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A simple preparation of half N-acetylated chitosan highly soluble in water and aqueous organic solvents

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Abstract

A simple and improved method of preparing highly soluble chitosan (half N-acetylated chitosan) was developed using a series of chitosan samples of low molecular weights, and the solubility of the half N-acetylated chitosan in water and organic solvents was investigated in detail. To reduce the molecular weight, chitosan was treated with $NaBO_3$ under the condition that chitosan was homogeneously dissolved in aqueous acetic acid. Weight-average molecular weights of the obtained chitosan samples were determined using a size-exclusion chromatography system equipped with a low-angle laser light-scattering photometer. Each chitosan sample was then N-acetylated with acetic anhydride under the condition that chitosan was homogeneously dissolved in aqueous acetic acid again. The water solubility of the half N-acetylated chitosan thus prepared increased with decreasing molecular weight. From ^{1}H NMR spectroscopy, it was suggested that the sequence of *N*-acetylglucosamine and glucosamine residues was random. The solubility of the half N-acetylated chitosan of low molecular weight was rather high even in aqueous dimethylacetamide and dimethylsulfoxide. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Chitin; Chitosan; Half N-acetylated chitosan; Solubility; Molecular weight; Degree of N-acetylation

1. Introduction

Chitin, poly-β-(1 → 4)-*N*-acetyl-D-glucosamine, is the second most abundant natural polysaccharide and exists largely in the shells of crustacea and insects. Chitosan, poly-β-(1 → 4)-D-glucosamine, can be readily obtained from chitin by deacetylation with alkali. The use of chitin and chitosan in various functional materials, including biomedical materials, have recently been developed [1,2]. We have also reported on the membrane applications of chitosan and its derivatives [3–5]. However, the applications of chitin and chitosan in biology, in which many enzyme assays are performed at neutral pH, is quite restricted, because they are essentially insoluble in neutral water. If water-soluble chitin and chitosan could be prepared in a simple manner, their biological and physiological applications would develop dramatically.

Chitin and chitosan become water soluble through a chemical modification in which the degree of substitution is controlled. For instance, it is known that water-soluble chitin with a deacetylation degree of about 50% can be obtained from chitin by hydrolysis with alkali (Fig. 1) [6,7]. It was suggested that the water solubility resulted from the random distribution of *N*-acetyl groups. On the other hand, water-soluble chitosan with about a

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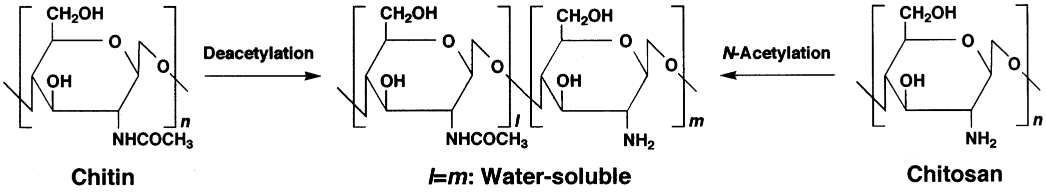

Fig. 1. Synthetic routes to water-soluble chitin and chitosan.

50% degree of N-acetylation was prepared from chitosan by N-acetylation with acetic anhydride (Fig. 1) [8]. However, a very long reaction time, i.e., over 2 days, was required in the former, and a complex solvent system, i.e., acetic acid–water–methanol–pyridine, was used in the latter. Furthermore, Aiba reported that reduction of the molecular weight did not influence the water solubility of the partially N-acetylated chitosan [9]. However, the relationship between molecular weight and such water solubility was not clear, since he used only two different molecular weights. In addition, there are few reports on the solubility of partially N-acetylated chitosan in common organic solvents.

In this paper, therefore, we report a simple and improved method to prepare highly water-soluble chitosan (half N-acetylated chitosan) using a series of chitosan samples of low molecular weights. Chitosan is previously degraded by treatment with $NaBO_3$ [10] and then N-acetylated with acetic anhydride in aqueous acetic acid. The relation between molecular weight and water solubility of the obtained N-acetylated chitosan is quantitatively investigated and the solubility of the half N-acetylated chitosan in some organic solvents is examined.

