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Selective N-deacetylation of *p*-nitrophenyl *N*,*N*′-diacetyl-β-chitobioside and its use to differentiate the action of two types of chitinases

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Abstract

We report the synthesis of a novel compound for chitinase assays, *p*-nitrophenyl 2-acetamido-4-*O*-(2-amino-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside [GlcNGlcNAc-pNP] by selective N-deacetylation of *p*-nitrophenyl 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside [$(GlcNAc)_2$-pNP] using a purified chitin deacetylase isolated from *Colletotrichum lindemuthianum* ATCC 56676. FABMS, ^1H NMR, and ^{13}C NMR analyses confirmed the structure of this new compound. This disaccharide derivative can be used to distinguish special chitinases that effectively remove partially deacetylated parts of substrates within a mixture of chitinases which degrades $(GlcNAc)_2$-pNP.

Keywords: Chitin deacetylase; *Colletotrichum lindemuthianum*; *p*-Nitrophenyl 2-acetamido-4-*O*-(2-amino-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside; β-*N*-Acetylhexosaminidase; Chitinase

1. Introduction

Chitin is among the most abundant biopolymers on earth and is a linear polymer of *N*-acetyl-D-glucosamine residues. It is found on the cell surface of fungal cell walls and is a component of arthropod integuments. Some of the GlcNAc residues in chitin are deacetylated in its natural form [1]. Chitinases are glycosyl hydrolases which hydrolyse the *N*-acetyl-β-D-glucosaminide bonds of chitin and its oligomers, and are responsible for a variety of phenomena such as (1) defense from invasion [2,3], (2) penetration of the cell wall for invasion [4,5], (3) control of cell surface structures [6], and (4) metabolism of chitin for utilization as energy, carbon and nitrogen sources [7]. It is known that chitinases greatly differ in their properties [8], and in order to elucidate their biological functions, research on the properties of chitinases has been performed intensively [8–10].

Chitinase activity has been estimated by methods such as the turbidimetric [11], viscometric [12], and reducing sugar assays [13]. In addition, the colorimetric method using the

Abbreviations: GlcN, 2-amino-2-deoxy-D-glucose; GlcNAc, 2-acetamido-2-deoxy-D-glucose; $(GlcNAc)_2$, *N*,*N*′-diacetylchitobiose; GlcNGlcNAc, $(GlcNAc)_2$ derivative which is N-deacetylated at the sugar residue on the non-reducing end; GlcNAcGlcN, $(GlcNAc)_2$ derivative which is N-deacetylated at the sugar residue at the reducing end; GlcNAc-pNP, *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside.

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chitobioside derivative, $(GlcNAc)_2$-pNP, was found to be useful for the facile and sensitive estimation of chitinase activity, especially for bacterial chitinases [13,14]. Herein, we have modified $(GlcNAc)_2$-pNP into a novel compound, GlcNGlcNAc-pNP, to remove background activity due to contaminating β-*N*-acetylhexosaminidases, which may degrade $(GlcNAc)_2$-pNP stepwise from its non-reducing end, finally liberating the yellow-colored *p*-nitrophenol. Furthermore, we found that apparently identical chitinases from one microorganism can be distinguished according to their specificities with this novel substrate.

2. Experimental

Materials.—$(GlcNAc)_2$-pNP and GlcNAc-pNP were purchased from Seikagaku Kogyo Co., Japan. Chitinase preparation from *Streptomyces griseus* and β-*N*-acetylhexosaminidase from *Penicillium oxalicum* were purchased from Sigma and Seikagaku Kogyo Co., respectively. GlcNAcGlcN was kindly donated by Dr K. Ohishi in the Numazu Industrial Research Institute of Shizuoka Prefecture [15]. All other chemicals were reagent grade.

