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Heterogeneous Distribution of Amino Groups in Partially *N*-Acetylated Derivatives of Chitosan

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Five derivatives of partially *N*-acetylated chitosan [degree of substitution (d.s.) for *N*-acetyl group: 0.05, 0.2, 0.4, 0.6 and 0.9] were prepared from chitosan by reaction with acetic anhydride (< 1 mol GlcN). Each derivative was exhaustively oxidized with $NaIO_4$, reduced with $NaBH_4$ and hydrolyzed with 0.5 N HCl by the conventional method. The reaction products were fractionated by gel chromatography using Bio-Gel P-2. Glycol aldehyde, *meso*-erythritol and a series of 2-acetamido-2-deoxy-β-D-glucopyranosyl(1-[→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl(1-]$_n$→2)-D-erthritols were detected in the hydrolyzates of all these derivatives, regardless of low d.s. for *N*-acetyl groups. These results indicate that free amino groups were heterogeneously present in the partially *N*-acetylated chitosans because chitosan was heterogeneously *N*-acetylated under the present conditions.

Chitosan[(1→4)-2-amino-2-deoxy-β-D-glucan] consists of *N*-deacetylated derivatives of chitin[(1→4)-2-acetamido-2-deoxy-β-D-glucan].[1] A small amount of free amino groups is generally present in naturally occurring chitin. Recently we prepared from chitosan a series of *N*-acyl derivatives (d.s. 1.0)[2] and partially *N*-acylated derivatives (d.s. < 1.0).[3] The partially *N*-acylated derivatives stoichiometrically form polyelectrolyte complexes with acidic polysaccharides,[4] and partially *N*-deacetylated chitins selectively aggregate leukemia cells, L 1210.[5] Furthermore, the gel formation of *N*-acetylchitosan has been shown to be inhibited when free amino groups are present.[6] The free amino groups present in these derivatives may play a principal role in the phenomena described above, but little is known about the distribution of amino groups in these derivatives.

We now report on the heterogeneous distribution of amino groups present in partially *N*-acetylated chitosans, as demonstrated by periodate oxidation, borohydride reduction and mild hydrolysis followed by gel chromatographic fractionation.

MATERIALS AND METHODS

Chitosan, $[\alpha]_D^{15}$ −4.5° (*c* 1.1, 10% acetic acid), was prepared from chitin (crab shells) by *N*-deacetylation with 40% NaOH in the presence of 0.01% $NaBH_4$.[6] The preparation showed no appreciable signals for NAc at ～2 ppm in the PMR spectrum (D_2O–DCO_2D, 9 : 1 v/v) and no absorptions for NAc at ～1650 and ～1550 cm^{-1} in the IR spectrum (KBr). TLC was performed on a Kieselguhr G-Kieselgel G (Type 60, Merck) plate, 1 : 3 w/w, and also on a commercial silica gel plate (Replate-26, Yamato Scientific Co., Ltd.), with solvent *A*, 1-propanol–ethanol–water, 16 : 29 : 5 v/v, and solvent *B*, 1-propanol–nitromethane–water, 7 : 2 : 1 v/v. Formaldehyde liberated was determined by the chromotropic method.[7] The other methods were as cited previously.[6]

RESULTS AND DISCUSSION

Preparation of Partially N-Acetylated Chitosans

Chitosan (2.00 g each) was dissolved in 2% acetic acid (40 ml) to afford a viscous solution, which was diluted with methanol (200 ml). Acetic anhydride (0.1～1.0 mol/GlcN) was added and the mixture was allowed to stand at room temperature overnight.[6] Partially *N*-acetylated derivatives were obtained as viscous solutions or soft gels, and isolated with good yields (Table I). D.s. for NAc was determined

TABLE I. ANALYSIS OF SEVERAL PARTIALLY *N*-ACETYLATED DERIVATIVES OF CHITOSAN

Derivative	Yield (%)	$[\alpha]_D^{17}$ degree (*c* 1.0, formic acid)	D.s. value of NAc		Acetic anhydride used (mol/GlcN)	Gelation[c]
			C/N[a]	PMR[b]		
1	82	−7.4	0.05	0.06	0.1	−
2	98	−6.5	0.24	0.2	0.25	−
3	90	−4.9	0.38	0.4	0.50	−
4	95	−6.0	0.62	n.d.	0.70	+
5	100	−4.8	0.86	n.d.	1.0	+