2. Experimental

Materials.—Commercially available chitosan from Tokyo Kasei Kogyo, Tokyo, Japan, was purified thrice by reprecipitation using aq AcOH (5%) and aq KOH in advance. It was finally washed with deionized water and acetone, and then dried in vacuo. Standard pullulans for size-exclusion chromatography (SEC) were purchased from Showa Denko, Tokyo, Japan. Acetic anhydride, acetone, *N*,*N*-dimethylacetamide (DMA), and Me_2SO from Wako Pure Chemical Industries, Osaka, Japan, were distilled just before use. All other chemicals were of reagent grade and used as received.

Table 1
Degradation conditions and weight-average molecular weight of chitosan

Sample code	Degradation condition			$\bar{M}_w$	$\bar{P}_n$ [a]
	$NaBO_3$ (%w/v)	Temperature (°C)	Time (h)		
600k	Untreated			6.0×10^5	1.4×10^3
380k	0.01	r.t.	1	3.8×10^5	9.3×10^2
340k	0.01	30	1	3.4×10^5	9.0×10^2
230k	0.01	50	1	2.3×10^5	5.9×10^2
150k	0.05	50	1	1.5×10^5	4.0×10^2
110k	0.5	50	1	1.1×10^5	3.1×10^2
78k	1.0	50	1	7.8×10^4	2.0×10^2
63k	2.0	50	1	6.3×10^4	1.7×10^2
33k	sat.	50	1	3.3×10^4	8.2×10
19k	sat.	50	2	1.9×10^4	5.1×10
12k	sat.	50	4	1.2×10^4	3.0×10
8.8k	sat.	50	8	8.8×10^3	2.3×10

[a] Number-average degree of polymerization calculated from $\bar{M}_n$; $\bar{M}_n = \Sigma H_i/\Sigma(H_i/M_i)$, where H_i is the sample concentration and M_i is the molecular weight of each fraction of SEC.

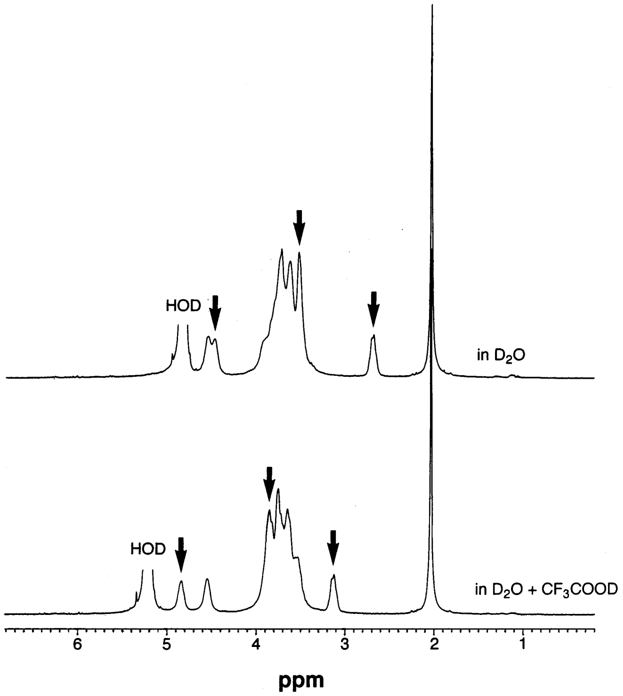

Fig. 2. ^{1}H NMR spectra of partially N-acetylated chitosan in D_2O (top) and in D_2O-containing CF_3COOD (bottom). The signals indicated by arrows are assigned to the H-2, H-3, and H-1 of the GlcN residue from the right.

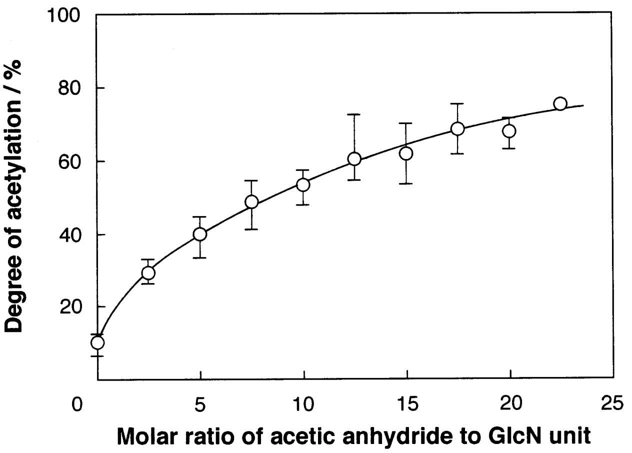

Fig. 3. Relationship between the degree of N-acetylation and the molar ratio of Ac_2O to GlcN units. The error bar represents the maximum and minimum of N-acetylation degrees, regardless of molecular weight.