Deacetylation of $(GlcNAc)_2$-pNP.—Chitin deacetylase (E.C.3.5.1.41) from *Colletotrichum lindemuthianum* (ATCC56676) was purified by the method of Tokuyasu and co-workers [16]. Deacetylation of $(GlcNAc)_2$-pNP was carried out by the method of Tokuyasu and co-workers [17] with slight modification. The reaction mixture (10 mL) contained $(GlcNAc)_2$-pNP as the substrate (0.2%, w/v), 20 mM (final) sodium tetraborate/HCl buffer (pH 8.5), purified chitin deacetylase solution (0.15 U/mL), and 0.15 mL/mL (wet volume, equilibrated with the same buffer) of anion-exchange resin (Q Sepharose Fast Flow, Pharmacia), which was added in order to remove chitin deacetylase from the reaction mixture for reuse. The deacetylation reaction was performed at 30 °C with stirring by a magnetic stirrer, and aliquots were sampled for monitoring by HPLC. Monitoring of the reaction was carried out on an HPLC system (Tosoh Co., Ltd.) equipped with a UV monitor (UV-8020, Tosoh Co., Ltd.), an integrator (PowerChrom 2.0.7, ADInstruments), and a prepacked column of Lichrospher 100 RP-18 (e) (Cica-Merck). The mobile phase was methanol/0.1% trifluoroacetic acid (7:93), the flow rate was 0.5 mL/min, and the operating temperature was 25 °C. Detection of each compound was performed by measuring absorbance at 300 nm. After the reaction was complete, stirring of the reaction mixture was stopped and the supernatant fraction was recovered. A part of the supernatant (5 mL) was then applied to a reverse-phase column (Sep-Pak plus C-18 (1 mL), Waters Corporation) equilibrated with water, and the column was eluted with 3 mL of methanol. The eluted fraction (3 mL) was evaporated, and the white solid residue (6.19 mg) was used in further studies.

Enzyme assays of glycosidases using pNP-derivatives as the substrates.—GlcNAc-pNP, $(GlcNAc)_2$-pNP, or GlcNGlcNAc-pNP was used as the substrate for chitinase and β-*N*-acetylhexosaminidase assays at the final concentration of 2 mM. The reaction mixture (20 μL) composed of the substrate (2 mM), 20 mM NaH_2PO_4–Na_2HPO_4 buffer (pH 6.0), and an aliquot of the enzyme was incubated for 60 min at 30 °C, and then 80 μL of 1 M Na_2CO_3 was added to stop the reaction and to increase the pH. Absorbance of the liberated *p*-nitrophenol was measured at 405 nm using a spectrophotometer (DU-600, Beckman) or a microplate reader (model 3550, Bio Rad). Estimation of the liberated sugar was carried out by HPAEC on a Dionex DX-300 equipped with a pulsed amperometric detector (Dionex Co.). The pre-packed column was a Dionex CarboPac™ PA1 analytical column (4 × 250 mm) accompanied by a CarboPac™ PA1 guard column (4 × 50 mm). The mobile phase was 15 mM NaOH, and the column was washed with 100 mM NaOH and a mixture of 1 M NaOAc and 100 mM NaOH after each analysis. The operating temperature was 25 °C, the flow rate was 1.0 mL/min, and the injection volume was 10 μL.

Separation of the chitinase preparation by gel filtration.—Chitinase preparation from *S. griseus* was loaded on a gel filtration column (Superose HR 12 10/30, Pharmacia Biotech.)

equilibrated with 20 mM NaH_2PO_4–Na_2HPO_4 buffer (pH 6.0) containing 150 mM NaCl, and eluted with the same solution (flow rate: 0.2 mL/min). The amounts of eluted proteins were estimated by measuring the absorbance at 280 nm. Fractions of 0.5 mL were collected and *p*-nitrophenol-liberating activities using GlcNAc-pNP, $(GlcNAc)_2$-pNP, and GlcNGlcNAc-pNP as substrates were measured in aliquots of the collected fractions.

Analytical methods.—The FAB mass spectrum of the reaction product was obtained using a JEOL JMS-SX102A mass spectrometer. 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 inverse 5 mm QXI ($^1H/^{13}C$, ^{15}N, ^{31}P; *xyz*-gradient) and QNP (X (^{13}C, ^{15}N, ^{31}P)/^1H; *z*-gradient) probeheads. DQF-COSY, TOCSY, HMQC and HMBC experiments were carried out to assign ^1H and ^{13}C signals, and all NMR data were reported in ppm (δ) downfield from Me_4Si. The purified sample (2 mg) was dissolved in D_2O (450 μL) for NMR analyses, and 2-methyl-2-propanol was used as the internal standard.

