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Chemical Modification of Chitosan. 14:[1] Synthesis of Water-Soluble Chitosan Derivatives by Simple Acetylation

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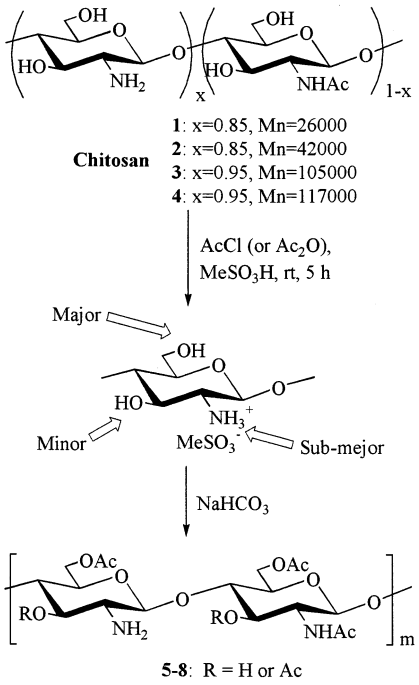
Chitin is a mucopolysaccharide composed of *N*-acetyl-D-glucosamine (GlcNAc) residue. Chitosan **1** is a *N*-deacetylated product of chitin mainly composed of D-glucosamine (GlcN) residue. Chitin and chitosan are attractive materials owing to their biological properties such as immunological activity[2] or wound healing.[3] Although chitin and chitosan are attractive biomacromolecules, these are water-insoluble materials because of their rigid crystalline structures. To obtain a water-soluble property is an important step toward the further application of chitin and chitosan as biomaterials. Some works have been reported to obtain water-soluble derivatives by *N*-acetylation of chitosan in aqueous medium, only around 50% DDA (degree of deacetylation) of the chitosan dissolved in water.[4] In view of developing materials with advanced functions, many attempts have been made to modify the molecular structure of chitin and thereby to improve or control the properties such as carboxymethyl, dihydroxyethyl, sulfuryl, or phosphoryl groups, etc.[5] In the case of cellulose chemistry, moreover, partial substitution of noncharged functional groups such as methyl or acetyl group to cellulose gives water-soluble properties. Despite the fact that chitin and chitosan are structurally similar to cellulose, there is no report about obtaining water-soluble derivatives by the substitution of methyl or acetyl groups.

Herein we report the successful preparation of water-soluble chitosan derivatives by simple *N*,*O*-acetylation in $MeSO_3H$ as solvent. A noteworthy point is that both moderate substitution of *N*,*O*-acetyl groups and moderate molecular weight (MW) are important factors in obtaining water-soluble chitosan derivatives.

Actually there is one report on the selective *O*-acylation of chitosan with salt by protection of the amino group in $MeSO_3H$ solvent, but it is insufficient and vague, especially for the chemical structure.[6] We used four kinds of chitosan to estimate the effect of the degree of deacetylation (DDA), the number-average molecular weight (M_n), and a detailed chemical structure of the acetylated product. The reaction is shown in Scheme 1, and the results are summarized in Table 1. By using an excess amount (10 equiv) of AcCl, the degree of substitution (DS) of the NHAc group was 0.15−0.29 and that of the OAc group was around 1.0. If AcCl and Ac_2O are compared as acetyl reagents, the acetylation efficiency of Ac_2O to hydroxyl and amino groups was slightly higher than that of AcCl. Reaction with Ac_2O, however, gave partly water-insoluble products (**7b** and **8c**) when high MW chitosan was used. The water insolubility for product **7c** would be due to the slightly high molecular weight (DP = 82, listed in Table 3). In another case, water-soluble derivatives were independently obtained on the order of the DDA and MW of original chitosan in moderate yield (44−72%). From the ^{13}C NMR analysis, the *O*-acetyl groups of these products were mainly substituted at the C-6 position. From these results, the chemical structure of water-soluble *N*,*O*-acetylchitosan was estimated as NHAc = ca. 0.2−0.3 and OAc = 1.0.

