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N-Acetyl group distribution in partially deacetylated chitins prepared under homogeneous conditions

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ABSTRACT

The distribution of *N*-acetyl group in partially deacetylated chitin (DA-chitin) was investigated by nitrous acid deamination. Most deamination products of various DA-chitins (over 50% of deacetylation), prepared under homogeneous conditions, were oligomers of less than six units. These results would suggest a random distribution of *N*-acetyl groups in the DA-chitin molecule.

INTRODUCTION

Chitin, a (1 → 4)-linked polysaccharide composed of 2-acetamido-2-deoxy-β-D-glucopyranosyl residues, is widely distributed in Nature as skeletal materials of crustaceans, insects, mushrooms, and cell walls of bacteria. It has been shown that partially deacetylated chitins (DA-chitins) have potent immunological activities[1], such as the activation of peritoneal macrophages in vivo, the suppression of growth of Meth-A tumor cells in syngeneic mice, and the stimulation of nonspecific host resistance against *Escherichia coli* infections. These activities were not observed for chitin itself[1]. As the DA-chitin ~ 70% deacetylated (DAC-70) was reported to be the most effective immunoadjuvant among several DA-chitins (0, 30, and 90% of deacetylation)[2], studies of the retention of DAC-70 in the animal body and the molecular composition, such as the charge distribution along the polysaccharide chain, are required to clarify the mechanism of immunoadjuvant activity by DAC-70.

Several methods were reported for the preparation of DA-chitins under heterogeneous or homogeneous conditions[3–6]. Kurita and assoc.[4,7] reported that 45–55% deacetylated chitins, prepared by the homogeneous deacetylation of chitin, were soluble in water. Aiba[8] also reported that DA-chitins (< 50% of deacetylation)

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prepared by the homogeneous *N*-acetylation of chitosan were stable even in alkaline solution. On the other hand, DA-chitins, prepared by the heterogeneous deacetylation of chitin, were insoluble in water or alkaline solution. Rinaudo and Domard[9] also observed that the various chitosans obtained from natural chitin were characterized by their fraction consisting of 2-acetamido-2-deoxy-D-glucose units in the molecule and that the solubility must depend on the *N*-acetyl group distribution.

We reported that the distribution of *N*-acetyl group in several DA-chitins, prepared under heterogeneous conditions, was random along the molecule[10], and we report herein the distribution of *N*-acetyl groups in various DA-chitins prepared under homogeneous conditions, which was investigated by nitrous acid deamination and statistical analyses according to the method of Bernoulli.

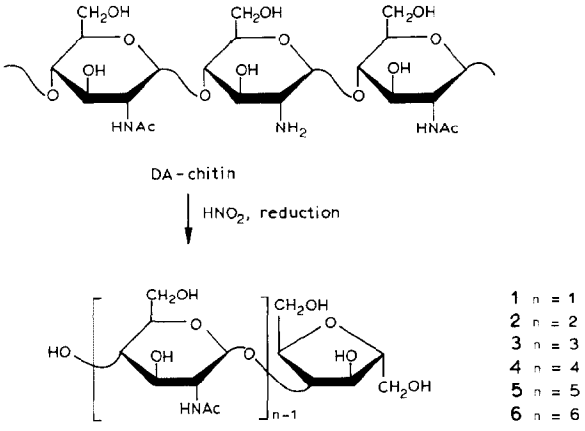
EXPERIMENTAL

Materials.—Chitin from shrimp shell, sodium nitrate, and other reagents of reagent grade were purchased from Wako Pure Chemical Industries Ltd, and used without further purification. 2-Acetamido-2-deoxy-D-glucose oligomers $(GlcNAc)_n$ (n = 2–6) were purchased from Seikagaku Kogyo Co., Ltd.

Preparation of partially deacetylated chitins.—DA-chitins (DACs) were obtained from an alkaline chitin solution by the homogeneous deacetylation of chitin according to the method of Kurita et al.[4]. DA-chitins (NA-DACs) were prepared by the homogeneous *N*-acetylation of chitosan (91% of deacetylation) according to the method of Hirano and Yamaguchi[5]. The degree of deacetylation was evaluated by elemental analysis and IR spectroscopy[11].

Deamination of partially deacetylated chitins.—The deamination of DA-chitins was performed according to the previously mentioned procedure[10]. A typical procedure for the deamination of DA-chitin is as follows: To a 10% AcOH solution (50 mL) of DA-chitin (0.5 g) was added 5% aq $NaNO_2$ (15 mL), and the mixture was stirred for 3 h at 2–4°C, followed by standing for 40 h at room temperature. Insoluble materials were removed by centrifugation (15000 rpm). The pH of the supernatant was adjusted to 5.5 with Amberlite IRA-400 (OH^-, 50 mL) resin. To the supernatant was added 6% aq $NaBH_4$ (5 mL), and the solution was stirred for 1 day at room temperature, followed by desalting with Amberlite IR-120B (H^+, 100 mL) and Amberlite IRA-400 (OH^-, 100 mL) resins. The desalted solution was concentrated to 20 mL for GPC analysis.