3. Results and discussion

Previously we found that the chitin deacetylase from *C. lindemuthianum* deacetylated only the *N*-acetyl group of the GlcNAc residue at the non-reducing end of $(GlcNAc)_2$ to give GlcNGlcNAc [17]. Here we focussed on using $(GlcNAc)_2$-pNP as the substrate for enzymatic deacetylation, expecting to convert it into a novel compound, GlcNGlcNAc-pNP. Indeed, the chitin deacetylase quantitatively converted the substrate into a new compound giving a single HPLC peak, which differed from the peak of $(GlcNAc)_2$-pNP (data not shown). The reaction product was degraded by a chitinase preparation from *Streptomyces griseus*, and the liberated sugar was estimated using HPAEC (Fig. 1). Under the HPAEC conditions, a single peak having the same retention time as GlcNGlcNAc was detected (Fig. 1(b)). GlcNAcGlcN eluted at around 23 min (data not shown) under the HPAEC conditions, so the possibility that the reaction product was GlcNAcGlcN was eliminated.

The structure of the deacetylation product was determined by FABMS and NMR spectroscopy: FABMS (glycerol) *m/z* 526 $[M+Na]^+$, 504 $[M+H]^+$, 365 $[M-pNP]^+$; ^1H NMR (600.13 MHz, D_2O) δ 1.230 (3 H, s, acetyl), 2.688 (1 H, dd, *J* 9.7, 8.2 Hz, H-2′), 3.382 (1 H, dd, *J* 9.7, 8.5 Hz, H-3′), 3.410 (1 H, dd, *J* 9.4, 8.5 Hz, H-4′), 3.503 (1 H, ddd, *J* 9.4, 5.9, 2.2 Hz, H-5′), 3.741 (1 H, dd, *J* 12.4, 5.9 Hz, H-6′), 3.823-3.879 (3 H, m, H-3, H-4, H-5), 3.918 (1 H, dd, *J* 12.4, 2.2 Hz, H-6), 3.976 (1 H, br d, *J* 12.0 Hz, H-6), 4.045–4.081 (1 H, m, H-2), 4.515 (1 H, d, *J* 8.2 Hz, H-1′), 5.330 (1 H, d, *J* 8.5 Hz, H-1), 7.172–7.199 (2 H, m (A_2X_2 type), H-pNP-3), 8.230–8.257 (2 H, m (A_2X_2 type), H-pNP-2); ^{13}C NMR (150.90 MHz, D2O) δ 23.78 (acetyl methyl), 56.68 (C-2), 58.40 (C-2′), 61.71 (C-6), 62.35

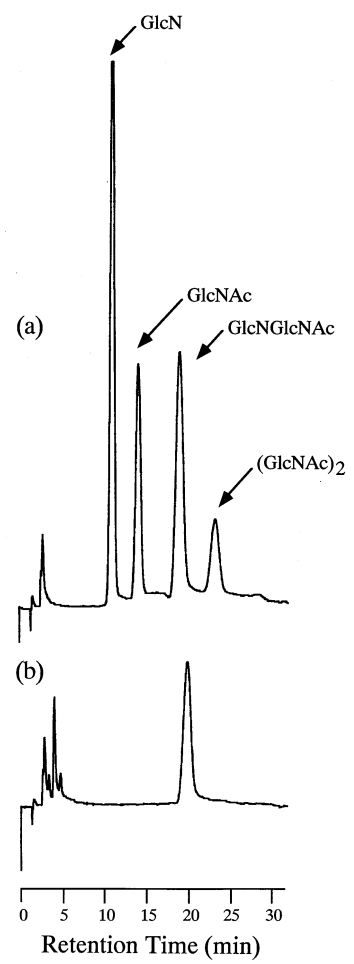

Fig. 1. (a) HPAEC profiles of standard sugars and (b) the compound formed by chitinase treatment of the novel substrate prepared from $(GlcNAc)_2$-pNP.