Scheme 1



To clarify the effect of NHAc and OAc on the water solubility, the complete *N*-acetylation or *O*-deacetylation was tested (Scheme 2). From product **6a**, water solubility was observed to be insoluble for both *N*-acetylation and *O*-deacetylation reactions. These results suggest that partially remaining amino group and *O*-acetyl group substituted around DS = 1 are important parameters for the water solubility of derivatives. Table 2 shows the effect of the amount of AcCl on the chemical structure of product. The protection effect of amino groups by salt formation with $MeSO_3H$ was much associated with the amount of AcCl. A noteworthy fact is that high protection of amino groups was observed at a low amount of AcCl, although this protection effect gradually decreased along with increasing the amount of AcCl. Since all of products listed in Table 2 were dissolved in water, water-soluble *O*-acetylchitosan could be prepared in the range of DDA = 21−81%. The molecular weight of original chitosan and *N*,*O*-acetylchitosan are shown

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Table 1. *N,O*-Acetylation of Varioius Chitosans **1**−**4** in $MeSO_3H$[a]

				yield, %		functional group				
chitosan	DDA, %	M_n	reagent	sol	insol	OAc	OH	NHAc	NH_2	product
1	85	26 000	AcCl	52	0	0.84	1.16	0.74	0.26	**5a**
1	85	26 000	Ac_2O	54	0	1.19	0.81	0.77	0.23	**5b**
2	85	42 000	AcCl	66	0	0.83	1.17	0.79	0.21	**6a**
2	85	42 000	Ac_2O	44	0	1.04	0.96	0.86	0.14	**6b**
3	95	105 000	AcCl	45	0	0.98	1.02	0.81	0.19	**7a**
3	95	105 000	Ac_2O	27		1.13	0.87	0.79	0.21	**7b**
					43	0.98	1.02	0.68	0.32	**7c**
4	95	117 000	AcCl	72	0	0.98	1.02	0.71	0.29	**8a**
4	95	117 000	Ac_2O	12		0.68	1.32	0.85	0.15	**8b**
					53	0.79	1.21	0.85	0.15	**8c**

[a] Reagent, 10 equiv/hexosamine unit. Yield was estimated on the bases of the weight of chitosan. Key: sol, H_2O-soluble fraction; insol, H_2O-insoluble fraction.

Table 2. Effect of the Amount of AcCl on the Acetylation

AcCl,		functional group				protection
equiv	OAc	OH	NHAc	NH_2	product	of NH_2,%[a]
1	0.54	1.46	0.19	0.81	**5c**	95
3	0.79	1.21	0.46	0.54	**5d**	64
5	0.80	1.20	0.60	0.40	**5e**	47
10	0.84	1.16	0.74	0.26	**5a**	31

[a] Protection of NH_2/% = [DS(NH_2)/0.85] × 100.

Table 3. Molecular Weight of Chitosan and *N,O*-Acetylated Chitosan

compd	FW[a]	M_n	M_w	DP[a]
1	167	26 000	75 000	156
2	167	42 000	100 000	251
3	163	105 000	251 000	644
4	163	117 000	325 000	718
5a	229	7600	17 100	33
5b	241	6400	14 100	27
6a	227	6500	14 700	29
6b	243	5800	11 300	24
7a	236	18 000	45 000	76
7b	242	12 000	29 000	50
7c	231	19 000	38 000	82
8a	232	13 000	29 000	56

[a] FW = 161 + DS × 42; DP = M_n/FW.

in Table 3. Despite the degree of polymerization (DP) was much decreased from the original chitosan during the acetyl reaction owing to the strong acidic condition in $MeSO_3H$, the moderate MW (DP = 24−76) of products was retained. Since a slightly high MW for product (**7c**: DP = 82) caused insolubility in H_2O, the moderate MW (DP < 76) is also an important factor in giving a water-soluble product in addition to the moderate *N,O*-acetyl substitution.