Gel permeation chromatography (GPC).—The composition of deamination products was determined by GPC with 2-acetamido-2-deoxy-D-glucose oligomers and 2,5-anhydro-D-mannitol as standards on a Shimadzu LC-6A apparatus, equipped with a Shimadzu RID-6A RI detector (column, Hitachi GL-W520, 1.07 × 30 cm; eluent, distilled water; flow rate, 1 mL/min; column temperature, 50°C). The structures of compounds **1**, **2**, and **3** were assigned by LC–MS analysis, which gave *m/z* 165 ($M + H^+$), 368 ($M + H^+$), and 571 ($M + H^+$), respectively.



Scheme 1.

RESULTS AND DISCUSSION

Nitrous acid deamination.—The nitrous acid deamination of mucopolysaccharides[12–16] converts the 2-amino-2-deoxy-D-glucose unit of DA-chitins into 2,5-anhydro-D-mannose, but the 2-acetamido-2-deoxy-D-glucose unit is stable under the reaction conditions[17,18]. As shown in Scheme 1, the deamination of DA-chitins with nitrous acid gives oligomers of various sizes, thus allowing an estimation of the distribution of *N*-acetyl groups (block or random).

The weight fraction, $W_n(\%)$, of deamination products was determined from the peak area in the gel permeation chromatogram according to the method described earlier[10], is given by eq *1*

$$W_n(\%) = \frac{\text{Peak area}}{\text{Total peak area}} \times 100 \qquad (1)$$

and the results are summarized in Table I. Oligomers of less than six units were obtained by the deamination of DA-chitins prepared by both the homogeneous *N*-acetylation of chitosan (NA-DACs) and the homogeneous deacetylation of

TABLE I

Deamination of various DA-chitins

DA-chitin	W_n [a] (wt%) of compounds					
	6	**5**	**4**	**3**	**2**	**1**
NA-DAC-50	(5)	7(8)	10(13)	21(20)	35(25)	26(23)
NA-DAC-63		9(3)	9 (8)	19(16)	26(31)	37(41)
NA-DAC-78			9 (3)	12(11)	21(29)	57(57)
DAC-49	10(5)	12(8)	13(13)	13(20)	16(25)	36(23)
DAC-82			(1)	14(6)	21(25)	66(69)

[a] The weight fraction (W_n) was calculated from the peak area of GPC. The values in parentheses give the theoretical values according to Bernoullian statistics.

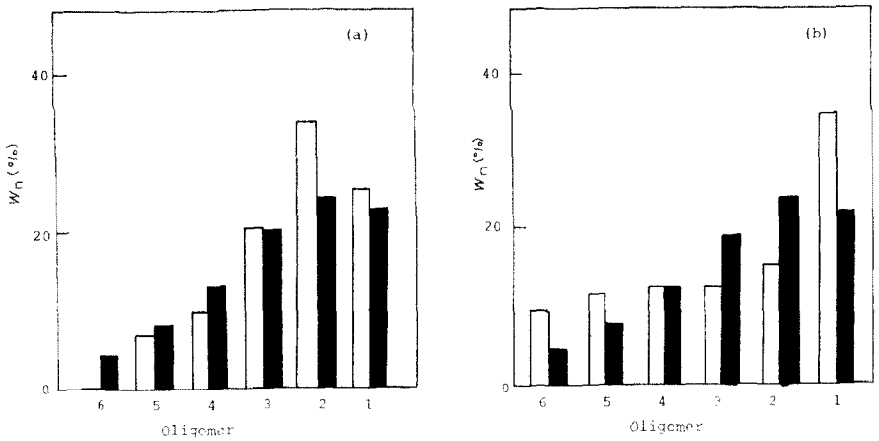


Fig. 1. Comparison between the theoretical values obtained by Bernoullian statistics from eq 2 (■) and the experimental values (□) of deamination products of NA-DAC-50 (a) and DAC-49 (b).

chitin (DACs). The main products were monosaccharide **1**, disaccharide **2**, and trisaccharide **3**. Most oligomers formed by this reaction were soluble in water. In some cases, insoluble materials were formed, but their yields were negligible (< 3 wt% from the starting material). Therefore, the procedure described herein gave quantitative results. It is interesting that the distribution of *N*-acetyl groups was found to be random in a molecule of DA-chitin prepared both under homogeneous and heterogeneous[10] conditions.

