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Note

Synthesis of a chitosan tetramer derivative, β-D-GlcNAc-(1→4)-β-D-GlcNAc-(1→4)-β-D-GlcNAc-(1→4)-D-GlcN through a partial N-acetylation reaction by chitin deacetylase

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Abstract

We have synthesized β-D-GlcNAc-(1→4)-β-D-GlcNAc-(1→4)-β-D-GlcNAc-(1→4)-D-GlcN (**2**) through a partial N-acetylation reaction of chitosan tetramer **1** by a chitin deacetylase from *Colletotrichum lindemuthianum* ATCC 56676. The compound was purified from the mixture of acetylation products of **1** using cation-exchange column chromatography and amine-adsorption column chromatography, and its structure was estimated by ^{1}H NMR and FABMS analyses. The enzymatic reaction allows a regioselectivity that is hard to achieve by chemical N-acetylation.

Keywords: Chitin deacetylase; *Colletotrichum lindemuthianum*; N-acetylation

Synthesis of partially deacetylated chitin oligomers has been drawing considerable interest because the products exhibit elicitor activity for plants [1–3]. However, partially deacetylated chitin oligomers have usually been synthesized as a mixture of components with heterogeneous degrees of polymerization and heterogeneous distribution of acetyl groups in the molecules, which causes difficulty in identifying structural features of the components that possess strong biofunctions.

On the other hand, the syntheses of partially deacetylated chitin oligomers with a defined distribution of acetyl groups make the screening of their biofunction possible. Drouillard and co-workers established methods for the production of a series of monodeacetylated chitin oligomer derivatives with GlcN residues at the non-reducing ends,

Abbreviations: GlcN, D-glucosamine; GlcNAc, *N*-acetyl-D-glucosamine.

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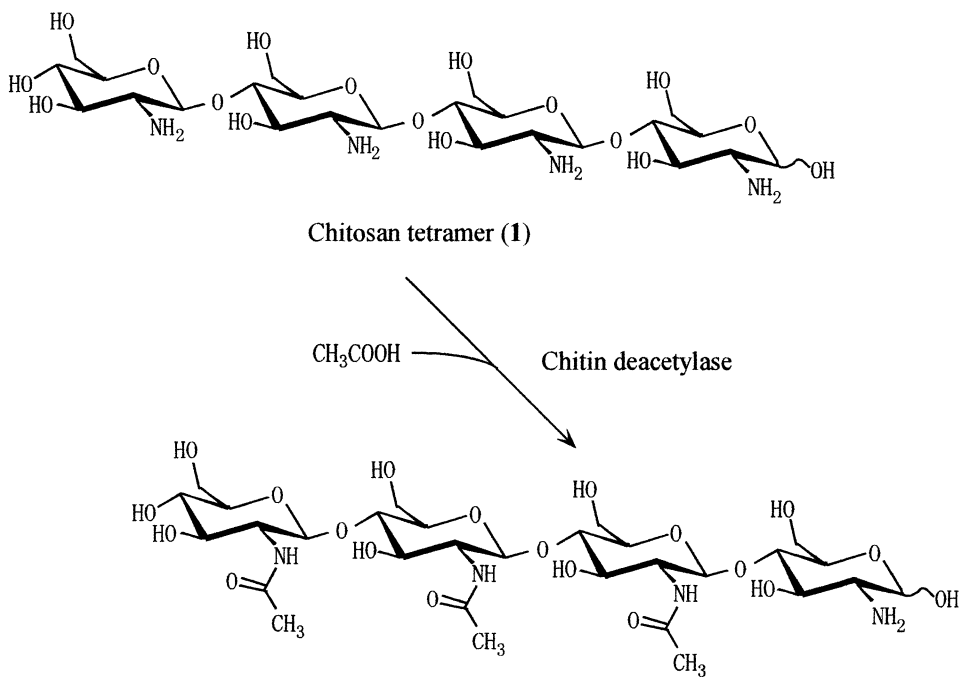
and they found that these oligomers exhibit an inhibitory activity for a chitobiase [4,5]. For the purpose of the screening of functional materials in the mixture of partially deacetylated chitin oligomers, we have been working on the establishment of methods for preparing partially deacetylated chitin oligomers with a defined distribution of acetyl groups. We have established a method to efficiently synthesize β-D-GlcN-(1→4)-D-GlcNAc using a chitin deacetylase from *Colletotrichum lindemuthianum* [6]. Recently, we found that the chitin deacetylase can also acetylate substrates in the presence of 3 M sodium acetate, and β-D-GlcNAc-(1→4)-D-GlcN can be synthesized from chitosan dimer by a reverse hydrolysis reaction [7].

