Recognition of Chitooligosaccharides and Their *N*-Acetyl Groups by Putative Subsites of Chitin Deacetylase from a Deuteromycete, *Colletotrichum lindemuthianum*†

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ABSTRACT: The reaction pattern of an extracellular chitin deacetylase from a Deuteromycete, *Colletotrichum lindemuthianum* ATCC 56676, was investigated by use of chitooligosaccharides [$(GlcNAc)_n$, $n = 3-6$] and partially *N*-deacetylated chitooligosaccharides as substrates. When 0.5% of $(GlcNAc)_n$ was deacetylated, the corresponding monodeacetylated products were initially detected without any processivity, suggesting the involvement of a multiple-chain mechanism for the deacetylation reaction. The structural analysis of these first-step products indicated that the chitin deacetylase strongly recognizes a sequence of four *N*-acetyl-D-glucosamine (GlcNAc) residues of the substrate (the subsites for the four GlcNAc residues are defined as −2, −1, 0, and +1, respectively, from the nonreducing end to the reducing end), and the *N*-acetyl group in the GlcNAc residue positioned at subsite 0 is exclusively deacetylated. When substrates of a low concentration (100 μM) were deacetylated, the initial deacetylation rate for $(GlcNAc)_4$ was comparable to that of $(GlcNAc)_5$, while deacetylation of $(GlcNAc)_3$ could not be detected. Reaction rate analyses of partially *N*-deacetylated chitooligosaccharides suggested that subsite −2 strongly recognizes the *N*-acetyl group of the GlcNAc residue of the substrate, while the deacetylation rate was not affected when either subsite −1 or +1 was occupied with a D-glucosamine residue instead of GlcNAc residue. Thus, the reaction pattern of the chitin deacetylase is completely distinct from that of a Zygomycete, *Mucor rouxii*, which produces a chitin deacetylase for accumulation of chitosan in its cell wall.

Amino sugars are widely found in a variety of organisms in all kingdoms as components of both polysaccharides and oligosaccharides. Most of the amino sugar residues exist as *N*-acetylated forms; however, in some cases they are *N*-unsubstituted (*1*−*4*), and some of the *N*-unsubstituted ones are further modified by sulfation (*5*), acylation (*6*), or lactamization (*7, 8*). It has been suggested that the phenomena result in the modification of the functions of the sugar chains such as the acquisition of resistance from stresses (*2, 9*) and the control of their metabolism (*4, 8*). Amino sugar *N*-deacetylases play crucial roles for the modification, and several kinds of them have been investigated intensively (*10*−*18*).

The activities of amino sugar *N*-deacetylases are known to be controlled by various mechanisms, such as the control of the expression of the genes in the course of development (*19*), the activation or repression of the enzyme activities by cofactors (*20*) or reaction products (*12*), and the control of the positions of deacetylation in substrates by the specific recognition by the enzymes (*11, 21, 22*). Analysis of substrate recognition by the enzymes provides significant information on both their possible roles in vivo and the structure−function relationships of the enzymes. However, there is a large difficulty in the structural analysis of a mixture of partially deacetylated products, and so far only a few *N*-deacetylases have been characterized in terms of substrate recognition.

Chitin, a linear polymer of *N*-acetyl-D-glucosamine $(GlcNAc)$[1] residues, is a component of fungal cell walls or arthropod integuments. *N*-Acetyl groups of chitin in the cell walls of some fungi are recognized by themselves as removable parts in the sugar chains, and two types of chitin deacetylases have been exclusively investigated; chitin deacetylases from Zygomycetes and those from Deuteromycetes. Zygomycetes accumulate chitosan, a highly *N*-deacetylated form of chitin, in the cell walls, and Davis and Bartnicki-Garcia (*1*) hypothesized that the biosynthesis of chitosan of a Zygomycete, *Mucor rouxii*, is performed through the tandem action of a chitin synthase and a chitin deacetylase; the chitin deacetylase removes *N*-acetyl groups of a nascent chitin just synthesized by the chitin synthase.

