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Preparation of *N*-acetylchitooligosaccharides from lysozymic hydrolysates of partially *N*-acetylated chitosans

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Abstract

The conditions for preparing partially *N*-acetylated chitosans (PNACs) by reaction of chitosan with acetic anhydride in homogeneous solution were investigated. The following conditions are recommended: chitosan, 1%; acetic acid, 0.4%; methanol, 50%; at room temperature. About 80% of the acetic anhydride reacted with amino groups and the degree of *N*-acetylation (da) of PNACs was easily controlled by the amount of acetic anhydride. PNACs were hydrolysed by lysozyme at 37°C for 6 days and hydrolysates were analysed by HPLC. 2-Acetamido-2-deoxy-D-glucose and *N*-acetylchitooligosaccharides [$(GlcNAc)_n$ (n = 2–4)] together with heterooligosaccharides composed of 2-acetamido-2-deoxy-D-glucose and 2-amino-2-deoxy-D-glucose were detected. The latter were *N*-acetylated by acetic anhydride, to yield $(GlcNAc)_n$. *N*-Acetylation of the hydrolysates doubled the amount of total $(GlcNAc)_n$ and $(GlcNAc)_3$, and increased the amount of $(GlcNAc)_4$ ninefold. The amount of $(GlcNAc)_n$ after the hydrolysis and the *N*-acetylation increased with the increase of da of PNACs. The optimum da for the formation of oligomers was ca. 70%. The yields of $(GlcNAc)_n$ (n = 2–5) after gel filtration chromatography were 16.3, 10.3, 18.0, and 1.7 mg, respectively, from the hydrolysates of 72% *N*-acetylated chitosan (100 mg). This procedure is useful for the production of $(GlcNAc)_2$, $(GlcNAc)_3$, and $(GlcNAc)_4$.

1. Introduction

Chitin and chitosan are polysaccharides which attract much attention in the biomedical, pharmacological, agricultural, and biotechnological fields [1–4]. In addition, chitooligosaccharides [$(GlcN)_n$)], *N*-acetylchitooligosaccharides [$(GlcNAc)_n$], and heterooligosaccharides composed of GlcN and GlcNAc are of special interest in agriculture and medicine. They have activities as elicitors [5–8], antibac-

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terial agents [9,10], immuno-enhancers [11], and lysozyme inducers [12]. These oligosaccharides have been prepared by chemical [13–15], enzymic [16–19], and fermentation [20,21] methods.

We found previously that the lysozyme digestibility of partially *N*-acetylated chitosans (PNACs) increased with the increase of the degree of *N*-acetylation (da) of PNACs [22]. In this paper, we report the preparation of $(GlcNAc)_n$ ($n = 2–5$) by *N*-acetylation of lysozymic hydrolysates of PNACs. In addition, this paper describes the reproducible preparation of PNACs.

2. Results and discussion

In a series of studies of chitosan, the properties [23,24] and reactivites [22,23,25–27] of PNACs have been investigated. However, there are few data useful for preparing PNACs. When the *N*-acetylation of chitosan in aqueous methanolic acetic acid under homogeneous conditions was conducted with an excess of acetic anhydride, the da was more than 90% and the mixtures formed gels [28–30]. Hirano et al. [31], Miya et al. [32], Roberts and Taylor [33,34], and Yabuki et al. [35] reported partial *N*-acetylation, but the reaction conditions were not studied systematically. We have therefore studied the effect of the concentration of acetic acid, methanol, and chitosan (1H-6 in Table 1). Fig. 1 shows the effect of the concentration of acetic acid and methanol on da. The chitosan concentration was 0.3% and the molar ratio of acetic anhydride to amino groups was 0.605. The da increased with the increase of methanol concentration. Above 40% (v/v) of methanol, the da is linearly proportional to the methanol concentration and increases with the decrease of acetic acid concentration. The highest

Table 1
Characterization of chitosans

Sample	M_v [a] ($\times 10^{-6}$)	M_w [b] ($\times 10^{-6}$)	Degree of *N*-acetylation [c] (%)
Chitosans			
1H-6	1.8	1.3	6
2H-5	1.2	0.83	5
3H-5	0.98	0.63	5
M-30	1.8	1.8	30
Partially *N*-acetylated chitosans (PNAC)			
2H-5-49		0.84	49
3H-5-59		1.0	59
M-30-64		1.5	64
M-30-72			72
M-30-75			75

[a] Viscosity average molecular weight [36].