In conclusion, for the chemical reaction, the difference of acetyl reagents (AcCl and Ac_2O) was not essential for this reaction system. In comparison with AcCl, Ac_2O gave a slight advantage on the high reactivity to OH group but a disadvantage for stronger depolymerization (Table 3) and the production of a water-insoluble fraction using high MW chitosan (**3** and **4**).

Figure 1 shows the time courses on the biodegradation of original chitosan **2** and *N,O*-acetylchitosan **6a** by standard activated sludge metabolism owing to evaluate the biodegradable property in nature. Chitosan **2** was insoluble in this aqueous medium, but **6a** was soluble in medium. Chitosan

Scheme 2

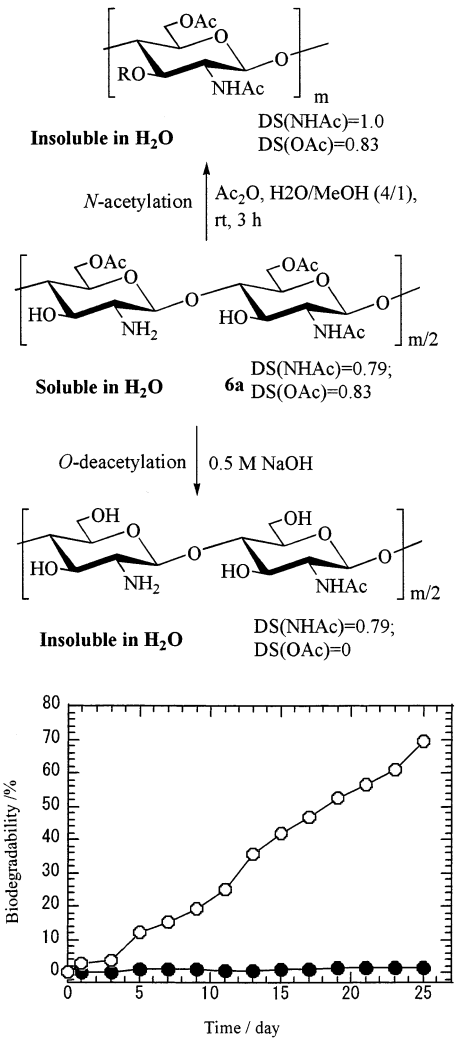

Figure 1. Time courses on the biodegradation of chitosan and *N,O*-acetylchitosan **6a**. Sample, 50 mg: (●) chitosan **2**; (○) *N,O*-acetylchitosan **6a**.

2, which is essentially degraded by enzymes such as chitosanase, lysozyme, etc.,[7] showed less biodegradability. Since chitosan **2** used in this test was a flake type solid, the insoluble property in aqueous medium and high degree of crystallinity of chitosan **2** would affect biodegradability. Moreover, less existing of bacteria producing chitosanase in this sludge would also affect poor biodegradation. On the

other hand, *N,O*-acetylchitosan **6a** showed high biodegradability, which would be caused by the hydrolysis of ester linkages and then free acetyl moiety was contributed in biodegradation. Other *N,O*-acylchitosans (**5a**, **6b**, and **7a**) also showed similar biodegradation as **6a** (data not shown). It is true that further detailed study in correlation of substrates and enzymes will be necessary to clarify the biodegradation mechanism; however, *N,O*-acetylchitosan **6a** showed enough biodegradability by standard activated sludge metabolism.

The freeze-dried products of water-soluble *N,O*-acetylchitosans were stable and remained water soluble for over 6 month. Despite the fact that the *N*-acetyl group of chitosan have been pointed out as having an important role on the biological activity,[2,3] the role for the *O*-acetyl group in chitosan is quite unclear as of now. Further work on the biological properties for the water-soluble *N,O*-acetylchitosans are interesting and will be studied in near future.

Experimental Section

Materials. Several chitosans were purchased from each company (**1**, Flonac C from Kyowa Technos; **2**, SK-10; **3**, DAC-95 from Koyo Chemical; **4**, chitosan 10B from Katokichi Co.).