Degradation of chitosan.—Purified chitosan (0.5 g) was dissolved in 5% aq AcOH (30 mL), and aq $NaBO_3$ (100 mL) of a prescribed concentration was added. After stirring at a predetermined temperature for a predetermined time, the reaction mixture was cooled, and aq KOH was added until the pH reached 10–11. The precipitate obtained was centrifuged and washed with deionized water until it was neutral, rinsed with acetone, dried in vacuo at room temperature (rt), and dried further under vacuum at 50 °C.

Determination of molecular weight.—Weight-average molecular weights of the degraded chitosan samples were determined using a Tosoh SEC system (column: TSKgel $GMPW_{XL}$, 7.8 mm i.d. × 30 cm) equipped with a low-angle laser light-scattering photometer (LS-8000) and a differential refractometer (RI-8011). Each sample was dissolved in acetate buffer (1 M, pH 4.8), which was the eluent, and filtered through a microfilter (0.45

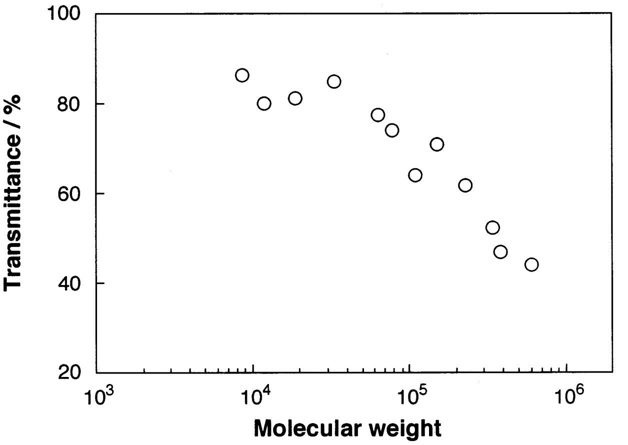

Fig. 4. Molecular-weight dependence of water solubility of half N-acetylated chitosan. Water solubility was characterized as transmittance of the dispersions at 600 nm.

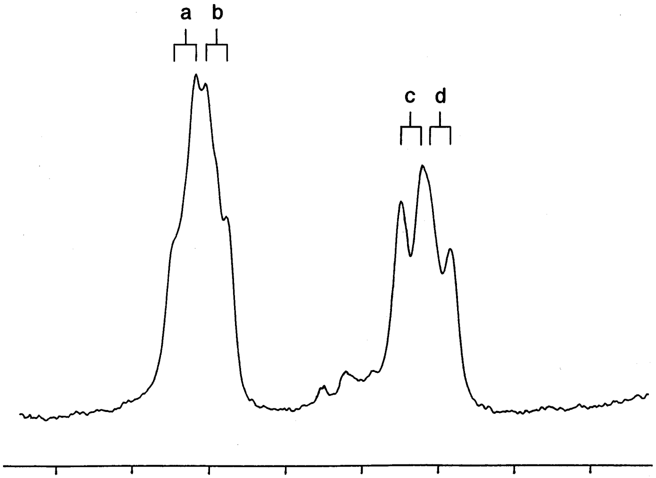

Fig. 5. ^{1}H NMR spectra in the region for resonances of anomeric protons of partially N-acetylated chitosan in D_2O containing DCl at 80 °C: (a) GlcN–GlcN, (b) GlcN–GlcNAc, (c) GlcNAc–GlcN, and (d) GlcNAc–GlcNAc.

Table 2
Fractions of four possible disaccharides for water-soluble half N-acetylated chitosan

Sample code	Degree of N-acetylation (%)	$F_{\mathrm{GlcN-GlcN}}$	$F_{\mathrm{GlcN-GlcNAc}}$ ($F_{\mathrm{GlcNAc-GlcN}}$)	$F_{\mathrm{GlcNAc-GlcNAc}}$
600k-50	50	0.24	0.26	0.24
380k-54	54	0.20	0.26	0.28
340k-52	52	0.23	0.25	0.26
230k-52	52	0.22	0.26	0.26
150k-50	50	0.24	0.26	0.24
110k-51	51	0.22	0.27	0.23
78k-51	51	0.23	0.26	0.25
63k-48	48	0.28	0.24	0.24
33k-47	47	0.26	0.27	0.20
19k-51	51	0.22	0.27	0.24
12k-48	48	0.24	0.28	0.20
8.8k-53	53	0.21	0.26	0.27

µm). The final concentration of the samples was 1.0 g/L. The column temperature was 40 °C, the flow rate was 1.0 mL/min, and the injected sample size was 100 µL.

