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Reverse hydrolysis reaction of chitin deacetylase and enzymatic synthesis of β-D-GlcNAc-(1→4)-GlcN from chitobiose

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Abstract

We found that a chitin deacetylase from *Colletotrichum lindemuthianum* could acetylate free amino sugar residues into N-acetylated forms in the presence of 3.0 M sodium acetate. The result was analyzed using a β-*N*-acetylhexosaminidase-coupled assay system with *p*-nitrophenyl 2-amino-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside as the substrate, and the liberation of *p*-nitrophenol was observed as a consequence of enzymatic N-acetylation of the glucosamine residue at the nonreducing end of the substrate. The chitin deacetylase also acetylated chitobiose and chitotetraose as substrates, which was evidenced by the decrease in the amount of free amino sugar residues in the chitooligosaccharides. The reaction product of chitobiose after the acetylation reaction was exclusively 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-amino-2-deoxy-D-glucose [GlcNAcGlcN], the structure of which was determined by FABMS and NMR analyses. This study offers a novel method for enzymatic N-acetylation of amino sugars, and especially with chitobiose as substrate, a selectively N-acetylated product, GlcNAcGlcN, can be synthesized.

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

1. Introduction

Chitin is among the most abundant biopolymers on the earth, and it is a linear polymer of β-(1→4)-linked 2-acetamido-2-deoxy-β-D-glucose residues. It is commercially derived from food wastes such as crab and shrimp shells, and the main target product for the conversion is its N-deacetylated form, namely, chitosan, which can be utilized as a material for the food and pharmaceutical industries [1]. In addition, chitosan oligomers have been attracting a keen interest in chitin utilization because they have been reported to possess physiological activities such as antitumor activity [2] and elicitor activity for plants [3]. There has been considerable intensive research on the efficient production of chitosan oligomers [4–6].

Recently, it has been reported that partially N-deacetylated chitin oligomers also possess physiological activities [7,8], and now they are

Abbreviations: GlcN, 2-amino-2-deoxy-D-glucose; GlcNAc, 2-acetamido-2-deoxy-D-glucose; $GlcN_2$, chitobiose; $GlcN_4$, chitotetraose; $(GlcNAc)_2$, *N*,*N*′-diacetylchitobiose; GlcNGlcNAc, $(GlcNAc)_2$ derivative which was N-deacetylated at the sugar residue in the nonreducing end; GlcNAcGlcN, $(GlcNAc)_2$ derivative which was N-deacetylated at the sugar residue in the reducing end; $(GlcNAc)_2$-pNP, *p*-nitrophenyl *N*,*N*′-diacetyl-β-chitobioside; GlcNGlcNAc-pNP, *p*-nitrophenyl 2-amino-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside.

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regarded as another set of targets for the utilization of chitin. They are usually prepared by partial hydrolysis of partially N-deacetylated chitin polymer, and Akiyama et al. have proposed an alternative chemoenzymatic synthesis method using lysozyme and chitotriose derivatives [9]. However, the products from either of the reactions are a mixture of randomly deacetylated chitin oligomers, which causes difficulty in identifying structural features of the components that possess the strongest biofunctions.

On the other hand, the characterization of chitooligosaccharide deacetylases (NodB proteins) from leguminous bacteria [10,11] and recent progress in the techniques of genetic engineering have realized a large-scale preparation of partially deacetylated chitin oligomers with rigid structures, which have only one unsubstituted glucosamine residue at the nonreducing end of the compounds [12]. Establishment of this preparative method resulted in the rapid discovery of chitobiase-inhibitory activity of the new materials [13], which accelerates the utilization of chitin on a broader scale.