Herein, starting from chitosan tetramer **1**, we have succeeded in the synthesis of β-D-GlcNAc-(1→4)-β-D-GlcNAc-(1→4)-β-D-GlcNAc-(1→4)-D-GlcN (**2**) through the reverse hydrolysis reaction (Scheme 1). The preparation of **2** was accomplished through three steps: (1) partial acetylation of **1** using the chitin deacetylase; (2) separation of triacetylated products with cation-exchange column chromatography, and (3) separation of the chitosan tetramer derivative, which is deacetylated only at the reducing end with amine-adsorption column chromatography. Through the first step with enzymatic acetylation, a reaction mixture with 68% of the acetylated amino sugar residues was obtained. We have also tried partial acetylation of **1** with an acetic anhydride–methanol system and obtained a reaction mixture with 76% of the amino sugar residues acetylated. Each reaction mixture was run onto a cation-exchange column in order to separate the triacetylated chitosan tetramer derivatives according to the numbers of unsubstituted amino groups in the compounds. Then the third step for the purification was performed using amine-adsorption column chromatography (High-Performance Carbohydrate Column). The column possesses a unique property in that the retention times (t_R) of partially deacetylated chitin oligomers with deacetylated reducing-end residues are longer than those with acetylated reducing-end residues (M. Mitsutomi, unpublished data). In this report we applied this unique specificity for the purification of **2** ($t_R = 14$ min) from the other three triacetylated chitosan tetramer derivatives with the reducing-



Scheme 1. Synthesis of β-D-GlcNAc-(1→4)-β-D-GlcNAc-(1→4)-β-D-GlcNAc-(1→4)-D-GlcN (**2**) from chitosan tetramer **1**.

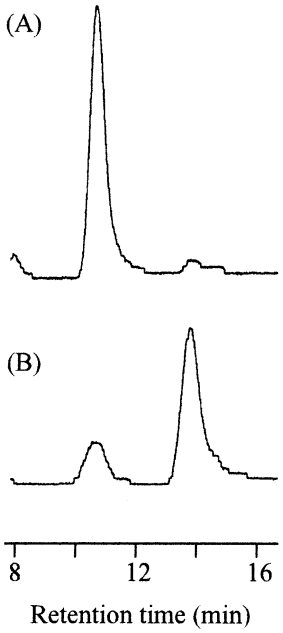


Fig. 1. Amine-adsorption column chromatography profiles of triacetylated chitosan tetramer derivatives through partial acetylation of chitosan tetramer: (A) the chemical acetylation method; (B) the enzymatic acetylation method.

end residues acetylated ($t_R = 11$ min). Fig. 1 shows the profiles on the amine-adsorption column chromatograph of the triacetylated products of enzymatic or chemical N-acetylation of **1**. The peak of the target compound at t_R of 14 min was detected in the enzymatically acetylated products, but not in the chemically acetylated products, suggesting that the regioselectivity during the partial acetylation of **1** differs between the two methods. For the purpose of obtaining **2**, the enzymatic acetylation method seemed to be more desirable compared with the alternative chemical acetylation method.

