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Note

Preparation of *N*-acetylchitooligosaccharides by hydrolysis of chitosan with chitinase followed by *N*-acetylation

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Chitin and chitosan are polysaccharides that attract much attention in the biomedical, pharmacological, agricultural, and biotechnological fields [1–5]. In addition, chitooligosaccharides [$(GlcN)_n$], *N*-acetylchitooligosaccharides [$(GlcNAc)_n$], and heterochitooligosaccharides composed of GlcN and GlcNAc are of special interest in agriculture and medicine. They have activities as elicitors [6–10], antibacterial agents [11,12], immuno-enhancers [13], and lysozyme inducers [14]. Oligosaccharides larger than the hexamer show high activity at a very low concentration [10]. These oligosaccharides have been prepared by chemical [15–17] and enzymic [18–21] methods.

From studies of the enzymic digestibility of chitosan [22–25] and from related work [26–29], the following results were obtained: lysozyme [EC 3.2.1.17] and chitinase [EC 3.2.1.14] can hydrolyse chitin and produce $(GlcNAc)_n$. However, the hydrolysis rate is low because of the heterogeneous conditions, and higher oligomers are not obtained in good yields. Lysozyme hydrolyses partially *N*-acetylated chitosans (PNACs) under homogeneous conditions. The lysozyme digestibility of PNACs increases with the increase of the degree of *N*-acetylation (da) of PNACs because lysozyme recognises a GlcNAc sequence with more than three residues [22]. *N*-Acetylation of the lysozymic hydrolysates from a PNAC with a da of 72% produced mainly dimer, trimer, and tetramer of GlcNAc, all in yields of more than 10% [23]. On the other hand, chitinase recognises a single GlcNAc residue in chitosan [24]. The hydrolysis rate by chitinase increases with increased da. The oligomers produced from a PNAC with high da will

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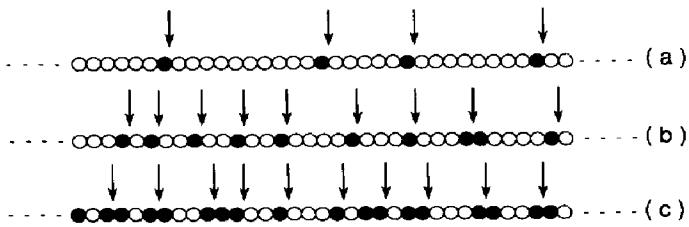

Fig. 1. Speculative representation of the action of chitinase on chitosans having various da: ○, 2-amino-2-deoxy-D-glucose; ●, 2-acetamido-2-deoxy-D-glucose; (a), chitosan with da of 10%; (b), chitosan with da of 30%; (c), chitosan with da of 50%. The arrows show the action of chitinase.

have low degrees of polymerisation but products from a PNAC with low da will be higher oligomers as speculatively represented in Fig. 1. In this paper, we report the preparation of *N*-acetylchitooligosaccharides [$(GlcNAc)_n$ ($n = 2–6$)] from chitosan by chitinase hydrolysis followed by *N*-acetylation with acetic anhydride.

In a previous paper [24], the chitinase digestibility of chitosan was studied. However, the reaction time used was shorter than 8 h and hydrolysates were not analysed. The hydrolysates are now analysed with respect to the degree of polymerisation of the oligosaccharides. Firstly, the hydrolysates produced by *Streptomyces griseus* chitinase were *N*-acetylated with acetic anhydride and the mixtures were directly analysed by HPLC using an Asahipak NH2P-50 column. The $(GlcNAc)_n$ from dimer to heptamer were clearly and easily separated as had been shown in previous work on the *N*-acetylated products from lysozymic hydrolysates [23] and in related work [19,26,27].

Secondly, the effect of the da on the yields of $(GlcNAc)_n$ was studied using chitosans of various da listed in Table 1. In this paper, the yield is defined as a percentage weight ratio of each oligosaccharide to chitosan. Fig. 2 shows the yields of the oligomers produced after hydrolysis by *S. griseus* chitinase followed by *N*-acetylation. The yields were relatively low when chitosan with da of 5% was used as a substrate. Higher yields were obtained from chitosans with more than 10% da. The major products were trimer,

Table 1
Characterisation of chitosans

Sample	M_v [a] ($\times 10^{-6}$)	M_w [b] ($\times 10^{-6}$)	Degree of *N*-acetylation [c] (%)
Chitosans			
2H-5	1.2	0.83	5
3H-5	0.98	0.63	5
M-13	1.4		13
Partially *N*-acetylated chitosans (PNAC)			
2H-5-19			19
2H-5-23		0.75	23
2H-5-30		0.90	30

[a] Viscosity-average molecular weight [31].
[b] Weight-average molecular weight determined by gel-permeation chromatography with pullulan standards.
[c] Averaged values of data obtained by IR spectroscopy, colloid titration, and elemental analysis.