[a] From elemental analysis.
[b] From the proton intensity ratio of NAc to CH_2 and CH of the sugar in the PMR spectra (D_2O–DCO_2D, 9:1 v/v).
[c] Key: +, gels formed; −, gels did not form.

from the proton intensity ratio of NAc to methine and methylene of the sugar in the PMR spectra (D_2O–DCO_2D, 9:1 v/v) and from the C/N ratio determined by elemental analysis.

Periodate oxidation

Each derivative was dissolved or suspended in 10% acetic acid (100 ml), and further diluted with distilled water (630 ml). The solution was adjusted to pH 4.2 and an ionic strength of 0.06 by addition of 2 N NaOH solution (30 ml). Excess $NaIO_4$ (5.309 g) was added in several portions at 5°C, and the mixture was allowed to stand with stirring in the dark at 5°C. After 120 hr, a constant end-value of periodate consumption was observed for each of the reactions under the present conditions.[8] Excess $NaIO_4$ was decomposed by addition of 2 M ethylene glycol (24 ml), followed by standing at 5°C for 1 hr. The reaction mixture was dialyzed against running water for two days and then in distilled water for one day. The nondialyzable solution was concentrated at ~35°C *in vacuo* to a ~500 ml-volume, and the pH was adjusted to 7.5 by addition of 2 N NaOH. To the solution was added $NaBH_4$ (500 mg), and the mixture was allowed to stand at room temperature for 16 hr. Excess $NaBH_4$ was decomposed by addition of 10% acetic acid (final pH ~5), and the solution was concentrated to a syrup. The syrup was suspended in methanol (100 ml) containing 1% acetic acid, and the mixture was concentrated to a syrup. Finally, the reaction products were dissolved in 0.5 N HCl (100 ml) and the solution was allowed to stand at 13~15°C for 16 hr. Precipitates produced during the mild hydrolysis were collected by centrifugation: 8.8 mg (0.4%), 5.2 mg (0.3%) and 226 mg (11%) for **2**, **3** and **4** (see Table I), respectively. The precipitates showed $[\alpha]_D^{15}$ −10~14° (*c* 0.4, formic acid). IR spectra (KBr): 3500~3100 (OH, NH), 2950~2900 (CH), 1650 and 1560 (C=O and NH of NAc), 1160~1000 (C–O) and 900 cm^{-1} (β-D-). The precipitates are the depolymerized products of *N*-acetylchitosan (d.s. 1.0) containing D-erythritol at reducing end groups, but the detailed d.s. was not determined. The precipitate yields were shown to increase with high d.s. for *N*-acetyl groups.

Then, the supernatant solution obtained above was diluted with distilled water (100 ml), neutralized with Amberlite IRA-400 (OH^-) resins, concentrated to a ~30 ml-volume, and lyophilized to afford amorphous products: 0.8 g (40%), 1.1 g (55%) and 1.7 g (85%) for **2**, **3** and **4**, respectively.

Gel chromatography

Each lyophilized product (300 mg) obtained above was dissolved in distilled water (5 ml) and applied to a column (1.8×227 cm) of Bio-Gel P-2, which was eluted with distilled water at a flow rate of 20 ml/hr and 5 ml fractions were collected. An aliquot (1 ml) was withdrawn from each fraction, diluted with water (2 ml) and *N*-acetylated sugars were traced by

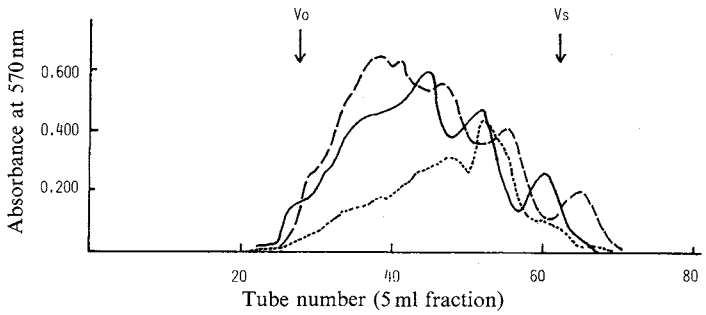

FIG. 1. Elution Profiles of the Oxidized Products of Three Partially *N*-Acetylated Chitosans on a Column (1.8 × 227 cm) of Bio-Gel P-2.

