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Determination of degree of deacetylation of chitosan by ^{1}H NMR spectroscopy

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Summary

This paper describes a novel method to determine the degree of deacetylation of chitosan by ^{1}H NMR spectroscopy. Measurements were carried out at 70°C by using 2 wt% CD_3COOD/D_2O and 2 wt% DCl/D_2O as solvents for chitosan. In the case of DCl/D_2O system, effect on hydrolysis of chitosan should be taken into consideration, and the pulse repetition delay required for 45° pulse is 40s. Whereas, in regard to CD_3COOD/D_2O system, 6s pulse repetition delay is enough for 45° pulse, and moreover, high magnetic field NMR apparatus is necessary, because signal of CD_2H residue in CD_3COOD overlaps with that of CH_3 residue in N-acetyl residue and determination of the difference of two spectra is required. The proposed method is more effective, precise and simple, comparing with the conventional colloid titration and elemental analysis methods. Assignments of ^{1}H and ^{13}C NMR spectra of chitosan are also reported.

Introduction

Chitosan is a polysaccharide obtained by partial or complete deacetylation of chitin, and its molecular structure and properties are largely affected by the degree of deacetylation. For this reason, it is needed to establish a method to determine exactly the degree of deacetylation. Conventionally, the degree of deacetylation has been determined from amino residue analysis by colloid titration (1), carbon/nitrogen ratio by elemental analysis, and absorbance ratio of infrared spectra(2). Chitosan is extremly hygroscopic, hence it is very difficult to eliminate completely the effect of moisture in these conventional methods. Moreover, in the case of colloid titration, a large amount of sample is to be used. Contrary, very few sample (ca. 5mg) is sufficient with ^{1}H NMR method. In this work, we report a novel method to determine the degree of deacetylation of chitosan by ^{1}H NMR spectra. In addition, assignments of ^{1}H and ^{13}C NMR spectra of chitosan by two dimentional NMR method are described.

Experimental

Materials

Chitosan sample(TK1) was purchased from Tokyo Kasei Ltd. Sample TK1 was immersed in 50 wt% NaOH aqueous solution, and deacetylated for 1hr at 110°C in argon atmosphere. After filtration, the product was further deacetylated under the same condition, and filtered, washed with distilled water, and vacuum dried. Thus, highly deacetylated sample (designeted as TK2) was prepared. Three kinds of commercial chitosan (KB004, 8B, and 7B) purchased from Katokichi Ltd. were used for experiments without further treatment.

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NMR measurements

^{1}H and ^{13}C NMR measurements were performed on a JEOL JNM-GX400 NMR spectrometer under a static magnetic field of 9.4T at 70°C. For ^{1}H NMR measurement, ca. 5 mg sample vacuum dried at 50°C for 2 days was introduced into a 5mmϕ NMR test tube, and further vacuum dried at 50°C for 2 days, to which 0.5 ml of 2 wt% CD_3COOD/D_2O solution or of 2 wt% DCl/D_2O solution was added, and finally the test tube was kept at 70°C to dissolve the polymer in solution. 45° single pulse sequence was used for FID accumulation. 45° pulse widths were 5.8 μs and 9.6 μs for 2 wt% CD_3COOD/D_2O and 2 wt% DCl/D_2O solutions, respectively. The pulse repitition delays were 6s and 40s for CD_3COOD/D_2O and DCl/D_2O solutions, respectively. The spectral width and data points were 6000 Hz and 32 K points, respectively. ^{1}H chemical shifts were expressed in ppm downfield from the signal for sodium 3-(trimethyl silyl) propane sulfonate (TSP) as an external reference. The ^{1}H spin-lattice relaxation time, T_1, was measured by means of inversion recovery ($180°-\tau-90°$) pulse sequence. For ^{13}C NMR measurements, a 10 mmϕ test tube was used, and the polymer concentration in the tube was 50 mg/ml.

The $^{1}H-^{1}H$ chemical shift correlation spectrum (COSY)(3) was obtained using a 512 x 1024 data matrix size and 48 transients for each t_1 value. The digital resolution of 2D matirx was 2.4 Hz. $^{13}C-^{1}H$ chemical shift correlation spectrum (4) was recorded by using a 512 x 2048 data matrix size and 544 scans for each t_1 value. The digital resolution of ^{13}C axis was 15.6 Hz and that of ^{1}H axis was 3.7 Hz.