We have been studying the preparation of partially N-deacetylated chitin oligomers or their derivatives with rigid structures by using a chitin deacetylase from *Colletotrichum lindemuthianum* [14,15]. Previously, we reported that the enzyme efficiently produces a partially N-deacetylated chitin dimer, GlcNGlcNAc from $(GlcNAc)_2$ [14]. Herein, we report that the chitin deacetylase can also acetylate substrates in the presence of 3 M sodium acetate, and GlcNAcGlcN can be synthesized from $GlcN_2$ by a reverse hydrolysis reaction.

2. Experimental

Materials.—Chitin deacetylase (EC 3.5.1.41) from *C. lindemuthianum* (ATCC 56676) was purified as described [16]. $(GlcNAc)_2$, $GlcN_2$, $GlcN_4$, $(GlcNAc)_2$-pNP, and β-*N*-acetylhexosaminidase from *Penicillium oxalicum* were purchased from Seikagaku Kogyo Co. Japan. GlcN and GlcNAc were purchased from Sigma Chemical Co., USA. GlcNGlcNAc-pNP was prepared from $(GlcNAc)_2$-pNP using a chitin deacetylase from *C. lindemuthianum* [15]. All other chemicals used were of reagent grade.

Detection of acetylation reaction using GlcN-GlcNAc-pNP.—The reaction mixture (18 μL) contained 1 mM GlcNGlcNAc-pNP as the substrate, 3.0 M sodium acetate (pH adjusted to 7.0 with AcOH), β-*N*-acetylhexosaminidase (2.8 mU), and purified chitin deacetylase (0.10 U). Control reactions were also performed using heat-denatured chitin deacetylase (100 °C, 20 min) instead of active chitin deacetylase. The reaction mixture was incubated at 30 °C, and the reaction was stopped by adding 182 μL of a 1 M Na_2CO_3 solution. The acetylation reaction was monitored by measuring the absorbance at 405 nm of liberated *p*-nitrophenol, using a Beckman DU 650 spectrophotometer.

Enzymatic acetylation of $GlcN_2$ and $GlcN_4$.—The reaction mixture (72 μL) contained 0.2% $GlcN_2$ or $GlcN_4$ as the substrate, 3.0 M sodium acetate (pH adjusted to 7.0 with AcOH), and purified chitin deacetylase (0.40 U). The reaction mixture was incubated at 37 °C and aliquots (5.0 μL) of the reaction mixture were sampled followed by the addition of 395 μL of 33% AcOH solution. The number of unsubstituted GlcN residues was estimated using the spectrophotometric method of Dische and Borenfreund [17].

Preparation of GlcNAcGlcN.—The reaction mixture (990 μL) contained 10 mg $GlcN_2$, 3.0 M sodium acetate (pH adjusted to 7.0 with AcOH), and purified chitin deacetylase (5.5 U). It was incubated at 37 °C for 24 h. The reaction mixture was then run onto a column (15×60 mm) of 10 mL (wet volume) of cation-exchange resin (Amberlite CG-120, Orugano Co., Ltd., Japan), with a flow rate of 1.0 mL min^{-1}. After washing the column with water (30 mL), the bound GlcNAcGlcN was eluted with 0.5 N HCl (50 mL). The quantity of unsubstituted GlcN residues in each fracion (5 mL) was estimated by a spectrophotometric method [17]. The fractions (nos. 8–13) were combined and adjusted to pH 5.5 by addition of NaOH. Then the sample (50 mL) was concentrated to 25 mL by evaporation under reduced pressure below 35 °C and dialyzed with an electric dialyzer (Micro Acilyzer S1,

Asahikasei Kogyo Co., Ltd., Japan). The desalted sample was then lyophilized to obtain white flakes (4.41 mg), and it was then stored at −20 °C.

Estimation of sugars was carried out using HPAEC on DX-300 (Dionex Co., USA) equipped with a Pulsed Amperometric Detector (Dionex Co.). The pre-packed column was a CarboPac™ PA1 analytical column (4 × 250 mm, Dionex) with CarboPac™ PA1 guard column (4 × 50 mm, Dionex). The mobile phase was 15 mM NaOH, and the flow rate was 1.0 mL min^{-1}. A part of the sample was diluted 400 times with water prior to the injection (10 μL).