N-Acetylation of chitosan.—Chitosan (0.3 g) was dissolved in 10% aq AcOH (50 mL), and Ac_2O was added. After stirring at ambient temperature for 5 h, aq NaOH was added until the pH reached 8–9 in order to stop the reaction. The reaction mixture was dialyzed against deionized water for 2 days to remove any microions and lyophilized. The acetylated chitosan was then treated with methanolic KOH for 5 h at rt and repeatedly washed with MeOH using a centrifuge. Finally, it was dissolved in deionized water and lyophilized.

NMR spectroscopy.—^{1}H NMR spectra were recorded on a Bruker ARX 300 spectrometer in D_2O at 25 °C. The partially N-acetylated chitosan was lyophilized twice from D_2O solution before preparing the samples for NMR analysis. All samples were dissolved in 5 mm diameter tubes, and the sample concentrations were 20 mg/mL. ^{1}H NMR spectra were also recorded in the presence of 6% CF_3COOD. The chemical shifts were given on the δ scale relative to Me_4Si. The measurement conditions were as follows: a spectral window of 8064 Hz, 32k data points, a pulse angle of 30°, an acquisition time of 2.03 s, and 32 scans with a delay of 1 s between scans. In order to decrease the line width of the anomeric proton signals, each sample of the half N-acetylated chitosan was dissolved in D_2O containing 1% DCl and the ^{1}H NMR spectra were recorded at 80 °C.

Estimation of solubility.—Water solubility of the half N-acetylated chitosan was evaluated from the turbidity. After being pulverized gently, the half N-acetylated chitosan (50 mg) was dissolved in deionized water (5 mL), and the transmittance of the solution was recorded on a Hitachi U-1000 spectrophotometer using a quartz cell with an optical path length of 1 cm at 600 nm. The pH dependence of the water solubility of the half N-acetylated chitosan was also estimated from the transmittance of the solution, which was previously prepared with aq AcOH (10%), by the stepwise addition of concd NaOH. The sample concentrations were 0.5%. The solubility of the half N-acetylated chitosan in aq organic solvents was similarly estimated from the

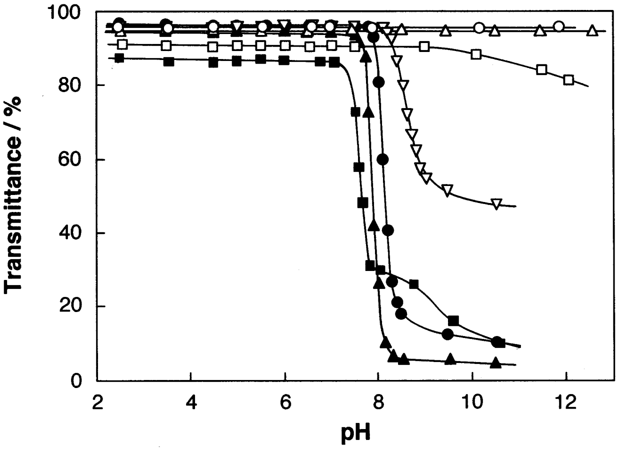

Fig. 6. pH dependence of water solubility of half N-acetylated chitosan: (○) 8.8k-53, (△) 19k-51, (□) 33k-47, (▽) 78k-51, (●) 150k-50, (▲) 340k-52, and (■) 600k-50. Sample codes correspond to those in Table 2.

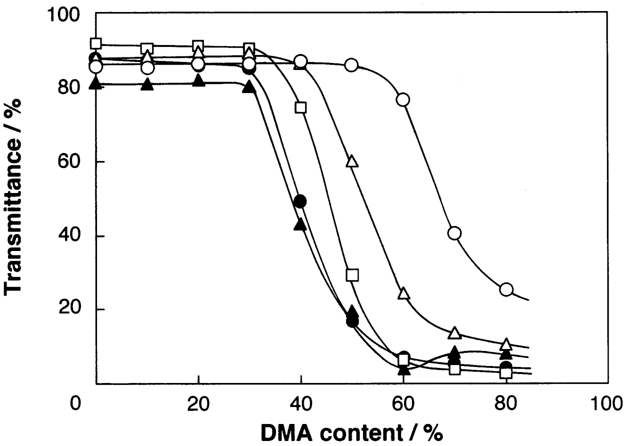

Fig. 7. Dependence of solubility of half N-acetylated chitosan on DMA content: (○) 8.8k-53, (△) 12k-48, (□) 19k-51, (●) 33k-47, and (▼) 63k-48. Sample codes correspond to those in Table 2.