The structure of the acetylation product was determined by FABMS and NMR spectroscopy. High-resolution FABMS (glycerol): Calcd for $C_{30}H_{53}N_4O_{20}$ ($[M+H]^+$), 789.3253. Found, 789.3289. ^{1}H NMR (500.13 MHz, D_2O): δ 2.03–2.04 (9 H, m, acetyl methyl), 2.99 (0.4 H, dd, J 10.5, 8.5 Hz, H-2 (β anomer)), 3.28 (0.6 H, dd, J 10.5, 3.5 Hz, H-2 (α anomer)), 3.42–3.91 (22.4 H, m, H-3 (β anomer), H-4, 5, 6, 2′, 3′, 4′, 5′, 6′, 2″, 3″, 4″, 5″, 6″, 2‴, 3‴, 4‴, 5‴, 6‴), 3.98 (0.6 H, dd, J 8.9, 10.5 Hz, H-3 (α anomer)), 4.52–4.53 (3 H, m, H-1′, 1″, 1‴), 4.91 (0.4 H, d, J 8.5 Hz, H-1 (β anomer)), 5.39 (0.6 H, d, J 3.5 Hz, H-1 (α anomer)). High-resolution FABMS gave the molecular formula of $C_{30}H_{52}N_4O_{20}$, suggesting the triacetylated chitosan tetramer structure. In the ^{1}H NMR spectrum, the total intensity of *N*-acetyl protons, which appeared at 2.03–2.04 ppm, was calculated as 9 H, showing three acetyl groups. Although significant signal overlap made complete assignment difficult, partial assignment around the H-2 protons, employing a DQF–COSY experiment, revealed the acetylated positions on the chitosan tetramer chain. H-2 protons in the reducing-end residue were assigned by cross-peaks with the anomeric H-1 proton resonances. The other H-2 protons (shown as H-2′, H-2″ and H-2‴ in the above assignment) were observed at lower frequency between 3.42 and 3.91 ppm. Such an upfield shift of the H-2 signals on the reducing-end residue was interpreted as the lack of an electron-withdrawing acetyl group on the C-2 linked amino nitrogen, i.e., a free NH_2. In addition, these chemical shifts, assigned for H-2 with/without acetyl group, were in good agreement with those of β-D-GlcN-(1→4)-D-GlcNAc and β-D-GlcNAc-(1→4)-D-GlcN [6,7]. From the data presented above, the structure of the acetylated product was determined to be β-D-GlcNAc-(1→4)-β-D-GlcNAc-(1→4)-β-D-GlcNAc-(1→4)-D-GlcN. The yield of the compound **2** was 1.1 mg, starting from 20 mg of **1**.

The strategies for the preparation of partially deacetylated chitin oligomers with defined distribution of acetyl groups have been roughly categorized into three groups: (1) the enzymatic degradation of partially deacetylated chitin using a lysozyme, chitinase or chitosanase [8–10]; (2) the intracellular synthesis of β-D-GlcN-(1→4)-β-D-GlcNAc-(1→4)-β-D-GlcNAc-(1→4)-β-D-GlcNAc-(1→4)-D-GlcNAc using a recombinant *Escherichia coli* system harbouring *nod* B and *nod* C genes from *Rhizobium* sp. [5], and (3) the enzymatic deacetylation of chitin oligomers using *N*-deacetylases [6,11,12]. Recently, we have established a fourth strategy for the synthesis of partially deacetylated chitin oligomers using the reverse hydrolysis reaction of the chitin deacetylase [7], and in this report we have applied the strategy for the synthesis of **2**.

Amano and Ito reported on the detection of compound **2** in the hydrolysate of partially deacetylated chitin by a lysozyme [8]. The first strategy, however, comprises a problem that partially deacetylated chitin oligomers with different degrees of polymerization are simultaneously produced. Another novel method with the acetylation reaction gives partially acetylated products with only the defined degree of polymerization, which facilitates the separation of the target compound using amine-adsorption column chromatography.

1. Experimental

Materials.—Chitin deacetylase (EC 3.5.1.41) from *C. lindemuthianum* (ATCC 56676) was purified as described [13]. Compound **1** was purchased from Seikagaku Kogyo Co., Japan. CM Sephadex C-25 was obtained from Pharmacia Biotech, Sweden, and the High-Performance Carbohydrate Column (4.6 × 250 mm) was from Waters Co., USA. All other chemicals used were of reagent grade.