Elemental analysis

The elemental analyses were performed with a Yanagimoto CHN Corder MT-3 type apparatus.

Colloid titration

ca. 80mg chitosan sample was dissolved in a 200 ml 2 wt% acetic acid aqueous solution. Therefrom, 20 ml solution was transfered into a Erlenmeyer flask and titrated with N/400 polyvinyl potassium sulphate solution by using Toluidine Blue as an indicator, whose end point is determined by the color change from blue to reddish purple.

Results and Discussion

Figure 1 represents the 400 MHz ^{1}H NMR spectrum measured at 70 °C for chitosan TK2 (D_{deac} =97%) dissolved in 2 wt% CD_3COOD/D_2O. Details of assignment of each proton are commented later.

In the vicinity of 2 ppm, the resonance band due to the CD_2H residue of CD_3COOD overlaps with that due to CH_3 residue of N-acetyl. However, as is obvious from the magnification (Fig. 1(b)), CD_2H band can be separated from CH_3 band by drawing symmetric curve for CH_3 band. Thus, the degree of deacetylation (D_{deac}) is evaluated from eq(1) by using the integral intensity, I_{CH3}, of this CH_3 residue, and the sum of integral intensities, I_{H2-H6}, of H2, H3, H4, H5, H6, and H6' protons.

$$D_{deac}\ (\%) = \{\ 1 - (\ \frac{1}{3}\ I_{CH3}\ /\ \frac{1}{6}\ I_{H2-H6}\ )\} \times 100 \qquad (1)$$

In this treatment, base line was given by a straight line connecting the intensities at 2.5 ppm and 5.5 ppm. Proton bands of H3, H4, and H6 were separated from respective HOD bands by drawing smooth curve at low magnetic field side. The integral intensities were measured by weighing method.

The very small resonance line appeared near 4.6 ppm is to be assigned to H1' band due to acetamidoglucose residue. This assignment is supported from the fact that the 1' peak becomes larger with decreasing degree of

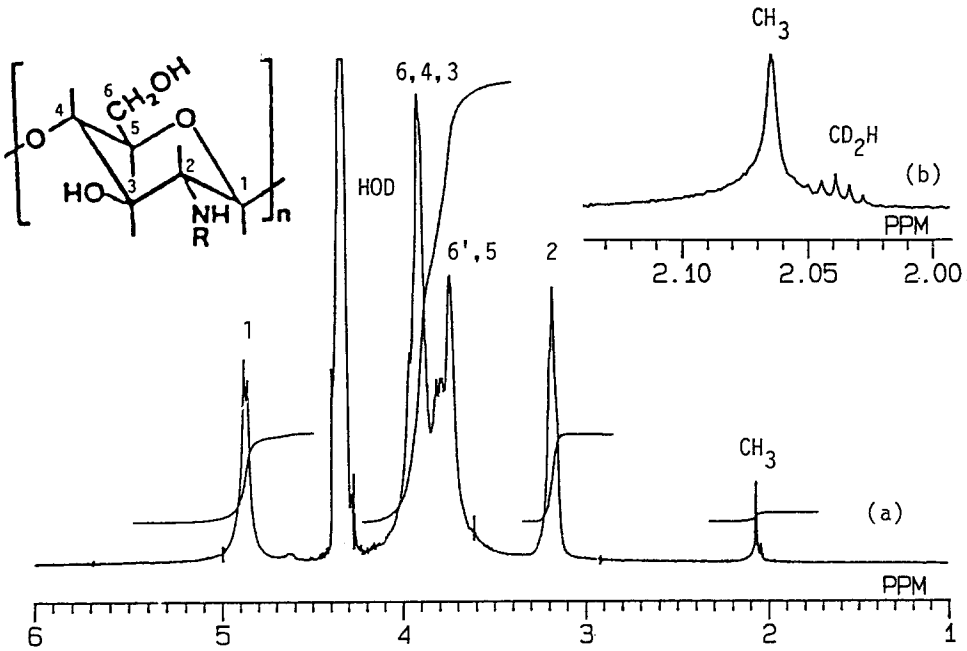

Figure 1. (a) 400 MHz ^1H NMR spectrum of chitosan TK2 (D_{deac} = 97%), in CD_3COOD/D_2O, at 70°C. (b) Magnification of spectrum in the vicinity of 2.00–2.13 ppm.