Analytical methods.—The FAB mass spectrum of the reaction product was obtained using a JEOL JMS-SX102A instrument. The positive-ion mode was used for the analysis, and glycerol was used as the matrix. NMR spectra were recorded on a Bruker DRX 600 spectrometer at 303 K using a 5-mm QNP (X (^{13}C, ^{15}N, ^{31}P)/^{1}H; *z*-gradient) probehead. DQF-COSY, HMQC and HMBC experiments were carried out to assign ^{1}H and ^{13}C signals, and all NMR data were reported in ppm (δ) downfield from Me_4Si. The purified sample (1 mg) was dissolved in D_2O (450 μL) for NMR analyses, and 2-methyl-2-propanol was used as the internal standard.

3. Results

First we established a β-*N*-acetylhexosaminidase-coupled assay system for the N-acetylation reaction of the chitin deacetylase from *C. lindemuthianum*, as shown in Fig. 1. As the β-*N*-acetylhexosaminidase from *P. oxalicum* cannot hydrolyze the GlcN-GlcNAc bond of GlcNGlcNAc-pNP, no liberation of *p*-nitrophenol is observed in the absence of the chitin deacetylase [15]. When the chitin deacetylase catalyzes the N-acetylation in the presence of sodium acetate to produce $(GlcNAc)_2$-pNP, the β-*N*-acetylhexosaminidase can remove GlcNAc one by one from the nonreducing end of the substrate to liberate *p*-nitrophenol. Fig. 2 indicates that *p*-nitrophenol was liberated only in the presence of the active chitin deacetylase.

Fig. 3 shows that the chitin deacetylase also acetylated chitosan oligomers, which was indirectly proved by a spectrophotometric quantitation of unsubstituted GlcN residues. It is known that chitin deacetylase can rapidly deacetylate chitin oligosaccharides with the degrees of polymerization of four and five [16], and here we found that the reverse hydrolysis reaction progresses more rapidly with $GlcN_4$ than with $GlcN_2$. While it is likely that the reaction product of $GlcN_4$ will be a mix-