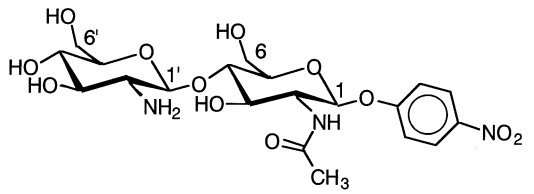

Fig. 2. Structure of the deacetylation product from (GlcNAc)$_2$-pNP.

(C-6′), 71.28 (C-4′), 73.60 (C-3), 76.71 (C-5), 77.29 (C-3′), 77.90 (C-5′), 79.66 (C-4), 100.16 (C-1), 104.44 (C-1′), 118.24 (C-pNP-3), 127.80 (C-pNP-2), 144.42 (C-pNP-4), 163.36 (C-pNP-1), 176.60 (acetyl carbonyl); $^{1}H-^{13}C$ HMBC experiment, H-1/C-5, H-1/C-pNP-1, H-2/C-3, H-6/C-5, H-6/C-4, H-1′/C-4, H-2′/C-1′, H-2′/C-3′, H-pNP-3/C-pNP-1. Thus, the compound was *p*-nitrophenyl 2-acetamido-4-*O*-(2-amino-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (Fig. 2).

The first application of GlcNGlcNAc-pNP was as a novel substrate that could not be hydrolysed by β-*N*-acetylhexosaminidases but could be hydrolysed by chitinases. Drouillard and co-workers [18] reported GlcNGlcNAc acted as an inhibitor of a chitobiase from *Serratia marcescens* (characterized as a β-*N*-acetylhexosaminidase), mainly because it lacked the ability to remove a GlcN residue at the non-reducing end of the substrate, and because the remaining GlcNAc residue bound at the active site of the enzyme. As GlcNGlcNAc-pNP has a deacetylated D-glucosamine residue at its non-reducing end, β-*N*-acetylhexosaminidases are probably unable to degrade it to liberate *p*-nitrophenol. Indeed, we determined that β-*N*-acetylhexosaminidase from *Penicillium oxalicum* did not liberate *p*-nitrophenol from GlcNGlcNAc-pNP. In contrast, when (GlcNAc)$_2$-pNP was used as the substrate with the same enzyme, *p*-nitrophenol was slowly liberated to give a yellow color in the reaction solution. As this exo-type enzyme is known as the one which can degrade (GlcNAc)$_2$ and (GlcNAc)$_3$ [19], it is reasonable to conclude that *p*-nitrophenol was liberated by the sequential removal of the saccharides from the non-reducing end of the substrate.

Substitution of the sugar residue at the non-reducing end of the oligomer can be used to design novel substrates that cannot be degraded by exo-type enzymes. For example, Usui and co-workers [20] enzymatically synthesized a novel compound, *p*-nitrophenyl 4^{5}-*O*-β-D-galactosyl-α-maltopentaoside, as a substrate for human alpha amylases in which a galactose residue was introduced at its non-reducing end to protect the structure from attack by exo-type glucosidases. The same effect can be expected when GlcNGlcNAc-pNP is used as a substrate for β-*N*-acetylhexosaminidases.

Conversely, we expected chitinases which degraded (GlcNAc)$_2$-pNP would degrade GlcNGlcNAc-pNP as well. When the chitinase preparation from *S. griseus* was used, it degraded GlcNGlcNAc-pNP to liberate GlcNGlcNAc and *p*-nitrophenol (data not shown). Ohtakara and co-workers [21] reported the degradation of partially N-acetylated chitosan by the same chitinase preparation after a partial purification, and reported the structures of the reaction products in terms of the distribution of GlcNAc residues and GlcN residues. In their report, it was shown that this enzyme liberated GlcNGlcNAc from polymeric chitinous compounds. In our case, the enzyme activity using GlcNGlcNAc-pNP as the substrate was estimated to be one-eighth the activity using (GlcNAc)$_2$-pNP as the substrate. To elucidate the reasons for the difference in the enzyme activity, further studies were performed using gel filtration chromatography.

