F_A 0.02) among the chitosan samples analyzed; its F_{AA} value was therefore difficult to measure owing to the diminished NMR AA signal. The signal-to-noise parameter (S/N) of signal AA was less than 3, and the correct integration of signals AD and DA was also impossible (S/N in both cases was 3). However, the resolution of a higher acetylated chitosan sample A (M_W 122 kg/mol) was still insufficient for an accurate determination of P_A. This means that, regardless of the number of acetylated residues along the chitosan chain, a clear pattern analysis using native samples is not possible at a molecular weight of 122 kg/mol or higher. It was demonstrated that accurate information about the pattern of acetylation of a native chitosan sample could be extracted only for a molecular weight < 41 kg/mol. Therefore, in order to achieve the same accuracy for chitosan preparations of higher molecular weights, the samples need to be depolymerized beforehand.

4. Conclusions

The pattern of acetylation P_A of commercially available low-molecular-weight chitosan preparations (<41 kg/mol) using ^{13}C NMR spectroscopy can be estimated without initial degradation of the native samples. The signal-to-noise ratios of the peaks DA, DD, AA and AD for the C-5 carbon in the ^{13}C spectra of the native chitosans used for P_A determination were 15–22, 97–192, 10–32 and 11–16, respectively. For P_A analysis of higher molecular weight samples (>41 kg/mol) limited degradation is recommended. P_A is an important parameter in the physicochemical characterization of chitosans [4,12,21–23] and should be included in the description of chitosan samples used in biomedical applications.

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