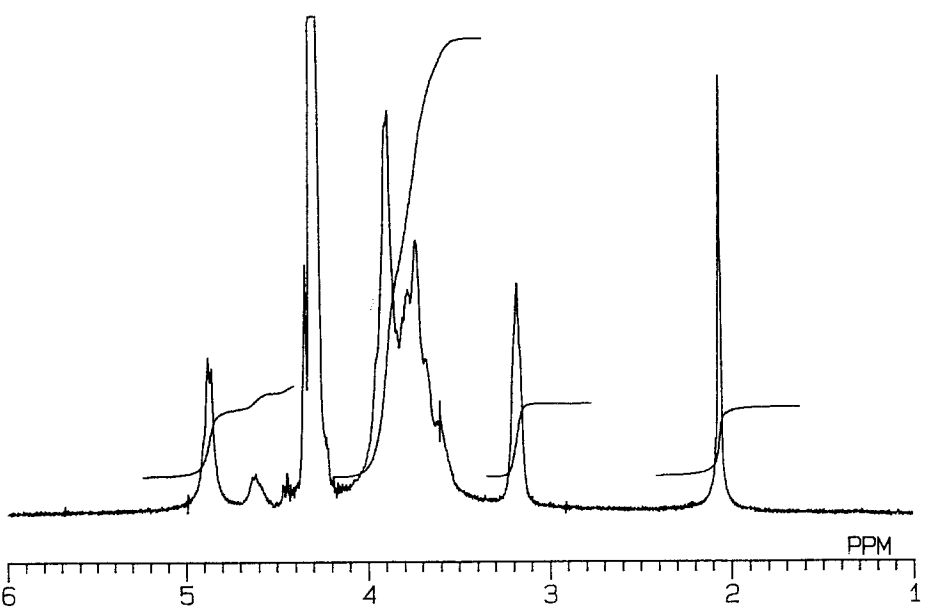

Figure 2. 400 MHz ^1H NMR spectrum of chitosan TK1 (D_{deac} = 77%), in CD_3COOD/D_2O, at 70°C.

deacetylation as is obvious from Figure 2. Another support is that the numerical value of H1' integral intensity divided by the sum of intensities of H1' and H1 is almost equal to the value of (1- D_{deac}/100) by using D_{deac} value calculated by eq (1). For calculation of D_{deac}, the total integral intensity of all the 7 protons included in a pyranose ring is divided by 7, but, in this work, 6 protons were used to avoid possible error in estimating 1' integral intensity. If we use the integral intensity shown in the figure, somewhat (2-3%) lower D_{deac} value may be obtained because the overlap portion due to HOD can not be evaluated.

Figure 2 shows the ^1H NMR spectrum measured at 70°C for chitosan TK1 (D_{deac} = 77%) dissolved in 2 wt% CD_3COOD/D_2O solution. The resonance line of CD_2H of CD_3COOD can not be separated because of the high content of CH_3 residue. Therefore correction for CD_3H was made using the result on TK2. The D_{deac} value thus obtained, however, was in accord with uncorrected value within 1% error.

In order to determine the pulse repitition delay (PD), ^1H spin-lattice relaxation times (T_1) of protons were measured for chitosan TK2 in 2 wt% CD_3COOD/D_2O solution. Results obtained are shown in Table 1. It is concluded that PD is enough with 6 s, because the T_1 value for N-acetyl residue is 1.70 s.

Table 1 ^1H spin-lattice relaxation times (T_1) for chitosan TK2 in 2 wt% CD_3COOD/D_2O measured at 400 MHz and 70°C

protons	chemical shift* (ppm)	T_1 (s)
H1	4.87, 4.85	0.96, 0.97
H2	3.18	1.23
H3,4,5,6,6'	3.90, 3.81, 3.78, 3.74	1.00, 0.77, 0.82, 0.92
N-acetyl	2.07	1.70

* TSP was used as external reference.