Oxidized products of the partially *N*-acetylated (derivatives: d.s. 0.2 (· · · · · ·); d.s. 0.4 (——); d.s. 0.6 (– – –). The column was eluted with distilled water at a flow rate of 20 ml/hr, and 5 ml fractions were collected and analyzed by the ninhydrin reaction after acid hydrolysis (see text). Essentially the same elution profiles were obtained on analysis of absorbance at 220 nm of these eluates without acid hydrolysis.

absorption at 220 nm for convenience. To another aliquot (0.2 ml) was added conc. HCl (0.5 ml), and the mixture was heated in a boiling water bath for 2 hr.[9] After cooling to room temperature, the hydrolyzates were mixed with 1.6 ml of 4 M acetate buffer solution (pH 5.51), and the ninhydrin reaction was performed. As shown in Fig. 1, the products were eluted in a wide range. Continuously *N*-acetylated sugar moieties [degree of polymerization (d.p.) > 8] appeared even in the hydrolyzate of **2** (d.s. 0.2) and increased with high d.s. (0.4 and 0.6) for *N*-acetyl groups. These results indicate that chitosan is heterogeneously *N*-acetylated with acetic anhydride under the present conditions.

*Isolation of 2-acetamido-2-deoxy-β-*D*-glucopyranosyl(1-[→4)-2-acetamido-2-deoxy-β-*D*-glucopyranosyl(1-]$_n$→2)-*D*-erythritols*

The products obtained from **3** (d.s. 0.4) contained at least ten compounds as shown by TLC, and the products were fractionated into five fractions by gel chromatography on a column (1.8 × 227 cm) of Bio-Gel P-2 (Table II). Each of the fractions was collected, concentrated and lyophilized. Fractions **a** and **b** were gelatinous, and **c** and **d** were amorphous.

TABLE II. FRACTIONATION OF HYDROLYZATES OF THE OXIDIZED PRODUCTS OF PARTIALLY *N*-ACETYLATED CHITOSAN (d.s. 0.4) ON A COLUMN (1.8 × 227 cm) OF BIO-GEL P-2

Fraction	Tube number[a]	Yield (%)	Average d.p. (n)[b]	*Rf* values with solvent *B*[c]
a	57 ~ 72	16	0	~0.67,[d] 0.80[e]
b	49 ~ 56	18	2	0.47, 0.59, ~0.67 (trace)
c	41 ~ 48	23	4	0.11, 0.19, 0.26, 0.37, 0.47 (trace)
d	36 ~ 40	15	6	0.04, 0.11, 0.19, 0.26, 0.37 (trace)
e	24 ~ 35	12	n.d.[f]	0.00 and tailing
f	Precipitates	0.3	n.d.	n.d.

[a] See Fig. 2.
[b] Based on *meso*-erythritol contents (see text). For the definition of n, see Fig. 3.
[c] Performed on a Kieselguhr G-Kieselgel G (Type 60) plate, 1 : 3 w/w, and detected by spraying with 10% H_2SO_4, followed by heating at ~200°C (see text for the solvent systems). With solvent *A*, **a** showed *Rf* ~0.75, 0.80; **b**, 0.54, 0.65, ~0.75 (trace); **c**, 0.10, 0.19, 0.28, 0.45, 0.54 (trace); **d**, 0.10, 0.19, 0.28, 0.45 (trace); and **e**, 0.00 and tailing.
[d] A mixture of *meso*-erythritol and 2-acetamido-2-deoxy-β-D-glucopyranosyl(1→2)-D-erythritol (see text).
[e] Glycol aldehyde.
[f] Not determined.