[b] Weight-average molecular weight determined by gel-permeation chromatography with pullulan standards.

[c] Averaged values of data obtained by IR spectroscopy and colloid titration.

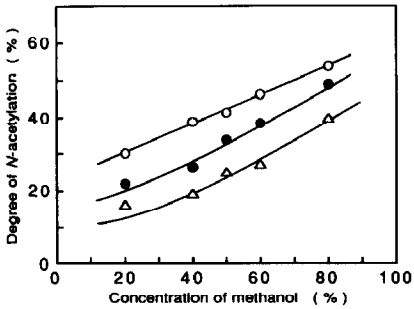

Fig. 1. Effect of the concentration of MeOH and AcOH on da: AcOH, 0.4% (○), 1% (●), and 3% (△); molar ratio of acetic anhydride to amino group, 0.605; chitosan, 0.3%.

efficiency of *N*-acetylation (81%) was attained at a methanol concentration of 80% and an acetic acid concentration of 0.4%. The acetylation efficiency is defined as the percentage of acetic anhydride which reacts with amino groups of chitosan.

The open circles in Fig. 2 show the relationship between da and the molar ratio of acetic anhydride to amino groups. The reaction was conducted so as to attain the highest acetylation efficiency. It is clear that the da can be controlled by the amount of acetic anhydride added. The chitosan solutions formed gels after *N*-acetylation when the methanol concentration was 80% and the molar ratio of acetic anhydride to amino groups was above 0.63. However, these gels could be dissolved by the addition of water when the da was below 70%. PNACs with more than 50% da were sparingly soluble in methanol-rich media.

The chitosan concentration of 0.3% was relatively low and large volumes of methanol were used. It is not suitable for PNAC production on a large scale. The effect of the amount of acetic anhydride on da was then studied at a chitosan concentration of 1%. In this case, a high concentration of methanol cannot be used because the original chitosan does not dissolve completely. The methanol concentration was therefore set at ca. 50%. The relationship was almost linear and the efficiency was ca. 80% (closed circles in Fig. 2). The lower concentration of

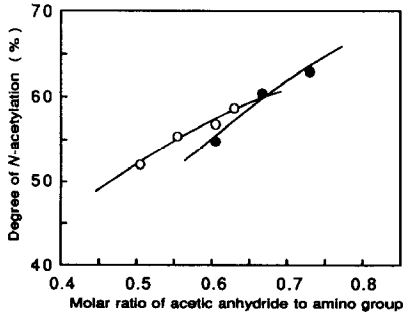

Fig. 2. Effect of the amount of acetic anhydride on da: chitosan, 0.3% (○) and 1% (●); MeOH, 80% (○) and 53% (●); AcOH, 0.4%.

methanol decreased the efficiency as seen in Fig. 1, but the decreased efficiency was compensated by the increase in the chitosan concentration. The mixtures did not form a gel at a da of 63% because the methanol concentration was not so high. In the IR spectrum of PNAC (Fig. 3), there is no absorption band for an ester group; only *N*-acetylation occurred under the conditions described here.

As described above, a higher efficiency of *N*-acetylation was attained at a higher concentration of methanol and lower concentration of acetic acid. The da could easily be controlled by changing the amount of acetic anhydride added. The recommended reaction conditions are as follows: chitosan, 1%; acetic acid, 0.4%; methanol, 50%.