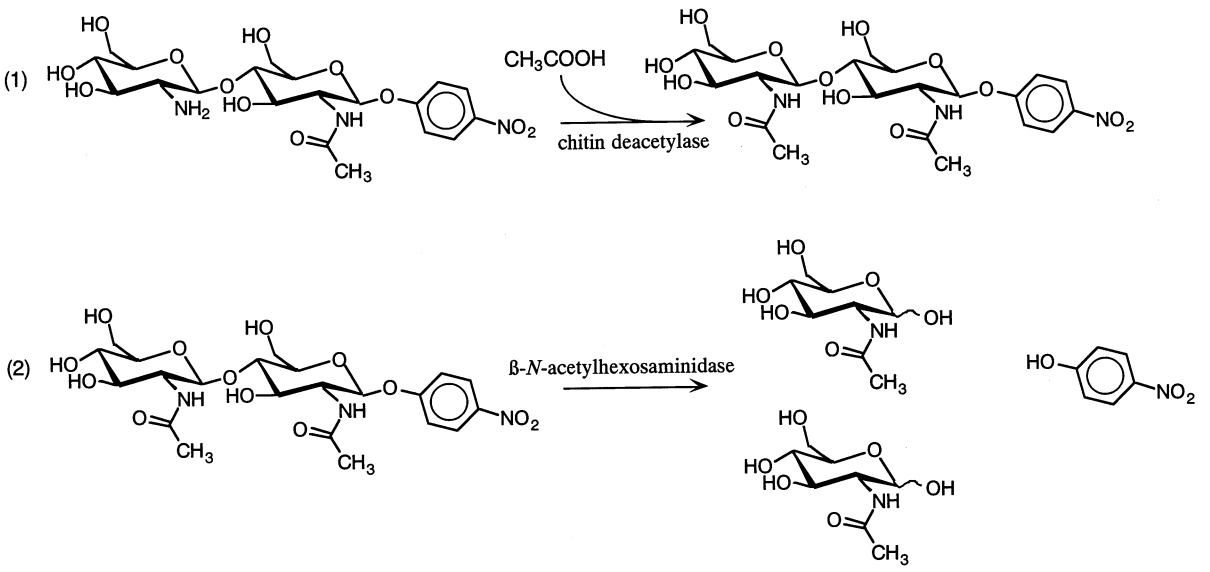

Fig. 1. Scheme of the β-*N*-acetylhexosaminidase-coupled assay system of reverse hydrolysis reaction by chitin deacetylase. (1) N-Acetylation of GlcNGlcNAc-pNP by chitin deacetylase; (2) cleavage of *N*-acetylhexosaminide bonds of $(GlcNAc)_2$-pNP by β-*N*-acetylhexosaminidase.

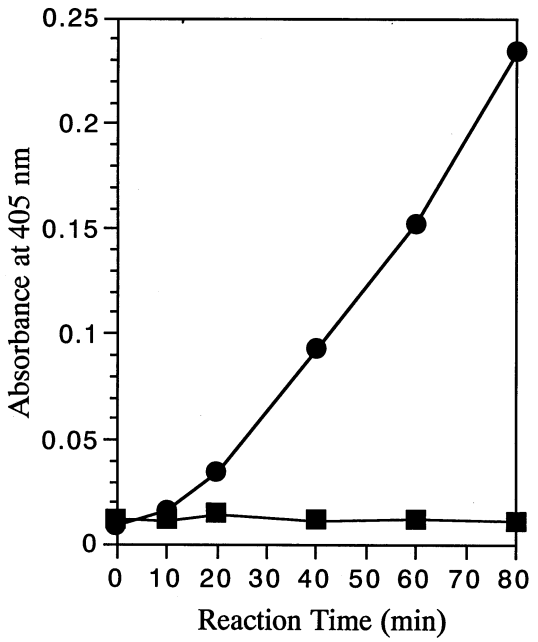

Fig. 2. Time course of *p*-nitrophenol liberation by the β-*N*-acetylhexosaminidase-coupled assay system. Active chitin deacetylase (●), heat-denatured chitin deacetylase (■).

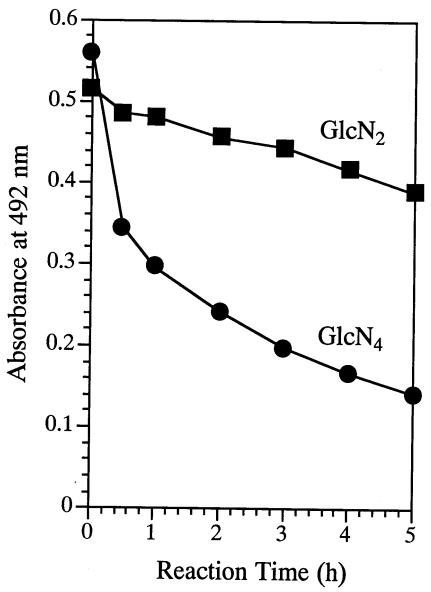

Fig. 3. Time course of enzymatic acetylation of $GlcN_2$ and $GlcN_4$. The amounts of free glucosamine residues were estimated as the absorbance at 492 nm using the spectrophotometric method of Dische and Borenfreund [17].

ture of partially acetylated compounds, the reaction product of $GlcN_2$ is expected to be a single compound, GlcNAcGlcN, which can be deduced from the deacetylation pattern of $(GlcNAc)_2$ by the enzyme [14]. So we moved on to the synthesis of GlcNAcGlcN using this