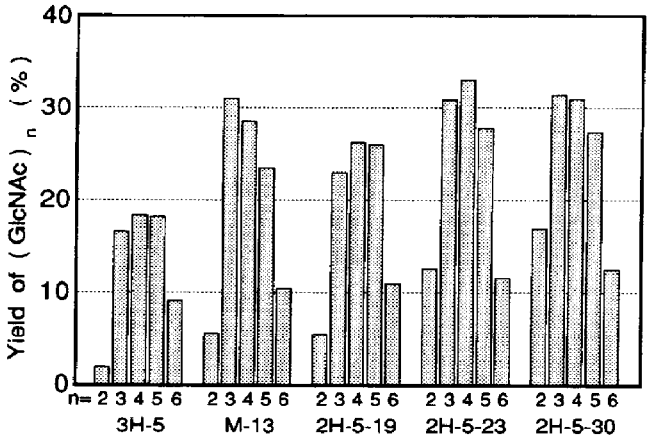

Fig. 2. Yields of $(GlcNAc)_n$ prepared from various chitosans by chitinase (*Streptomyces griseus*) hydrolyses and *N*-acetylation; chitosan–chitinase = 200:1 by weight, pH 5.4, 37°C, 5 days. Numerals along the *x*-axis are the degree of polymerisation of oligosaccharides.

tetramer, and pentamer. The yield of hexamer was ca. 10% when using chitinase from *S. griseus*, while less than 10% of hexamer was released by chitinases from *Bacillus* sp. and *Bacillus* sp. PI-7S. The total yield was relatively high when *Bacillus* sp. PI-7S chitinase was used. The production of $(GlcNAc)_n$ by *S. griseus* chitinase was then studied in detail.

The time course of $(GlcNAc)_n$ production from M-13 chitosan after hydrolysis and *N*-acetylation was studied. The total yield was 72% for a 4-day reaction and 94% after 8 days; the yield of hexamer increased to 14%. A 7-day reaction time is optimum.

For large-scale production, chitosan (M-13, 200 mg) was hydrolysed by *S. griseus* chitinase (1 mg) for 7 days. The solution of oligosaccharides, after *N*-acetylation, was concentrated and subjected to gel filtration chromatography. Fig. 3 shows the chro-

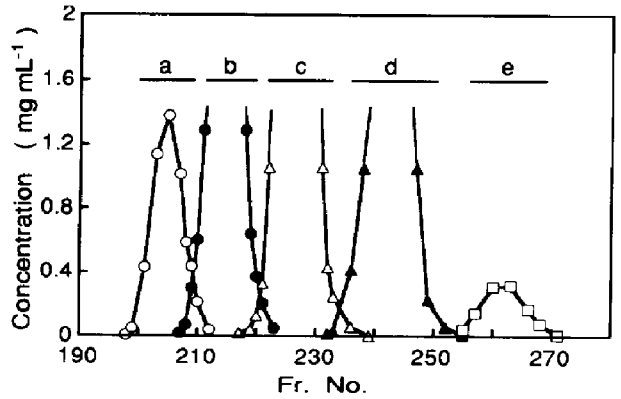

Fig. 3. Gel chromatographic separation of products from M-13 after hydrolysis and *N*-acetylation. The *N*-acetylated products were put on a Toyopearl HW-40S column (5×68 cm) and eluted with water (94 mL h^{-1}), and each fraction was analysed by HPLC: ○, $(GlcNAc)_6$; ●, $(GlcNAc)_5$; △, $(GlcNAc)_4$; ▲, $(GlcNAc)_3$; □, $(GlcNAc)_2$.

matogram in which each fraction was analysed by HPLC. The oligosaccharides were separated into 5 peaks as indicated by bars in Fig. 3. Five peaks a to e, corresponding to hexamer, pentamer, tetramer, trimer, and dimer of $(GlcNAc)_n$, respectively, were detected. The yields of $(GlcNAc)_n$ from dimer to hexamer were 1.9, 50.5, 50.9, 39.1, and 24.5 mg, respectively, after lyophilisation. The purities of lyophilised oligosaccharides, calculated from the peak areas of HPLC patterns, were 97, 98, 94, 92, and 96% for $(GlcNAc)_n$ from dimer to hexamer, respectively.