It was found previously that PNACs with a higher da were more digestible by lysozyme [22]. However, the low molecular weight materials in hydrolysates were not analysed. We have now analysed the hydrolysates and optimised the reaction conditions in order to obtain larger amounts of $(GlcNAc)_n$ with a higher degree of polymerisation. Initially, the hydrolysates were directly analysed by high performance liquid chromatography (HPLC) with a Tosoh Amide-80 column (Fig. 4). The major products were monomer and dimer (Fig. 4b). After *N*-acetylation of the hydrolysates, the amounts of trimer, tetramer, and pentamer were increased (Fig. 4c). Amano and Ito [37] reported that heterooligosaccharides composed of GlcN and GlcNAc were formed in lysozyme digests. However, the heterooligosaccharides were not eluted from the column because of adsorption. By contrast, part of the heterooligosaccharides were eluted from an Asahipak NH2P-50 column as shown in Fig. 5b, but oligomers with more than five residues were not eluted. After *N*-acetylation, oligosaccharides were clearly separated (Fig. 5c).

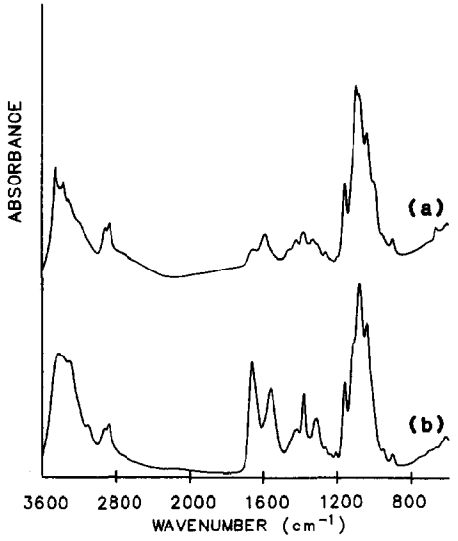

Fig. 3. IR spectra: (a) original chitosan (da, 5%); (b) PNAC (da, 63%).

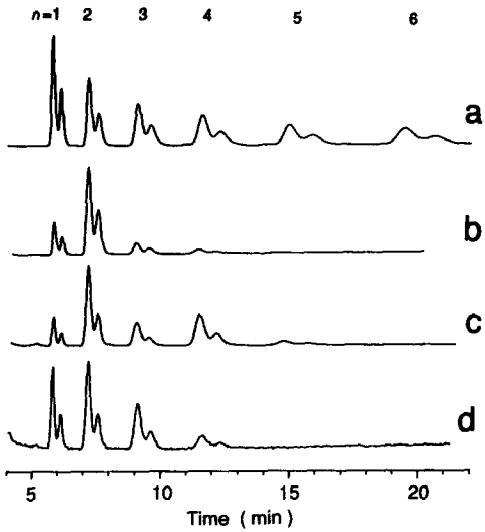

Fig. 4. HPLC profiles of $(GlcNAc)_n$ and hydrolysates: a, authentic $(GlcNAc)_n$ ($n = 1$–6); b, hydrolysates of M-30-75 before *N*-acetylation; c, hydrolysates of M-30-75 after *N*-acetylation; d, hydrolysates of colloidal chitin. The hydrolysates were put on a TSKgel Amide-80 column and eluted with 65:35 MeCN–water.

Secondly, the effect of the da of PNACs on yields of $(GlcNAc)_n$ was studied. In this paper, the yield is defined as a percentage weight ratio of each oligosaccharide to chitosan. Fig. 6 shows the yields of the oligomers before and after *N*-acetylation. The yields of oligomers from PNACs with less than 60% da were low. Higher yields were obtained from PNACs with more than 60% da. *N*-Acetylation increased the amount of total $(GlcNAc)_n$ twofold, $(GlcNAc)_3$ twofold, and $(GlcNAc)_4$ ninefold when M-30-75 was used. The major products were dimer and tetramer.