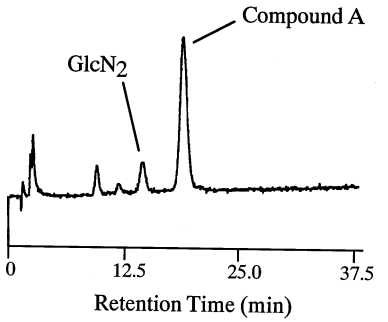

Fig. 4. HPAEC profile of the sugars in the reaction mixture after 24-h acetylation of $GlcN_2$.

enzymatic method. After 24 h reaction at 37 °C, 88% (according to the peak areas of compounds in the HPAEC profile shown in Fig. 4) of $GlcN_2$ was converted into the unknown compound (Compound A, Fig. 4) with a retention time at 19 min. A small amount of GlcN was produced and detected at 10 min, which was also detected with the control reaction mixture without the chitin deacetylase (data not shown).

The structure of the acetylation product was determined by FABMS and NMR spectroscopy: High-resolution FABMS (glycerol): Calcd for $C_{14}H_{27}N_2O_{10}$ ($[M+H]^+$), 383.1666; found, 383.1676. ^1H NMR (600.13 MHz, D_2O) α anomer: δ 2.052 (3 H, s, CO*Me*), 2.958 (1 H, dd, *J* 10.6, 3.6 Hz, H-2), 3.46–3.50 (2 H, m, H-4′, H-5′), 3.54–3.58 (2 H, m, H-4, H-3′), 3.652 (1 H, dd, *J* 12.1, 4.9 Hz, H-6), 3.72–3.75 (2 H, m, H-2′, H-6′), 3.773 (1 H, dd, *J* 12.1, 2.2 Hz, H-6), 3.80 (1 H, dd, *J* 11.5, 8.5 Hz, H-3), 3.877 (1 H, ddd, *J* 9.9, 4.9, 2.2 Hz, H-5), 3.929 (1 H, dd, *J* 12.3, 2.3 Hz, H-6′), 4.562 (1 H, d, *J* 8.5 Hz, H-1′), 5.268 (1 H, d, *J* 3.6 Hz, H-1), β anomer: δ 2.052 (3 H, s, CO*Me*), 2.679 (1 H, dd, *J* 9.6, 8.3 Hz, H-2), 3.46–3.52 (3 H, m, H-5, H-4′, H-5′), 3.54–3.59 (3 H, m, H-3, H-4, H-3′), 3.624 (1 H, dd, *J* 12.0, 5.2 Hz, H-6), 3.72–3.75 (2 H, m, H-2′, H-6′), 3.809 (1 H, dd, *J* 12.0, 2.0 Hz, H-6), 3.924 (1 H, dd, *J* 12.4, 2.3 Hz, H-6′), 4.554 (1 H, d, *J* 8.5 Hz, H-1′), 4.639 (1 H, d, *J* 8.3 Hz, H-1); ^{13}C NMR (150.13 MHz, D_2O) α anomer: δ 23.8 (CO*Me*), 57.3 (C-2′), 58.5 (C-2), 61.9 (C-6), 62.3 (C-6′), 71.5 (C-4′), 71.8 (C-5), 72.8 (C-3), 75.2 (C-3′), 77.6 (C-5′), 81.1 (C-4), 92.8 (C-1), 103.2 (C-1′), 176.3 (*C*OMe), β anomer: δ 23.8 (CO*Me*), 56.0 (C-2), 57.3

(C-2′), 61.7 (C-6), 62.3 (C-6′), 71.5 (C-4′), 75.2 (C-3, C-3′), 76.3 (C-5), 77.6 (C-5′), 81.1 (C-4), 97.4 (C-1), 103.2 (C-1′), 176.3 (*C*OMe). Thus, the compound was 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-amino-2-deoxy-D-glucose (GlcNAcGlcN).

