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Determination of the degree of *N*-acetylation and the distribution of *N*-acetyl groups in partially *N*-deacetylated chitins (chitosans) by high-field n.m.r. spectroscopy*

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ABSTRACT

The composition and sequence of 2-acetamido-2-deoxy-β-D-glucose (GlcNAc) and 2-amino-2-deoxy-β-D-glucose (GlcN) residues in partially *N*-deacetylated chitosans, prepared under homogeneous and heterogeneous conditions, have been determined by ^{1}H-n.m.r. spectroscopy. It was necessary to depolymerise the chitosan slightly by treatment with nitrous acid before spectroscopy. A sequence-dependent deshielding of H-1 of the GlcNAc residues made it possible to determine the proportions of the four possible diads. Chitosan prepared by *N*-deacetylation under homogeneous conditions gave values for the diad frequencies that were roughly consistent with a random distribution of the *N*-acetyl groups. Samples prepared under heterogeneous conditions have a frequency of the GlcNAc–GlcNAc diad slightly higher than for a random (Bernoullian) distribution. The chitosans, prepared under both homogeneous and heterogeneous conditions, with a degree of acetylation of 50% were soluble at neutral pH.

INTRODUCTION

Chitin, a (1→4)-linked 2-acetamido-2-deoxy-β-D-glucan that occurs mainly in insects, marine invertebrates, fungi, and yeasts, is a structural polysaccharide which is insoluble in most aqueous solvents. Chitosan, partially deacetylated chitin, contains 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc) and 2-amino-2-deoxy-β-D-glucopyranose (GlcN) residues and may be considered as a binary heteropolysaccharide. The GlcN units carry positive charges in acidic media. Commercial chitosans are water-soluble at an acidic pH and have numerous applications in industry, pharmacy, and biotechnology[1].

The degree of *N*-acetylation (d.a.) is an important characteristic of chitosans, and i.r.[2] and u.v.[3] spectroscopy and titration[4] have been used to determine the d.a. Recently, Neugebauer and co-workers[5] reported a method whereby the d.a. could be determined without solubilisation of the polysaccharide, which makes it suitable for application to highly *N*-acetylated chitosans that are insoluble in water. To our knowledge, the determination of d.a. values by n.m.r. spectroscopy has not been described.

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* High-field N.m.r. Spectroscopy of Partially *N*-Deacetylated Chitins (Chitosans), Part I.
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Sannan and co-workers[6] found that, for chitosans prepared by a treatment of chitin with alkali under homogeneous conditions, ~50% *N*-deacetylation results in solubility in neutral and basic aqueous media. Kurita and co-workers[7] suggested that *N*-deacetylation under homogeneous conditions gave a random-type copolymer of GlcNAc and GlcN, whereas, under heterogeneous conditions, products with a more blockwise distribution of the *N*-acetyl groups were obtained, but the evidence was indirect, since it was based on X-ray diffraction data.

High-field n.m.r. spectroscopy has been applied to characterise the composition and sequence of binary heteropolysaccharides, such as alginate[8,9], galactomannans[10], and pectins[11]. The method is simple, rapid, and suitable for routine use, and we now report its use to determine the d.a. and diad frequencies (nearest-neighbour probabilities) in partially *N*-deacetylated chitins (chitosans) obtained under homogeneous and heterogeneous conditions.

EXPERIMENTAL

Materials. — Chitin was isolated[12] from fresh shrimp shell and milled in a hammer mill to pass through a 0.5-mm sieve.

Preparation of chitosan fractions. — *N*-Deacetylation of chitin under homogeneous conditions involved the procedure of Sannan *et al.*[2]. For *N*-deacetylation under heterogeneous conditions, chitin (1.0 g) was treated with aqueous 50% (w/v) NaOH (100 mL) at 70° for 20, 40, and 70 min. Each solution was deaerated and stirred continuously.

Degradation of chitosan. — Solutions of chitosan (100 mg) in 0.07M HCl (10 mL) were gently shaken overnight at ambient temperature. A known amount (2–4 mg) of solid $NaNO_2$ was added, and each solution was stored at room temperature for 4 h, then lyophilised. Each residue was dissolved in D_2O and lyophilised, and the procedure was repeated three times in order to minimise the HOD signal (from the solvent).