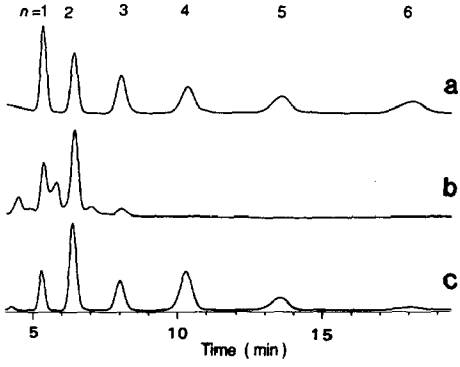

Fig. 5. HPLC profiles of $(GlcNAc)_n$ and hydrolysates: a, authentic $(GlcNAc)_n$ ($n = 1$–6); b, hydrolysate of M-30-75 before *N*-acetylation; c, hydrolysate of M-30-75 after *N*-acetylation. The hydrolysates were put on an Asahipak NH2P-50 column and eluted with 70:30 MeCN–water.

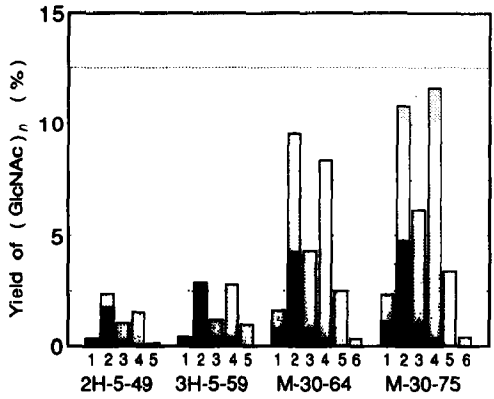

Fig. 6. Yield of $(GlcNAc)_n$ in lysozymic hydrolyses of PNACs: hatched columns, before *N*-acetylation; empty columns, increases after *N*-acetylation; numerals along the *x*-axis are the degree of polymerisation of oligosaccharides. The weight ratio of PNACs to lysozyme was 12.5:1.

The optimum da for the formation of oligomers was ca. 70%. As a control experiment, colloidal chitin was used as a substrate. The yields of monomer to tetramer were 1.6, 3.1, 2.4, and 0.7%, respectively, because of the low hydrolysis rate in suspension and the high rate of hydrolyses of the resulting water-soluble chitin oligomers in solution.

In the experiments of lysozymic hydrolyses of PNACs, the amount of lysozyme used was relatively small. The total yield was less than 41% after reaction for 6 days and increased to 75% after reaction for 13 days. The amount of lysozyme was therefore increased by 2.5 times. The yields of dimer and tetramer from the hydrolysate of M-30-72 became 26 and 24%, respectively, after 6 days, so a preparative experiment was conducted at this substrate–enzyme ratio.

Fig. 7 shows a gel filtration chromatogram of the hydrolysates produced from M-30-72. Pentamer, tetramer, trimer, and dimer were separated and the yields

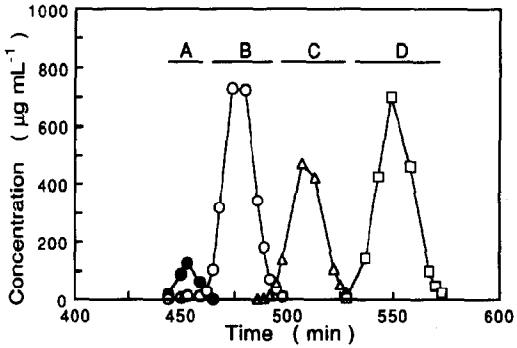

Fig. 7. Gel chromatographic separation of products from M-30-72 after lysozymic hydrolysis and *N*-acetylation: ●, pentamer; ○, tetramer; △, trimer; □, dimer; each fraction was analysed by HPLC. Combined fractions from A to D were lyophilised and the purity was checked by HPLC (Fig. 8).

after lyophilisation were 1.7, 18.0, 10.3, and 16.3 mg, respectively, from 100 mg of M-30-72 chitosan. The purity was analysed by HPLC and the chromatograms are shown in Fig. 8. The pentamer was contaminated with tetramer and the purity calculated from the peak area was ca. 72%. The purities of tetramer, trimer, and dimer were more than 93%.