*Acetylation of **1***

Enzymatic method. The reaction mixture (2 mL) contained 20 mg of **1**, 3.0 M sodium acetate (pH adjusted to 7.0 with AcOH), and purified chitin deacetylase (2.7 U). The reaction mixture was incubated at 37 °C for 16 h. Then the reaction mixture was run onto a column (15 × 60 mm) of activated charcoal (type F400, Toyo Calgon Co., Ltd., Japan), with a flow rate of 2.0 mL min^{-1}. After washing the column with water (50 mL), the bound materials were eluted with 3:2 EtOH–water (50 mL). Then the eluted sample was evaporated under reduced pressure below 35 °C and dissolved in 3 mL of 20 mM sodium acetate buffer (pH 5.0, Buffer A).

Chemical method. The reaction mixture (5 mL) contained 5 mg of **1**, 2 mL of 4% AcOH, 2 mL of MeOH and 1 mL of Ac_2O. The reaction mixture was incubated at 25 °C for 3 h. Then the mixture was evaporated under reduced pressure, and the pellet was dissolved in 1.1 mL of Buffer A. The number of unsubstituted GlcN residues was estimated by the spectrophotometric method of Dische and Borenfreund [14], using **1** as the standard.

Separation of triacetylated products.—The acetylation products in Buffer A (1 mL) were run onto a column (15 × 60 mm) of 1 mL (wet volume) of cation-exchange resin (CM Sephadex C-25, swelled with Buffer A) with a flow rate of 0.5 mL min^{-1}. After washing the column with the buffer (3 mL) and a linear gradient of NaCl (0–250 mM, 2 mL) in the buffer, the bound triacetylated products were eluted with a linear gradient of NaCl (250–460 mM, 7.5 mL) in the buffer. The elution of the acetylation products was detected by monitoring the absorbance at 220 nm (UVIDEC-100-III, Japan Spectroscopic Co., Ltd., Japan). The eluted sample was fractionated (1 mL each), and fractions 6–7 with triacetylated products were combined. The sample was desalted using an electric dialyser (Micro Acilyzer S1, Asahikasei Kogyo Co., Ltd., Japan) and then evaporated under reduced pressure below 35 °C.

*Purification of **2**.*—The dried sample obtained by the enzymatic acetylation of **1** was dissolved in 200 μL of water, and aliquots (20 μL) were run onto the High-Performance Carbohydrate Column with a flow rate of 1.0 mL min^{-1} and a mobile phase of 7:3 MeCN–water. The elution of the acetylation products was detected by monitoring the absorbance at 210 nm (UV-8020, Tosoh Co., Ltd., Japan). A peak around a t_R of 14 min after the injection was recovered and evaporated under reduced pressure below 35 °C. Then the dried sample was dissolved in 1 mL of Buffer A, and again purified with CM Sephadex C-25 column chromatography as mentioned above, in order to remove small amounts of contaminated materials after the amine-adsorption column chromatography. The dialysed sample was lyophilized to obtain white flakes, and stored at −20 °C.

Comparison of the HPLC profiles of monodeacetylated products.—The triacetylated products after either enzymatic or chemical acetylation were analysed using the High-Performance Carbohydrate Column, as mentioned above. Each of the desalted samples (3 μg) was injected after the first separation with CM Sephadex C-25.

Analytical methods.—The FAB mass spectrum (positive-ion mode, glycerol matrix) of the reaction product was obtained using a JEOL JMS-SX102A instrument. NMR spectra were recorded on a Bruker AVANCE 500 spectrometer at 298 K equipped with a 5-mm TBI ($^{1}H-^{13}C-BB$; *xyz*-gradient) probehead. A DQF–COSY experiment was carried out to assign ^{1}H signals, and all NMR data were reported in ppm (δ) downfield from tetramethylsilane using 2-methyl-2-propanol as the internal standard (δ 1.23).

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