Table 2 Degree of deacetylation (%) of chitosan samples determined by ^1H NMR spectroscopy, elemental analysis and colloid titration

sample	^1H NMR		elemental analysis	colloid titration
	DCl/D_2O	CD_3COOD/D_2O		
TK2	97	97	96	—
TK1	78	77	78	88
KB004	92	92	87	100
8B	—	71	—	78
7B	—	61	—	65

Table 2 gives the degree of deacetylation (%) determined from eq (1), together with the results obtained from elemental analysis and colloid titration methods. In the determination by ^1H NMR method, contribution of CD_2H residue should be eliminated for 2 wt% CD_3COOD/D_2O system. However, such consideration is not needed for 2 wt% DCl/D_2O system. The ^1H NMR spectrum and the ^1H spin-lattice relaxation time at 70°C for chitosan TK2 in 2wt% DCl/D_2O are shown in Figure 3 and Table 3, respectively.

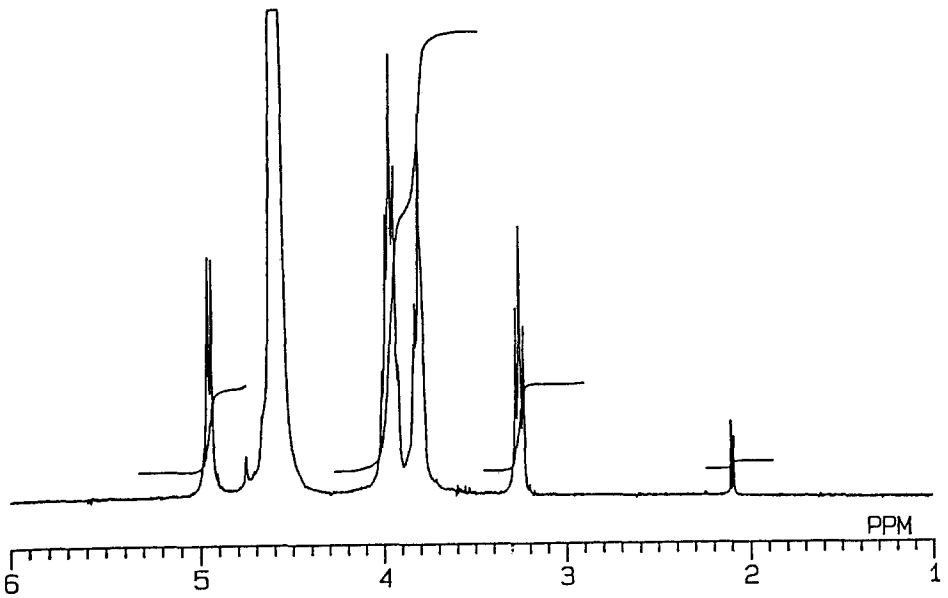

Figure 3. 400 MHz ^1H NMR spectrum of chitosan TK2 in DCl/D_2O at 70°C.

Table 3 ^1H spin-lattice relaxation times (T_1) for chitosan TK2 in DCl/D_2O measured at 400 MHz and 70°C

protons	chemical shift* (ppm)	T_1 (s)
H1	4.97, 4.95	0.92, 0.99
H2	3.28, 3.26, 3.24	1.69, 1.59, 1.71
H3,4,5,6,6'	3.99, 3.97, 3.95, 3.84, 3.81	1.07, 1.06, 0.92, 0.69, 0.76
N-acetyl	2.09	1.0
CH_3COOH	2.11	12

* TSP was used as external reference.

Two resonance lines appear at about 2.1 ppm. The upfield and downfield resonances were assigned respectively to CH_3 of N-acetyl residue and that of acetic acid produced by hydrolysis. The pulse repitition delay for this system is 40s, because the T_1 value for CH_3 residue of acetic acid is 12 s. Degree of deacetylation was calculated from eq(1) by using I_{CH3} and I_{H2-H6}. The former was defined as the integral intensity for CH_3 residues of N-acetyl and of acetic acid, and the latter as the integral intensity for 6 protons, H2-H6. The integral intensity was obtained as shown in the figure by means of computor attached to NMR apparatus. The results thus obtained were shown in Table 2. Obviously, this result coincides with that obtained for 2 wt% CD_3COOD/D_2O system. Attention should be called to the following facts: when the sample is kept at 70°C for a long time, acid hydrolysis is promoted, which results in the increase of CH_3COOH content,