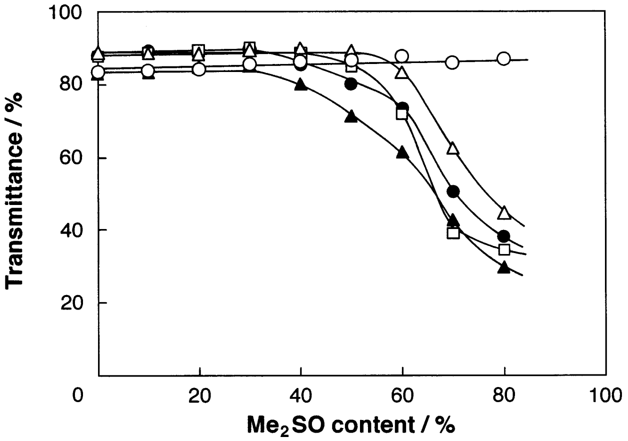

Fig. 8. Dependence of solubility of half N-acetylated chitosan on Me_2SO content: (○) 8.8k-53, (△) 12k-48, (□) 19k-51, (●) 33k-47, and (▼) 63k-48. Sample codes correspond to those in Table 2.

transmittance of the solution, which was prepared in advance with deionized water, by adding organic solvents stepwise. The sample concentrations were 1.0%.

3. Results and discussion

Although various methods are available for depolymerization of chitosan, we employed an oxidative degradation with $NaBO_3$ to save time. Also aqueous acetic acid was used as the solvent to achieve the reaction under homogeneous conditions. Consequently, the main chain of chitosan seems to be randomly cut up. Table 1 lists the degradation conditions along with the weight-average molecular weight and the number-average degree of polymerization for the chitosan samples obtained. The weight-average molecular weight varies according to the $NaBO_3$ concentration and the reaction time, and a series of chitosan samples of molecular weights from 8800 to 600,000 was obtained.

The depolymerized chitosan samples thus obtained were N-acetylated with acetic anhydride in aqueous acetic acid to enable the reaction to proceed under simple and homogeneous conditions, and then treated with methanolic potassium hydroxide [11]. The ^1H NMR spectrum of the partially N-acetylated chitosan in D_2O is shown in Fig. 2 (top). Although the spectrum gives complex signals because the partially N-acetylated chitosan is the copolymer of glucosamine (GlcN) and *N*-acetylglucosamine (GlcNAc), the resonance at 2.04 ppm is easily assigned to the *N*-acetyl protons and apparently there are no other acetyl protons such as *O*-acetyl protons. The ^1H NMR spectrum was also recorded in the presence of CF_3COOD as shown in Fig. 2 (bottom). It can be seen that the peaks at 2.72, 3.53, and 4.49 ppm in D_2O shift significantly downfield, as indicated by the arrows. These peaks are assigned to the H-2, H-3, and H-1 of the GlcN residue, respectively. The peak at 4.56 ppm corresponds to the H-1 signal of the GlcNAc residue, and the resonances of the H-2 and H-3 of the GlcNAc residue exist at 3.77 and 3.48 ppm, respectively. These assignments are supported by the 2D $^1H-^1H$ chemical shift correlation spectra reported previously [12]. The H-4, H-5, H-6a, and H-6b with resonances between 3.4 and 4.0 ppm were not assigned.

The degree of N-acetylation can be calculated from the ratio of the integral intensity of the H-1 of the GlcNAc residue to that of the GlcN residue for the ^1H NMR spectra in the presence of CF_3COOD. We can also estimate the N-acetylation degree from the ratio of the integral intensity of the *N*-acetyl protons to the sum of integral intensities of the H-2, H-3, H-4, H-5, H-6a, and H-6b as reported by Hirai et al. [13]. Fig. 3 shows the relationship between the degree of N-acetylation and the molar ratio of acetic anhydride to GlcN units. The derivatives with different N-acetylation degrees were prepared by changing the quantity of acetic anhydride, independently of the molecular weight. However, an excessively large amount of acetic anhydride was required when the acetylation was carried out in such an aqueous system as in this report; about 50% of the amino groups are acetylated with 8–9 equivalents of acetic anhydride.