As shown in Table II, the total yield was 84% (**c** > **b** > **a** > **d** > **e**). Fraction **a** showed two spots (*Rf* ~0.67 and 0.80) on Kieselguhr G-Kieselgel G plate (Table II) and the broad spot (*Rf* ~0.67) was a mixture of two compounds because **a** showed apparently three spots (*Rf* 0.90, 0.81 and 0.73) on a commercial plate of silica gel using solvent *B*. Two spots (*Rf* 0.9 and 0.81) were assigned to glycol aldehyde and *meso*-erythritol, respectively, on the basis of their mobilities. A hydrolyzate (2 N HCl, 100°C, 8 hr) of the other spot (*Rf* 0.73) contained 2-amino-2-deoxy-D-glucose and *meso*-erythritol as shown by TLC. The IR spectrum (KBr) of **a** indicated the absorptions of NAc (1630 and 1560 cm^{-1}) and the β-D-configuration (880 cm^{-1}). All of these results indicate that the third spot (*Rf* 0.73) is 2-acetamido-2-deoxy-β-D-glucopyranosyl (1→2)-D-erythritol. Fractions **b**~**e** showed identical IR abosrptions (KBr): 3500~3100 (OH, NH), 2900 (CH), 1650 and 1560 (C=O and NH of NAc), 1160~1020 (C–O) and 900 cm^{-1} (β-D-), and identical PMR signals (DMSO-d_6): δ 2.0 (NAc-Me), 3.0~4.1 (methine and methylene of the sugar), 4.6 and 5.1 (OH) and 7.8 (NH). The PMR signals at δ 4.6, 5.1 and 7.8 disappeared on addition of a drop of D_2O. Each of **b**~**e** was hydrolyzed with 2 N HCl at 100°C for 8 hr, and two spots appeared in the hydrolyzates and were assigned to 2-amino-2-deoxy-D-glucose and *meso*-erythrytol by TLC. Fractions **b**~**e** showed no appreciable reducing power (<0.01% against 2-acetamido-2-deoxy-α-D-glucose) as determined by a modified Schales method[10] and were not oxidatively degraded by reaction with $NaNO_2$[11] as determined by the anthrone method.[12] These results indicate that neither reducing end groups nor free amino groups are present in **b**~**e**.

One molar formaldehyde is produced by periodate oxidation from the erythritol moiety of one mol of 2-acetamido-2-deoxy-β-D-glucopyranosyl (1-[→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl(1-]$_n$→2)-D-erythritols. Therefore, the amounts of formaldehyde liberated correspond to *meso*-erythritol contents

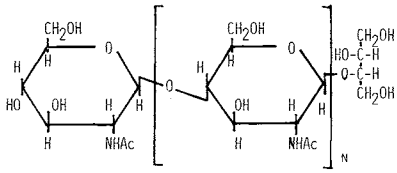

FIG. 2. 2-Acetamido-2-deoxy-β-D-glucopyranosyl(1-[→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl(−]$_n$→2)-D-erythritols.

Ac: CH_3CO–.

in these derivatives. The *meso*-erythritol contents are 15, 8.9 and 7.0% for **b**, **c** and **d**, respectively, and the average d.p. values of these fractions are 2, 4 and 6 for **b**, **c** and **d**, respectively, as expressed by *n* in Fig. 2. These d.p. values are in good agreement with those estimated from their elution profiles (Fig. 1).

Chitosan chains exist as unfolded forms in aqueous acetic acid because of repulsion of cationic amino groups, and selective *N*-acetylation begins not only on reducing and nonreducing end moieties but also on middle moieties of chitosan chains under the present conditions. Vicinal amino and hydroxyl groups, which are not acetylated, at C2 and C3 of hexosaminyl moieties are oxidized with $NaIO_4$ to afford a dialdehyde compound with release of NH_3.[13] The dialdehyde groups are reduced with $NaBH_4$, and mild hydrolysis of the products afords a series of the present compounds.[14] Not only glycol aldehyde and *meso*-erythritol but also a series of 2-acetamido-2-deoxy-β-D-glucopyranosyl(1-[→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl(1-]$_n$→2)-D-erythritols were detected in all the hydrolyzates, regardless of d.s. for *N*-acetyl groups. These results indicate that amino groups are heterogeneously present in the partially *N*-acetylated chitosans. Furthermore, the present method is useful for the preparation of a series of chito-oligosaccharides containing D-erythritol at reducing end groups.

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