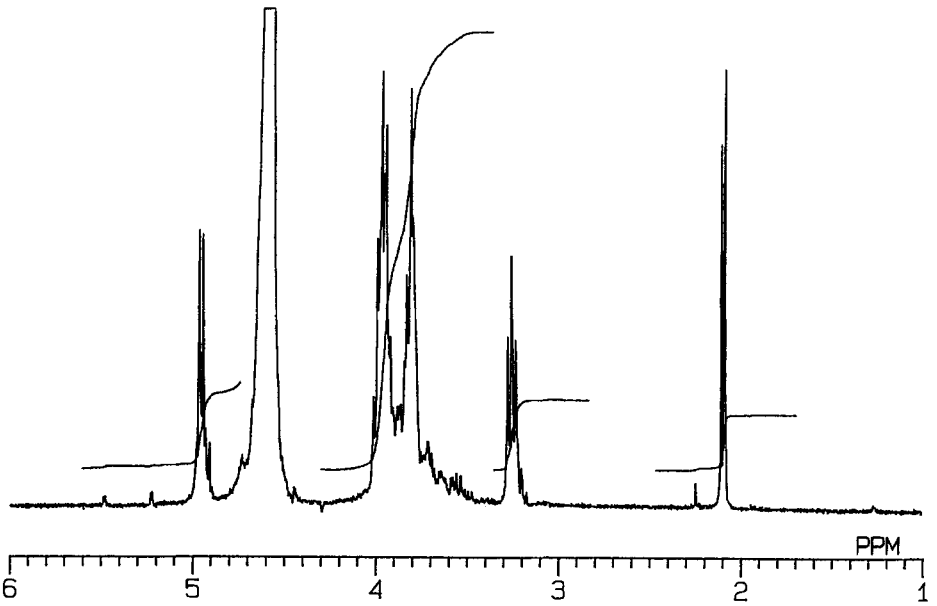

Figure 4. 400 MHz ^{1}H NMR spectrum of chitosan TK1 in DCl/D_2O at 70°C.

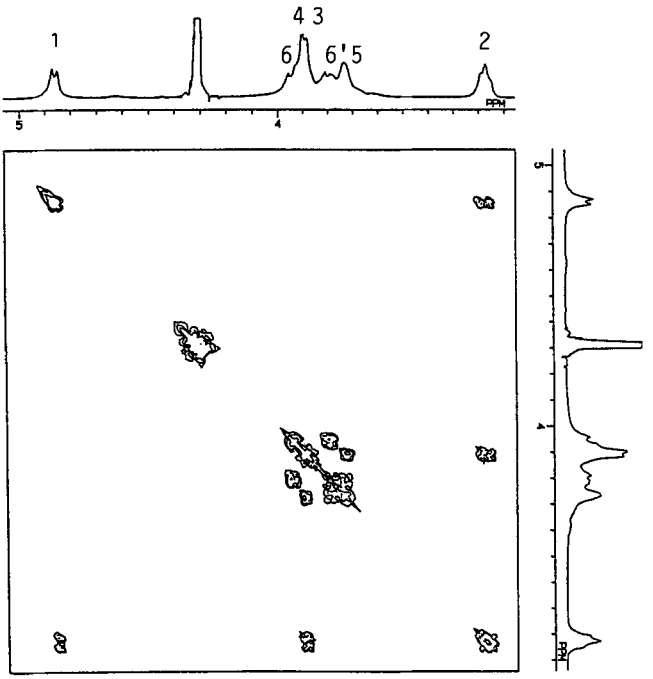

Figure 5. ^{1}H-^{1}H homonuclear chemical shift correlation (COSY) spectrum of chitosan KB004 (D_{deac} = 92%) in CD_3COOD/D_2O at 70°C.

decrease of N-acetyl content, and production of oligomers. Thus the spectra of protons of pyranose ring are complicated. As is shown in Figure 4, NMR spectrum of chitosan of low degree of deacetylation becomes more complex. At 20 hrs after dissolution of TK2 in DCl/D_2O, two resonance lines, one is for CH_3COOH and the other is for N-acetyl, are detected (see Figure 3), but at 30 hrs and 60 hrs after the dissolution, only one line due to CH_3COOH is obtained. However, the degrees of deacetylation are the same within 1% error for samples after 20 hrs, 30 hrs, and 60 hrs.