4. Discussion

We have investigated the properties of the chitin deacetylase that are potentially applicable to glycotechnology. Previously, we reported a unique property of the enzyme: it deacetylates only the *N*-acetyl group of the GlcNAc residue at the nonreducing end of $(GlcNAc)_2$ to give GlcNGlcNAc. Here we have focussed on the reverse hydrolysis reaction of the enzyme for its application in glycotechnology. Several deacetylases, such as chitin deacetylases from Zygomycetes [18,19] and a peptidoglycan deacetylase from *Bacillus cereus* [20], are known to be strongly inhibited in the presence of acetate. In contrast, chitin deacetylases from Deuteromyces are not inhibited by acetate [16,21], and the enzyme from *Aspergillus nidulans* is activated by the addition of acetate [22]. Thus, it is likely that only the enzymes from Deuteromyces can be used for the reverse hydrolysis reaction, which requires an excess of the acetate reagent.

The new detection method of the reverse reaction shown in Fig. 1 is about ten times as sensitive as the conventional spectrophotometric method used for Fig. 3. The time course of the reaction was observed as a curve in Fig. 2, presumably because the velocity of the hydrolysis reaction by the β-*N*-acetylhexosaminidase depends on the concentration of the substrate, $(GlcNAc)_2$-pNP, produced by the chitin deacetylase, which is zero at the beginning. The K_m value of the β-*N*-acetylhexosaminidase using *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside as the substrate was 0.48 mM [23], which supports the hypothesis. A slight increase in pH during the N-acetylation reaction due to the consumption of acetic acid can also be a reason for the curve, because the chitin deacetylase is activated by the slight increase in the pH around pH 7 [16]. As the concentration of *p*-nitrophenol liberated after 1 h of reaction was estimated to be 7 μM, using the value of 2.0×10^4 M^{-1} cm^{-1} as an extinction coefficient of *p*-nitrophenol at 405 nm, at least an equivalent amount of acetic acid should have been used for the reaction.

Using chitosan oligomers, it was proven that the reverse reaction can be used for the synthesis of N-acetylated compounds. Although a chemical method using acetic anhydride has been commonly used for the synthesis of N-acetylated compounds, this enzymatic method has an advantageous point in the regioselectivity. In this report a selectively acetylated compound, GlcNAcGlcN, could be exclusively synthesized. This method is also expected to be applicable for the N-acetylation of chitosan derivatives with reactive groups other than amino groups of GlcN residues. Mitsutake et al. studied the reverse hydrolysis reaction by a sphingolipid ceramide N-deacylase and its application for the synthesis of isotope-labelled ceramide [24]. The authors also indicated that various free fatty acids can be incorporated into sphingosine. As for the chitin deacetylase, the specificity for the donor fatty acids remains to be investigated.

GlcNAcGlcN was detected in a hydrolysate of partially deacetylated chitosan by a lysozyme, and also used as an acceptor for the transglycosylation reaction of the lysozyme to synthesize novel sugars [25]. Recently, Ohnishi et al. found a unique deacetylase from a chitinase-producing bacterium, *Vibrio alginolyticus*, which exclusively catalyzes the conversion of $(GlcNAc)_2$ into GlcNAcGlcN [26]. They reported that $(GlcNAc)_2$ could be the only substrate for the enzyme; however, the significance of the enzyme and the metabolite is unclear at present. Our method for the synthesis of GlcNAcGlcN will accelerate both the further synthesis of novel sugars using the disaccharide as a modification unit and the elucidation of the role of the compound in nature.

It will also help in the synthesis of partially N-deacetylated chitin oligomers with rigid distribution of N-acetyl groups, and we have established the methods to synthesize GlcN-

GlcNAc and GlcNAcGlcN, the smallest units of partially N-deacetylated chitin oligomers with rigid structures. It will give us a chance to assay their physiological activities, which accelerates their application in a broader range and potentially could lead to the sophisticated utilization of chitinous biomass.

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