Bernoullian statistics.—A statistical analysis of the weight fraction $W_n(\%)$ of deamination products (oligomers) of the DA-chitin that had a random distribution of *N*-acetyl groups was performed on the basis of Bernoullian statistics[19]. The theoretical weight fraction $W_n(\%)$ of each oligomer was obtained by eq 2,

$$W_n = n \times da^2 \times (1 - da)^{n-1} \times M_n \times (n \times M_O)^{-1} \quad (2)$$

where n is the number of residues contained in oligomers **1**–**6**, da is the degree of deacetylation, M_n is the molecular weight of the n'th oligomer, and M_O is the average molecular weight of the unit in DA-chitin.

Fig. 1 shows good agreement between experimental and theoretical values (Bernoullian statistics) of the weight fraction $W_n(\%)$ of deamination products (oligomers). As shown in Fig. 1a, the distribution of the deamination products (oligomers) of NA-DAC-50 was similar to that obtained by Bernoullian statistics. In the case of the DA-chitins listed in Table I other than DAC-49, the experimental values were also in good agreement with theoretical values. Hence, it may be concluded that the *N*-acetyl group is distributed randomly in these DA-chitins.

The distribution of the deamination products of DAC-49 (Fig. 1b), however, was different from that of NA-DAC-50 and that obtained by Bernoullian statistics. These results suggest that another type of distribution of *N*-acetyl group is present in the DAC-49 molecule. Moreover, the solubility of DAC-49 was different from that of NA-DAC-50. DAC-49 (MW, 50 000) dissolved in water, but not NA-DAC-50

(MW, 190 000). This difference in solubility depends not only on the molecular weight but also on the distribution of *N*-acetyl groups. DAC-49, furthermore, dissolved in water at pH 9–10, but not NA-DAC-50. The latter, however, dissolved in water at pH 9–10 when it was first dissolved in water at pH 5 containing acetic acid, followed by adjusting the pH of the solution to 9–10. These results suggest that the different solubilities are not affected by the salt form of the amino group.

Kurita et al.[4] reported that deacetylation under homogeneous conditions proceeded randomly to give random-type copolymers of GlcNAc and GlcN units. It was also reported that deacetylation under heterogeneous conditions proceeded preferentially in the amorphous region to give block-type copolymers of GlcNAc and GlcN units[3]. Our results, however, suggest that the *N*-acetyl groups are distributed randomly in both DA-chitins prepared under heterogeneous conditions[10] (DAC, > 66% of deacetylation) and under homogeneous conditions (DAC > 49% of deacetylation; NA-DAC, > 50% of deacetylation).

The random distribution of the *N*-acetyl groups in a DA-chitin molecule prepared under heterogeneous condition[10] suggests the following mechanism for the deacetylation process. In the first stage (probably up to 30–50% of deacetylation), the deacetylation under heterogeneous conditions proceeds preferentially in the amorphous regions to give block-type copolymers of GlcNAc and GlcN units[3]. In the case of DACs having a high degree of deacetylation (> 66% of deacetylation), however, the deacetylation would also proceed in the crystal region of DA-chitin to give random-type copolymers of GlcNAc and GlcN units.

Comparison of degree of deacetylation.—Table II shows the composition of GlcNAc segments in various DA-chitins; it was calculated from the weight fractions $W_n(\%)$ given in Table I and the degree of deacetylation (da evaluated by GPC), which was calculated from eqs *3* and *4*

$$A = \frac{203 \times (1 - \mathrm{da}/100)}{(161 \times \mathrm{da}/100) + 203 \times (1 - \mathrm{da}/100)} \times 100 \quad (3)$$

$$\mathrm{da}(\%) = \frac{203 \times (100 - A)}{203 \times 100 - 42 \times A} \times 100 \quad (4)$$

TABLE II

Composition of GlcNAc segments of DA-chitins

DA-chitin	Segment [a] (%)				da [b] (%)		
	$(GlcNAc)_4$	$(GlcNAc)_3$	$(GlcNAc)_2$	GlcNAc	GPC	EA	IR
NA-DAC-50	6	8	15	20	57	50	54
NA-DAC-63	8	7	14	14	62		63
NA-DAC-78		8	9	12	76	78	76
DAC-49 [c]	10	10	9	9	59	49	50
DAC-82			10	12	82	82	80

[a] Calculated from: $(GlcNAc)_{n-1}(\mathrm{wt}\%) = W_n(\%) \times [203(n-1)+1]/[203(n-1)+164]$. [b] Degree of deacetylation: GPC, calculated from eq *4*; EA, evaluated by the elemental analysis; IR, evaluated by IR spectroscopy. [c] Contains 9% of $(GlcNAc)_5$.

where da and A are the degree of deacetylation of DA-chitin evaluated by GPC and the summation of the composition (%) of GlcNAc segments, respectively. These deacetylation values (da evaluated by GPC) agree approximately with those evaluated both by elemental analysis and the IR method[11]. Hence, we would like to propose this procedure as one of the methods for estimating the degree of deacetylation of DA-chitins.

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