N.m.r. spectroscopy. — The samples of chitosan were each dissolved in D_2O in 5-mm tubes at pD 3–4. The deuterium resonance was used as a field-frequency lock and the chemical shifts were referenced to internal sodium 3-(trimethylsilyl)propionate-d_4. The ^{1}H-n.m.r. spectra were recorded with a Bruker WM-500 (500 MHz) or Jeol FX 100 (99.6 MHz) spectrometer at 90° in order to diminish the viscosity of the solutions and, thereby, the line width of the signals. The relative intensities of the resonances were determined by a computer program developed by O. E. Bakøy in our laboratory.

RESULTS AND DISCUSSION

Preliminary degradation of samples. — Limited depolymerisation of the samples of chitosan with nitrous acid diminished the viscosity of their solutions, improved the solubility, and narrowed the line widths of the signals. Nitrous acid attacks GlcN units but not GlcNAc units in the chitosan molecules, the following glycosidic linkage is cleaved, and a 2,5-anhydro-D-mannose residue is generated at the new reducing end[13].

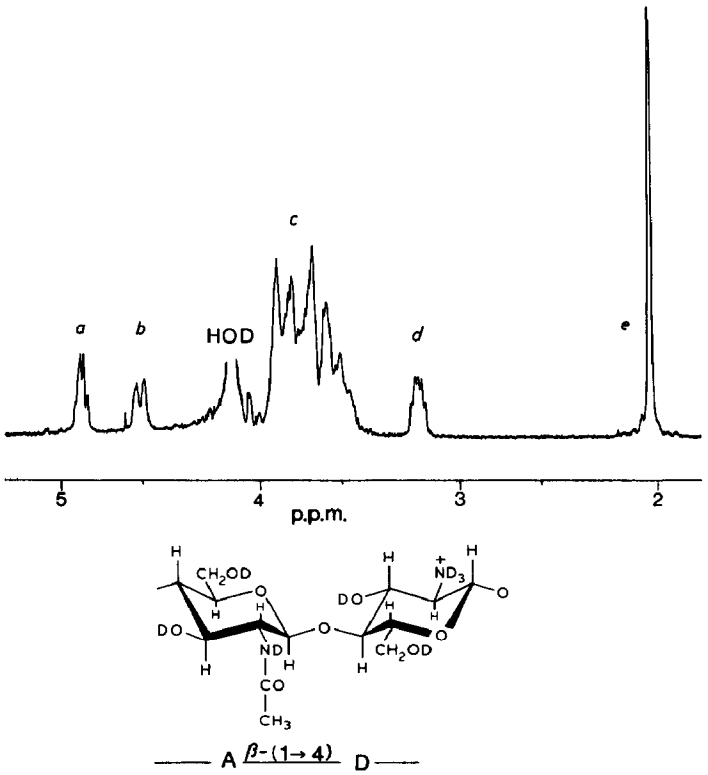

Fig. 1. ^{1}H-N.m.r. spectrum (500 MHz) of solutions of chitosan (10 mg/mL) in D_2O (at pD 3 and 90°).

Figure 1 shows the 500-MHz ^{1}H-n.m.r. spectrum of a chitosan with d.a. 47%.

Determination of the relaxation times and relative areas. — It is necessary to ensure that the relative areas of the observed resonances represent the relative amounts of protons involved. At 99.6 MHz, it was essential to eliminate the solvent peak (HDO) partly by employing a 180°–*t*–90° pulse sequence ($t \sim 3$ s) with a recycle time of ~ 7 s as reported[14]. This procedure was possible because the carbohydrate protons relaxed more rapidly ($T_1 \sim 0.3$ s, except for the NAc protons which had $T_1 \sim 0.6$ s) than the deuterons. Thus, the relaxation times were short enough to allow complete relaxation between pulses. Moreover, the internal consistency concerning the equal intensity of resonances from the same residue was satisfactory.

Initial degradation of the chitosan was necessary in order to obtain high-quality spectra; highly acetylated oligosaccharides (from the preliminary degradation) may precipitate from the solutions in D_2O at high concentrations. The ^{1}H-n.m.r. spectra of a chitosan fraction with d.a. of 50%, at five concentrations in the range 0.2–12%, showed no significant differences in the relative areas of the observed resonances. When this chitosan fraction was treated first with nitrous acid and then with lysozyme in order to degrade any highly acetylated oligosaccharides, the ^{1}H-n.m.r. spectrum of the product was not significantly different in d.a. or diad frequencies from that of the product obtained by treatment only with nitrous acid. Therefore, it was concluded that the relative areas of the peaks represented the relative occurrence in chitosan.