The D_{deac} value obtained by ^1H NMR for highly deacetylated sample agrees well with that obtained from elemental analysis. As mentioned earlier, chitosan sample is highly hygroscopic, and furthermore the amount of sample used for elemental analysis is very small (ca. 2 mg), therefore the accurate determination is very difficult. For samples of above 90% D_{deac}, Table II indicates that ^1H NMR method is most recommendable for obtaining the degree of deacetylation.

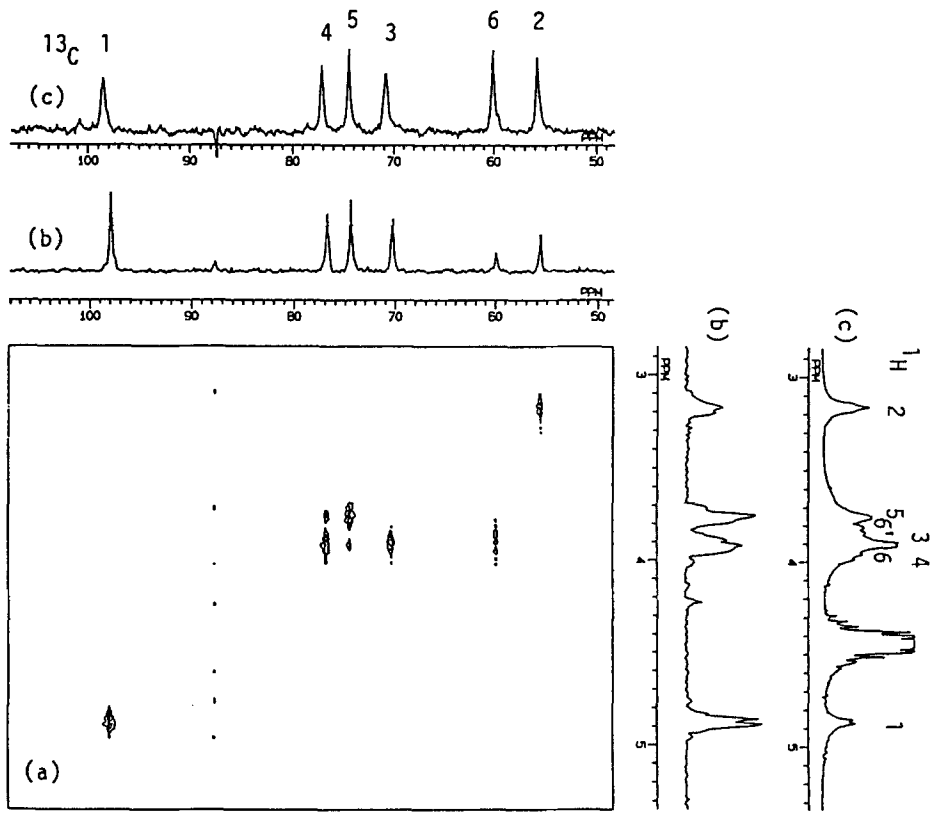

Figure 6. (a) ^{13}C-^1H heteronuclear chemical shift correlation spectrum of chitosan KB004 in CD_3COOD/D_2O at 70°C. (b) The projection of (a). (c) One dimensional ^1H and ^{13}C NMR spectra.

Assignments of ^{1}H and ^{13}C NMR spectra of chitosan were investigated by two-dimensional homonuclear and heteronuclear chemical shift correlation spectroscopy. Figures 5 and 6 indicate the ^{1}H-^{1}H chemical shift correlation spectrum (COSY) and the ^{13}C-^{1}H chemical shift correlation spectrum, both for chitosan KB004, respectively. The assignments were performed by taking into considerations the experimental results shown in Figures 5 and 6, data of Domard et al. (5), and chemical shift data on oligosaccharides (6). The assignments are shown in Figures 5 and 6. With regard to the ^{1}H chemical shift, our assignments of H3, H4, and H6 are different from those of Domard et al.

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