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[1] Abbreviations: GlcN, D-glucosamine; GlcNAc, *N*-acetyl-D-glucosamine; $GlcN_n$, β-(1−4)-linked *n*-mer of D-glucosamine; $(GlcNAc)_n$, β-(1−4)-linked *n*-mer of *N*-acetyl-D-glucosamine; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry. The sequences of the partially deacetylated chitooligosaccharides are abbreviated as such: GlcNGlcNAcGlcNAcGlcNAc, β-(1−4)-linked tetramer of GlcN and three GlcNAc residues, in the example the left GlcN is at the nonreducing end, and the right GlcNAc is at the reducing end.

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Recently, reaction patterns of the chitin deacetylase from *M. rouxii* have been estimated, and it was found that it exhibits a multiple attack reaction mechanism from the nonreducing end of the substrates (*21*, *22*). The mechanism supports a highly systematic, processive deacetylation of the nascent chitin molecule just after synthesis by the chitin synthase.

On the other hand, it has been known that some Deuteromycetes also produce chitin deacetylases. Except for a chitin deacetylase from *Aspergillus nidulans*, which works during its autolysis (*18*), chitin deacetylases of Deuteromycetes have been exclusively found from plant pathogens, and they are secreted during the penetration process into the plant cells (*13*−*17*). The significance of these enzymes is unclear, and both enzymological and physiological approaches have to be carried out in order to elucidate the significance.

We have been studying an extracellular chitin deacetylase from a Deuteromycete, *Colletotrichum lindemuthianum* ATCC 56676, and trying to approach the significance of the *N*-deacetylation of chitin by means of characterization of the enzyme (*17*). We previously found that this enzyme can fully deacetylate chitooligosaccharides whose degrees of polymerizations are greater than 3, while it can deacetylate only at the nonreducing end GlcNAc residue of chitobiose (*17*, *23*). However, these data only on the final reaction products are not sufficient to estimate the reaction patterns of this enzyme.

In this report, we succeeded in estimation of the deacetylation patterns of the chitooligosaccharides with the aid of three methods: exoglycosidase digestion, normal-phase column chromatography, and MALDI-TOF MS analysis. According to the data, we postulated that the enzyme exhibits a multiple chain mechanism. We also introduced the concept of subsites in the enzyme with the estimation of the substrate recognition by the putative subsites in the enzyme.

MATERIALS AND METHODS

Materials. $(GlcNAc)_n$ and $GlcN_n$ were purchased from Seikagaku Kogyo Co., Tokyo, Japan. GlcNGlcNAcGlcNAcGlcNAc, GlcNAcGlcNGlcNAcGlcNAc, and partially *N*-deacetylated chitooligosaccharides which were used for the estimation of the properties of a normal-phase column were prepared from a hydrolysate of partially deacetylated chitin (*24*−*26*). GlcNAcGlcNAcGlcNAcGlcN was synthesized through enzymatic acetylation of $GlcN_4$ (*27*). CM-Sephadex C-25 was obtained from Pharmacia Biotech, Uppsala, Sweden, and the High-Performance Carbohydrate column (HPC column, 4.6 × 250 mm) was from Waters Co., Milford, MA. All other chemicals used were of reagent grade.

Enzymes. Chitin deacetylase (EC 3.5.1.41) from *Colletotrichum lindemuthianum* (ATCC 56676) was purified from a culture filtrate of the fungus and estimated as described (*17*). *β-N*-Acetylhexosaminidase (*β*-GlcNAcase) from *Pycnoporous cinnabarinus* IFO 6139 was purified and estimated as described (*28*). *β*-Hexosaminidase (*β*-GlcNase) from *Penicillium* sp. AF9-P-128 was a kind gift from Professor Y. Uchida of Saga University, Japan, and the enzyme activity was estimated as described (*25*−*28*).