TABLE I

Chemical shifts (δ) of proton resonances for chitosan in D_2O at 90° (pD 3)

Residue	*Proton*			
	H-1	*H-2*	*H-2/6*	*Acetyl-H*
GlcNAc (A)	4.55–4.65		3.5–4.0	2.04
GlcN (D)	4.85	3.15	3.5–4.0	

Identification of resonances in the spectrum. — The 300-MHz ^{1}H-n.m.r. spectrum of a fully *N*-deacetylated chitosan has been reported[15]. In the 500-MHz ^{1}H-n.m.r. spectrum (Fig. 1), five resonances (*a–e*) were identified (Table I). The resonances of H-1 and H-2 of a GlcN unit occur at 4.8 (*a*) and 3.1 p.p.m. (*e*), respectively. The identification of the resonance at 4.6 p.p.m. (*b*) as due to H-1 of a GlcNAc unit is based on the reported spectrum of methyl 2-acetamido-2-deoxy-β-D-glucopyranoside[16]. These identifications were confirmed by a COSY experiment. The resonance at 2.04 p.p.m. (*e*) is due to the three NAc protons. No attempts were made to identify the resonances of H-2 of GlcNAc and H-3/6, which occur in the range 3.5–4.0 p.p.m.

Determination of the degree of N-*acetylation (d.a.).* — From the identification of the resonances (*a–e* in Fig. 1), it was possible to determine the d.a. In order to use all of the intensities of *a–e*, the following method was developed. Let f be defined as

(Total area of resonances)/(area of GlcNAc H-1 + NAc resonances)

$= (I_a + I_b + I_c + I_d + I_e)/(I_b + I_e)$.

If F_A is the mole fraction of *N*-acetylated units, then the mole fraction of *N*-deacetylated units is $1 - F_A$. Expressing f in terms of F_A gives

$f = (1 - F_A + F_A + 6 + 3F_A)/(F_A + 3F_A) = (7 + 3F_A)/4F_A$; [$I_c + I_d$ includes six protons from every monomer unit (GlcN or GlcNAc), and I_e is three times the mole fraction of the GlcNAc].

The mole fraction of *N*-acetylated units (F_A) may be expressed as

$F_A = 7(I_b + I_e)/[4(I_a + I_c + I_d) + I_b + I_e]$.

It is possible to determine the d.a. of chitosans at lower field (100 MHz), but not the diad frequencies. At 100 MHz, the d.a. can be determined either from the relative areas of the H-1 resonances or in combination with the area of the NAc resonances, as the HOD-signal (from the solvent) partly overlaps the resonances at 3.5–4.0 p.p.m.

Diad frequencies in homogeneous N-*deacetylated chitosans.* — In order to study the neighbouring-residue effect on the ^{1}H-n.m.r. resonances, a series of chitosan samples with degrees of *N*-deacetylation (d.d.) within the range from 51–96% (F_A 0.49–0.04) were prepared. Fractions of lower d.d. are insoluble in acidic media[2]. Figure 2 shows the region for the resonances of anomeric protons of these samples recorded at 500 MHz. The resonance of H-1 of a GlcNAc unit has two relatively well-resolved peaks that reflect two diad frequencies. These resonances suggest that the H-1 resonance of a GlcNAc unit is sensitive to a nearest neighbour, most probably to the following residue

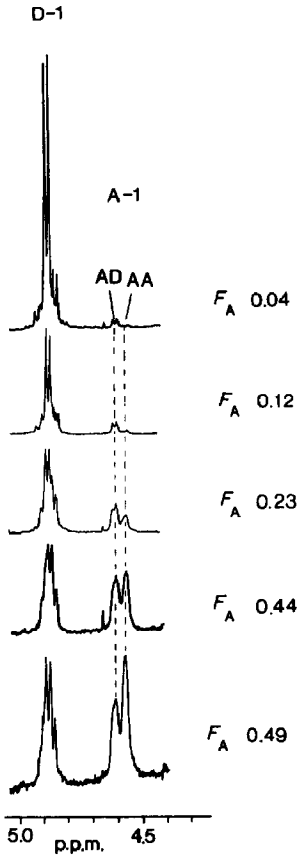

Fig. 2. ^{1}H-N.m.r. spectra in the region for resonances of anomeric protons of a series of chitosans.

in the chain. Consequently, the two diad frequencies must be AD (GlcNAc–GlcN) and AA (GlcNAc–GlcNAc). The AA diad preponderates at low d.d. and the AD diad at higher d.d. The H-1 resonance of a GlcNAc residue is relatively broad, which is probably due to its proximity to a rapidly relaxing nitrogen atom that is in a more asymmetric environment than in a GlcN unit.