Fig. 4 depicts the molecular-weight dependence of the transmittance of 1% aqueous solutions of the N-acetylated chitosan derivatives with about 50% degree of N-acetylation. The water solubility obviously depends on the molecular weight of chitosan, although some scatter in data, which is possibly due to the quality of samples, can be seen. According to Vårum et al., the H-1 signal of the ^{1}H NMR spectrum is very sensitive to the sequence of the GlcN and GlcNAc residues and it is feasible to decide the frequency of the four possible disaccharides: GlcN–GlcN, GlcN–GlcNAc, GlcNAc–GlcN, and GlcNAc–GlcNAc [14]. Fig. 5 shows the ^{1}H NMR spectrum in the anomeric proton (H-1) region of the partially N-acetylated chitosan in D_2O containing DCl at 80 °C. This spectrum is essentially similar to the bottom of Fig. 2. The H-1 resonance of the GlcN residue appears as two doublet peaks [15,16] and they correspond to two disaccharides GlcN–GlcN (a) and GlcN–GlcNAc (b), respectively. On the other hand, the H-1 resonance of the GlcNAc residue also shows two doublet peaks, which correspond to two disaccharides GlcNAc–GlcN (c) and GlcNAc–GlcNAc (d), respectively. The fractions of GlcN–GlcNAc or GlcNAc–GlcN are all in the range 0.24–0.28 (Table 2). The half N-acetylated chitosan derivatives prepared here strongly suggest that the *N*-acetyl groups randomly distribute and the deviation from randomness is very small, regardless of the molecular weight of chitosan. Accordingly, the decrease in water solubility of the half N-acetylated chitosan of high molecular weight is probably due to the high molecular weight itself, not the blockwise distribution of *N*-acetyl groups.

There is a possibility that the water solubility of the half N-acetylated chitosan derivatives obtained in this way might decrease in an alkaline solution [17], because they include about 50% of the GlcN residues whose pK_a is estimated as 6.6 [18]. Fig. 6 shows the pH dependence of the transmittance of the half N-acetylated chitosan solutions. When the half N-acetylated chitosan derivatives are dissolved in aqueous acetic acid, their solubility at neutral pH appears to be higher than that in pure water. The ionic strength might be a cause for this phenomenon. In the cases of the lower-molecular-weight derivatives, the water solubility is high and retained over a wide pH range, whereas in the cases of the higher-molecular-weight derivatives, it is high at acidic pH but abruptly decreases at a pH a little over neutrality. Especially, the derivatives of 8.8k-53, 19k-51, and 33k-47 give very high solubility, but the solubility of the rest decreases with increasing molecular weight in the alkaline region. It seems that the high water solubility of the half N-acetylated chitosan derivatives of low molecular weight is attributed to the decrease of intermolecular interactions, such as van der Waals forces; the lower the molecular weight, the lower the intermolecular attraction forces.

We also investigated the solubility of the half N-acetylated chitosan derivatives of low molecular weights in DMA and Me_2SO. DMA is known to be a good solvent for chitin and cellulose when it is used with LiCl [19] and Me_2SO is frequently used in enzyme assays. After the half N-acetylated chitosan was previously dissolved in deionized water, DMA (Fig. 7) or Me_2SO (Fig. 8) was gradually added; the lower the molecular weight, the higher the solubility in aqueous DMA. The solubility of the half N-acetylated chitosan derivatives in aqueous Me_2SO is slightly higher than that in aqueous DMA. The derivative of 8.8k-53, in particular, is markedly soluble in aqueous Me_2SO. Consequently, reducing the molecular weight prior to N-acetylation is one of the most effective methods of rendering chitosan soluble in aqueous organic solvents.

In conclusion, it was revealed that highly soluble half N-acetylated chitosan could be prepared through a simple and improved method: reduction of the molecular weight of chitosan, followed by N-acetylation in aqueous acetic acid. The half N-acetylated chitosan thus obtained had a random distribution of the *N*-acetyl groups, and the lower the molecular weight, the higher the water solubility. Furthermore, the solubility of the half N-acetylated chitosan in aqueous organic solvents also increased with decreasing molecular weight.

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