Fig. 3 shows the chromatograms after separation of the enzyme preparation from *S. griseus* by gel filtration. According to the profile of the colorimetric assay of (GlcNAc)$_2$-pNP degrading activity (the line with circles), it was found that the chitinase preparation was composed of a mixture of one broad group of chitinases and two smaller groups that eluted later. On the other hand, the profile of the colorimetric assay of GlcNGlcNAc-pNP degrading activity (the line with triangles) was different. In Fractions 12–23, there were only two sharp peaks of chitinases (Fractions 14 and 22) that degraded GlcNGlcNAc-pNP, which contrasted the broad peak of chitinases in the fractions that degraded (GlcNAc)$_2$-pNP. Chitinases that eluted between Fractions 16 and 21 could not degrade

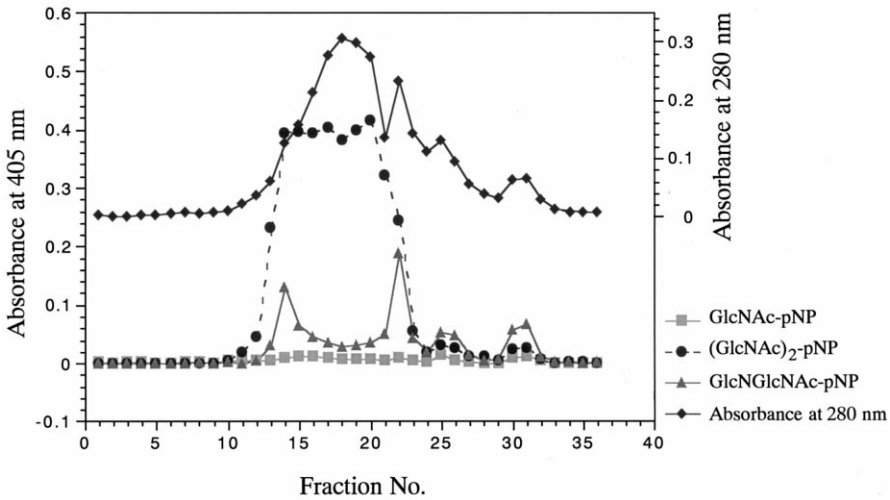

Fig. 3. Superose 12 column chromatography of chitinase preparation from *Streptomyces griseus* (Sigma). *p*-Nitrophenol liberating activity (estimated as the absorbance at 405 nm) of each fraction using GlcNAc-pNP, $(GlcNAc)_2$-pNP, or GlcNGlcNAc-pNP as the substrate was measured as well as the absorbance at 280 nm.

GlcNGlcNAc-pNP in spite of the fact that they effectively degraded $(GlcNAc)_2$-pNP. As for the two small peaks of chitinases (Fractions 25 and 30), the production of *p*-nitrophenol seemed to be more active from GlcNGlcNAc-pNP than from $(GlcNAc)_2$-pNP. The data indicate that the recognition of these substrates differs for the group of chitinases produced by this microorganism.

Although it is reported that some of the GlcNAc residues in chitin are deacetylated in its natural form [1], β-D-glucosaminidases, which remove the GlcN residue at the non-reducing end of the substrate, have been rarely reported [22,23]. It is suggested that another mode of removing N-deacetylated parts of chitin may be working instead of removing GlcN residues by β-D-glucosaminidases. Mitsutomi and co-workers [24] analyzed the reaction products of partially N-acetylated chitosan by two chitinases, chitinase A1 and chitinase D from *Bacillus circulans* WL-12. In their report, they found chitinase A1 degraded the substrate to produce GlcNGlcNAc, which was not detected in the reaction products after degradation by chitinase D. Their results suggested that the two chitinases can be distinguished according to their ability to remove GlcNGlcNAc residues at the non-reducing end of the substrate.

In this report, we provide a novel method to detect the removal of GlcNGlcNAc residues at the non-reducing end of the substrate, and provide proof that chitinases can be classified in two types according to their ability to remove $(GlcNAc)_2$ and GlcNGlcNAc residues at the non-reducing ends of substrates. The chitinases that can effectively degrade GlcNGlcNAc-pNP may be responsible for special functions, such as removing the N-deacetylated GlcN residues in chitin. This would facilitate the complete degradation of chitin by other chitinases produced by the organism.

Thus, the novel substrate, GlcNGlcNAc-pNP, has enabled us to observe a new aspect of chitinase action, and will offer a novel method for the classification of these enzymes.

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