The preparation and separation of $(GlcNAc)_n$ have been reported. Barker et al. [14] prepared $(GlcNAc)_n$ ($n = 2–7$) by acid hydrolysis of chitosan, followed by *N*-acetylation. The yields of dimer, trimer, and tetramer were 10.5, 11.3, and 7.6%, respectively. Rupley [13] produced $(GlcNAc)_n$ ($n = 2–5$) by acid hydrolysis of chitin. The yields of dimer, trimer, and tetramer were 7, 4.5, and 1.5%, respectively. Bosso et al. [15] reported fluorohydrolysis of chitin and obtained $(GlcNAc)_n$ ($n = 2–10$) in relatively high yields; the main product was the dimer (37%). The yields of trimer, tetramer, pentamer, and hexamer were 23, 10, 5, and 4.6%, respectively. Recently, Izume et al. [17] prepared $(GlcNAc)_n$ ($n = 2–7$) by hydrolysis of chitosan by chitosanase, followed by gel filtration and *N*-acetylation. The yields of dimer to heptamer were 5.2, 23.4, 14, 8.2, 12, and 9%, respectively. In our case, the pentamer and other higher oligomers were not obtained in high yield, but the dimer, trimer, and tetramer were prepared in high yields (16.3, 10.3, and 18%), and the purities were above 93% after only one chromatographic step. The hydrolysates were acetylated in one pot and the chromatography used only water, rendering desalination unnecessary. Purified lysozyme is commercially available and is much cheaper than chitinases. We recommend the procedures described here for preparing the dimer, trimer, and tetramer.

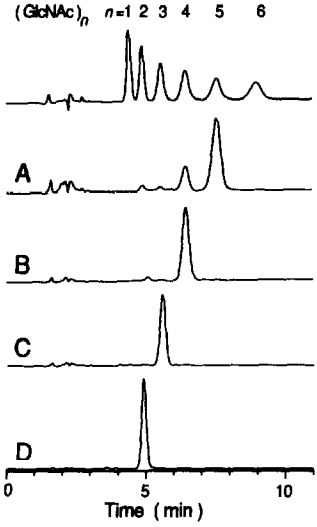

Fig. 8. HPLC profiles of *N*-acetylchitooligosaccharides after separation on a Toyopearl HW-40S column: the oligosaccharides were separately put on an Asahipak NH2P-50 column and eluted with 66:34 MeCN–water. A, B, C, and D are the combined fractions in Fig. 7.

3. Experimental

Materials.—Chitosan samples with various degrees of *N*-acetylation were obtained from Katakura Chikkarin Co., Japan. Some chitosan samples were heated at 110°C in aq 47% NaOH for 1 h under N_2 to afford 1H-6, 2H-5, and 3H-5 samples in Table 1. M-30 chitosan was purified as follows: a solution of a crude chitosan sample in aq 2% acetic acid was centrifuged to remove insoluble materials. The supernatant solution was poured dropwise into aq 4% NaOH, and the precipitate was thoroughly washed with water and dried. PNACs were prepared by *N*-acetylation under homogeneous conditions according to the method described below. The molecular weights [36] and the degrees of *N*-acetylation are summarised in Table 1. Colloidal chitin was prepared using methanesulfonic acid according to the method of Hirano and Nagao [38]. Hen egg-white lysozyme (EC 3.2.1.17) and $(GlcNAc)_n$ ($n = 1$–6) were purchased from Seikagaku Kogyo Co., Japan and used without further purification.