General Methods. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL A-500 NMR spectrometer. Molecular weight was determined by means of GPC using pullulan as standards (column, Tosoh TSK Gel G4000pwxl and G3000pwxl; eluent, 0.5 M AcOH-0.5 M AcONa buffer; temp, 40 °C; flow rate, 1.0 mL/min; detection, RI).

Typical Procedure. Chitosan **1** (2 g) was dissolved in $MeSO_3H$ (20 mL) at room temperature for 1 h. To this solution was added acetyl chloride (10 equiv/hexosamine unit of chitosan). After the reaction was stirred at room temperature for 5 h, ca. 50 g of ice was added to stop the reaction. The acidic aqueous solution was dialyzed for 1 day to remove most of acid, followed by neutralizing remained acid and removing counteranion from chitosan salt with $NaHCO_3$. Finally the solution was dialyzed again for more than 2 days and lyophilized. The DS value of *N,O*-acetylchitosan was estimated by ^{1}H NMR in 0.5 M DCl/D_2O from the peak area at δ 1.9−2.2 (CH_3 of NHAc and OAc) against 3.2−4.2 (H-2,3,4,5,6 of GlcN and GlcNAc). The DS of NH_2 was also estimated by ^{1}H NMR from the peak area at δ 3.2 (H-2 of GlcN residue) against 3.5−4.2 (H-2 of GlcNAc and H-3,4,5,6 of GlcN and GlcNAc). Selected data for **6a**: ^{1}H NMR: δ 1.9−2.2 (br m, 4.86 H, CH_3 of NHAc and OAc), 3.2 (br s, 0.21 H, H-2 of GlcN residue), 3.5−4.2 (br m, 5.79 H, H-2 of GlcNAc and H-3,4,5,6 of GlcN and GlcNAc), 4.58 (br, 0.79H, H-1 of GlcNAc); ^{13}C NMR: δ 23.7, 23.9, and 25.0 (OAc and NHAc), 56.8−58.0 (C-2 of GlcN and GlcNAc), 61.8 (C-6), 64.3 (C-6 substituted), 73.4−74.5 (C-3), 76.3−76.6 (C-5), 79.2−81.1 (C-4), 102.5−103.1 (C-1), 175.4−176.3 (NHCO and OCO).

Biodegradation of *N,O*-Acetylchitosan by Activated Sludge. The biodegradation of original chitosan **2** and *N,O*-acetylchitosan **6a** was evaluated by BOD tester 100F (TAITEC Co.) as follows. Carbon-free inorganic buffer solution (1 L, pH 7.0) containing $MgSO_4$ (7.2 mg), $CaCl_2$ (27.7 mg), $(NH_4)_2SO_4$ (39.6 mg), $FeCl_3$ (0.6 mg), KH_2PO_4 (17 mg), K_2HPO_4 (43.5 mg), $Na_2HPO_4\ 7H_2O$ (66.8 mg), and NH_4Cl (3.4 mg) was prepared. The supernatant of standard activated sludge (30 mL), buffer solution (270 mL), and sample (50 mg) were placed in a fermentor at 25 °C using. A powder (1.0 g) of $Ca(OH)_2$ was used as a trap of simultaneous producing CO_2 gas. At the prescribed time, the amount of consumed O_2 gas was measured by observing the scale of the additional cylinder. The fermenteter, which did not include sample, was used as a control. The net amount of consumed O_2 gas (mL) was evaluated as the difference of sample and control. Each element (C, H, N, O) in a sample was converted according to chemical combustion as follows.

$$C + O_2 \rightarrow CO_2$$

$$4H + O_2 \rightarrow 2H_2O$$

$$2N + 2H + 3O_2 \rightarrow 2HNO_3$$

From these equations, the theoretical amount of consumed O_2 (mL at 25 °C), which means complete degradation, could be calculated. Finally, the biodegradability (%) was calculated as follows.

$$\text{biodegradability (\%)} = [\text{experimentally consumed } O_2 \text{ (mL)/theoretical } O_2 \text{ (mL)}] \times 100$$

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