Since $F_{AD} = F_{DA}$ for long chains, the DD (GlcN–GlcN) sequence may be calculated from the relationship $F_D = F_{DA} + F_{DD}$. Table II gives the diad frequencies for chitosan fractions obtained under homogeneous and heterogeneous conditions of *N*-deacetylation. From the diad frequencies, the number-average block length ($\overline{N}$) can be calculated, which is independent of any statistical model[8]. There is a tendency for the GlcNAc and GlcN units to occur in blocks, reflected by the lower transition frequency (AD and DA) compared to a Bernoullian distribution. However, the accuracy in the determination of the relative intensities of the partly overlapping resonance lines at 4.5 and 4.6 p.p.m. is $\pm 10\%$, which means that the block tendency in the homogeneous deacetylated chitosan fractions may not be significant. Since the fractions were prepared by deacetylation under homogeneous conditions, it was expected that the diad fre-

TABLE II

Distribution of diad frequencies in partially deacetylated chitosans as determined by ^{1}H-n.m.r. spectroscopy

Sample	F_{AA}	$F_{AD} = F_{DA}$	F_{DD}	$\overline{N}_A$	$\overline{N}_D$
Deacetylation under homogeneous conditions (aqueous 10% NaOH, 25°)					
1	0.29	0.21	0.30	2.3	2.4
random (F_A 0.49)	0.24	0.25	0.25	2.0	2.0
2	0.23	0.22	0.35	2.0	2.5
random (F_A 0.44)	0.19	0.25	0.31	1.8	2.2
3	0.08	0.14	0.63	1.6	5.5
random (F_A 0.23)	0.05	0.18	0.60	1.3	4.3
4	0.03	0.09	0.80	1.3	9.8
random (F_A 0.12)	0.01	0.10	0.78	1.2	8.8
5	0.01	0.03	0.92	1.3	32.0
random (F_A 0.04)	0.00	0.04	0.91	1.0	24.0
Deacetylation under heterogeneous conditions (aqueous 50% NaOH, 70°)					
20 min	0.31	0.21	0.27	2.5	2.3
random (F_A 0.52)	0.27	0.25	0.23	2.0	1.9
40 min	0.14	0.19	0.48	1.7	3.5
random (F_A 0.33)	0.11	0.22	0.44	1.5	3.0
60 min	0.10	0.17	0.55	1.6	4.2
random (F_A 0.28)	0.08	0.20	0.52	1.4	3.6

quencies would be close to the calculated random (Bernoullian) diad distribution. Kurita and co-workers[7] suggested that chitosans obtained by deacetylation under homogeneous conditions had a random distribution of GlcNAc units.

The resonance of H-1 of a GlcN unit also contains information on sequence. However, because the resonances are not well resolved and the sequence information is complicated by different line widths, depending on the neighbouring residues, no attempt was made to identify the resonances.

Diad frequencies in chitosans obtained by N-*deacetylation under heterogeneous conditions.* — Kurita and co-workers[7] reported that chitosans of this type had a blockwise distribution of NAc groups and were insoluble in water. Samples of chitosan with d.d. 50–95% were all soluble in dilute acid, and those with d.d. 50% were soluble also at neutral pH. Table II shows that the GlcNAc units of chitosans prepared by *N*-deacetylation under heterogeneous conditions have a slightly more blockwise distribution than those prepared under homogeneous conditions. However, the deviation from a random distribution is not high (22–27% for the AA diad), and the solubility at neutral pH of the fraction with d.a. 50% prepared under heterogeneous conditions suggested that the structure was not very different from that of chitosan with d.a. 50% prepared under homogeneous conditions. The chitosans prepared by *N*-deacetylation under heterogeneous conditions involved finely ground chitin and may not be directly comparable with the commercial chitosans.

The ^{13}C-n.m.r. data for chitosans will be reported separately.

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