N-*Acetylation of chitosan.*—The *N*-acetylation of chitosan was conducted generally as follows: chitosan (30 mg) was dissolved in aq 10% AcOH and water, and diluted with MeOH to a final volume of 10 mL. The composition of AcOH and MeOH was adjusted by the volumes of water, MeOH, and aq AcOH. The desired amount of Ac_2O was added whilst the solution was well stirred. After stirring at room temperature overnight, 1 M NaOH was added to precipitate PNAC. The precipitates were washed well with aq MeOH and dried under vacuum. In the case of 1% concentration, chitosan (1.5 g) was dissolved in aq 2% AcOH (30 mL) and water (40 mL), and diluted with MeOH (80 mL). The desired amount of Ac_2O was added, and the mixture was treated as described above. IR spectra were recorded on a Nicolet 20DXB FTIR spectrometer with a resolution of 2 cm^{-1}. The da was estimated by colloid titration.

Hydrolysis of PNACs by lysozyme and N-*acetylation of hydrolysates.*—Method *A* (small amount of lysozyme): PNAC (50 mg) was dissolved in 1 M AcOH (2 mL), aq 1% NaN_3 (0.5 mL), and water (5.79 mL), and the pH of the solution was adjusted to 5.4 by 1 M NaOH (1.71 mL). A lysozyme solution (2 mg mL^{-1}, 1 mL) was added and the mixture was incubated at 37°C for 3 days. More lysozyme solution (1 mL) was added and the mixture was incubated for 3 days. A portion (0.5 mL) of the mixture was taken out and diluted with MeOH (0.5 mL). Ac_2O (0.08 mL) was added for *N*-acetylation and the mixture was stirred at room temperature for 2 h. The products in the supernatant solution were analysed by HPLC.

Method *B* (large amount of lysozyme): PNAC (M-30-72, 100 mg) was dissolved as described above, to afford a 0.5% solution. Lysozyme (20 mg) was added and the mixture was incubated at 37°C. At intervals, a portion (0.5 mL) was taken out, acetylated, and analysed as described above.

Hydrolysis of colloidal chitin by lysozyme.—A colloidal chitin suspension (0.5% w/v, 10 mL) in 0.2 M acetate buffer (pH 5.4) with 0.05% NaN_3 was incubated with lysozyme (2 mg mL^{-1}, 1 mL) at 37°C for 3 days and then more lysozyme solution (1 mL) was added. After 3 days the suspension was centrifuged and the products in the supernatant solution were analysed by HPLC.

Analysis of hydrolysates by HPLC.—$(GlcNAc)_n$ were analysed by two sets of HPLC. The first set comprised a Tosoh TSKgel Amide-80 column (4.6 × 250 mm; flow rate, 1.0 mL min^{-1}; eluent, 65:35 MeCN–water). The second set comprised an Asahipak NH2P-50 column (4.6 × 250 mm; flow rate, 1.0 mL min^{-1}; eluent, 70:30 MeCN–water). Quantitative analysis was done using an authentic mixture of $(GlcNAc)_n$, and a UV detector at 210 nm.

Separation of $(GlcNAc)_n$ by gel filtration chromatography. — PNAC (M-30-72, 100 mg) was dissolved in 1 M AcOH (4 mL), 1% NaN_3 (1 mL), and water (11.6 mL), and the pH of the solution was adjusted to 5.4 by 1 M NaOH (3.4 mL). Lysozyme (20 mg) was added and the mixture was incubated at 37°C for 7 days. The mixture was concentrated to 7 mL and diluted with MeOH (28 mL), and Ac_2O (0.5 mL) was added. The mixture was stirred at room temperature for 2 h and then 1 M NaOH (3 mL) was added. The solution was centrifuged and the supernatant solution was concentrated to 1 mL. The precipitate was extracted with water (20 mL), and the extract was combined with the concentrated supernatant. The mixture was concentrated again to 17 mL and put onto a Toyopearl HW-40S column (5 × 70 cm) previously equilibrated with water. The column was eluted with degassed water at a flow rate of 91.6 mL h^{-1}. Each fraction (4.6 mL) was analysed by HPLC. Four combined fractions indicated by bars in Fig. 7 were lyophilised and the purity was checked by HPLC (Fig. 8).

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