$(GlcNAc)_n$, from trimer to hexamer, could be obtained in high yield and purity. In the case of hexamer, the yield was 12.3%. The purities of $(GlcNAc)_n$ were $> 92\%$ after only one chromatographic step. This procedure has two advantages: the chitosan used here can easily be obtained from crab shell chitin by *N*-deacetylation under heterogeneous conditions and a highly deacetylated sample is unnecessary. Secondly, chitinase is commercially available as a reagent. The amount of chitinase was 1 mg for 200 mg of chitosan.

The yields of pentamer and hexamer by acid hydrolysis were less than 7% [15–17]. Izume et. al. prepared $(GlcNAc)_n$ $(n = 2–7)$ by chitosanase hydrolysis of chitosan followed by *N*-acetylation [19]. The yields of tri-, tetra-, penta-, and hexa-saccharides of GlcNAc were 23.4, 14, 8.2, and 12%, respectively, on the basis of the weight of chitosan used. In our case, these yields were 23.5, 25.5, 19.6, and 12.3%, respectively. The yield of hexamer is comparable and those of tetramer and pentamer are higher than those reported by Izume et al. Hydrolysis of chitosans with da of 10-30% by chitinase is a very suitable method for preparation of higher *N*-acetylchitooligosaccharides.

1. Experimental

Materials.—Chitosan samples were obtained from Katakura Chikkarin Co., Japan. Two chitosan samples were heated at 110°C in aq 47% alkali for 1 h under N_2, to afford 2H-5 and 3H-5 samples in Table 1. PNACs were prepared by *N*-acetylation under homogeneous conditions according to the method of Hirano et al. [23,30]. The molecular weights [31] and da are summarised in Table 1. Chitinases from *Streptomyces griseus* (0.86 U mg^{-1}, Sigma), *Bacillus* sp. (0.045 U mg^{-1}, Wako Pure Chemical Industries, Japan), and *Bacillus* sp. PI-7S (0.053 U mg^{-1}, Pias, Japan) are commercial products and were used without further purification. Authentic $(GlcNAc)_n$ $(n = 2–6)$ were purchased from Seikagaku Kogyo Co., Japan.

Hydrolysis of chitosans by chitinase followed by N-*acetylation of the hydrolysates.*—Chitosan (10 mg) was dissolved in 1 M AcOH (0.4 mL), 1% NaN_3 (0.1 mL), and water (1.06 mL). The pH of the solution was adjusted with 1 M NaOH to 5.4 for *S. griseus* and *Bacillus* sp. PI-7S chitinases or 4.5 for *Bacillus* sp. chitinase. A chitinase solution (0.1 mL) was added and the mixture was incubated at 37°C for the desired time. The amounts of chitinases from *S. griseus*, *Bacillus* sp., and *Bacillus* sp. PI-7S were 0.05, 0.1, and 0.1 mg, respectively. A portion (0.3 mL) of the mixture was taken out and diluted with MeOH (0.3 mL). Acetic anhydride (0.03 mL) was added for *N*-acetylation and the mixture was stirred at room temperature for 1 h. The products in the supernatant solution were analysed by HPLC on an Asahipak NH2P-50 column (4.6×250 mm;

eluent, 66 : 34 MeCN-water; flow rate, 1.0 mL min^{-1}). Quantitative analysis was done, using authentic $(GlcNAc)_n$, by a UV detector at 210 nm.

Separation of $(GlcNAc)_n$ by gel filtration chromatography.—Chitosan (M-13, 200 mg) was dissolved in 1 M AcOH (5 mL), aq 1% NaN_3 (1.25 mL), and water (14.73 mL), and the pH of the solution was adjusted to 5.0 with 1M NaOH (3.52 mL). Chitinase from *S. griseus* (1 mg/0.5 mL) was added and the mixture was incubated at 37°C for 7 days. The mixture was concentrated to 8 mL and diluted with MeOH (24 mL). Acetic anhydride (1 mL) was added, the mixture was stirred at room temperature for 5 h, and 1 M NaOH (5 mL) was added. The mixture was centrifuged and the supernatant solution was concentrated to ca. 5 mL. The precipitates were extracted with water (20 mL) and the extract was combined with the concentrated supernatant. The mixture was concentrated to 13 mL and poured onto a Toyopearl HW-40S column (5 × 68 cm). The column was eluted with degassed water at a flow rate of 94 mL h^{-1}. Each fraction (3.13 mL) was analysed by HPLC. Five combined fractions, indicated by bars in Fig. 3, were lyophilised and their purity was checked by HPLC.

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