Deacetylation of $(GlcNAc)_4$ for the Analysis of Reaction Pattern. Reaction mixture (6 mL) contained 0.5% $(GlcNAc)_4$, 20 mM sodium tetraborate/HCl buffer (pH 8.5, buffer A) and the purified chitin deacetylase (4.4 units), and was incubated at 40 °C. Samples (1 mL) were taken from the mixture after 10, 20, 120, 240, and 720 min of incubation time, and the deacetylation reaction was stopped by the addition of 0.5 mL of 33% acetic acid followed by boiling for 3 min. The sample was then desalted with an electric dialyzer (Micro Acilyzer G1, Asahikasei Kogyo Co., Ltd., Kanagawa, Japan), and an equal amount of 40 mM sodium acetate buffer (pH 5.0) was added to it. The bufferized sample was run on a cation-exchange column of CM-Sephadex C-25 (26 × 55 mm) equilibrated with 20 mM sodium acetate buffer (pH 5.0, buffer B), with a flow rate of 20 mL h^{-1}. After the column was washed with buffer B (90 mL), partially deacetylated products were eluted with a linear gradient of NaCl (0−2 M, 270 mL) in the same buffer. Eluted sample was fractionated (2 mL each), and the elution of the deacetylation products was detected by the measurement of reducing sugars with GlcN as the standard (*29*). The fractions indicated in Figure 1 were combined, and each combined sample was desalted with Micro Acilyzer S1 and then lyophilized for further structural analysis.

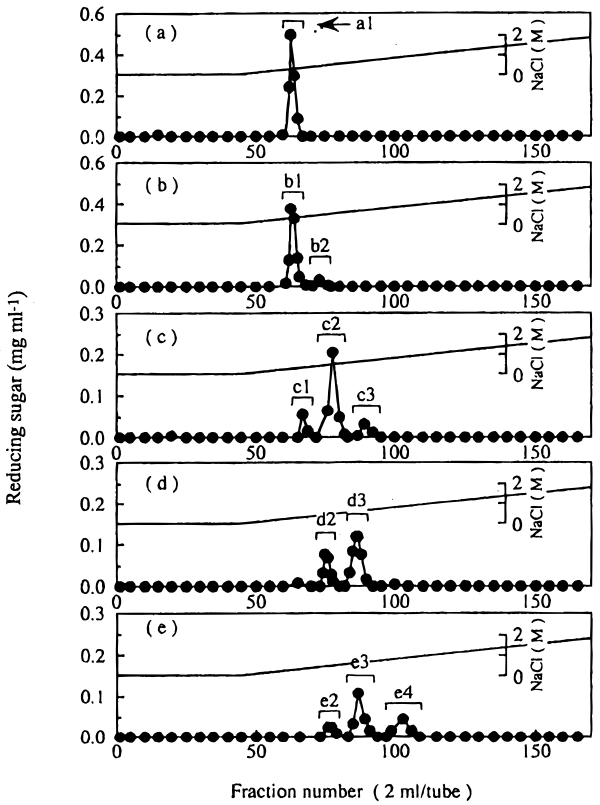

FIGURE 1: Time course of the chromatogram of hydrolysates of $(GlcNAc)_4$ by the chitin deacetylase on CM-Sephadex C-25. The reaction products of $(GlcNAc)_4$ with the chitin deacetylase for 10 min (a), 20 min (b), 120 min (c), 240 min (d), and 720 min (e) were run on a CM-Sephadex C-25 column. Fractions indicated by bars (a1, b1, c1, c2, d2, d3, e3, and e4) were combined. Symbols: (●) reducing sugar; (—) gradient of NaCl.

Separation of Initial Deacetylation Products of $(GlcNAc)_3$, $(GlcNAc)_5$, and $(GlcNAc)_6$. Reaction mixture (1 mL) contained 0.5% $(GlcNAc)_3$, $(GlcNAc)_5$, or $(GlcNAc)_6$ as the substrate, 20 mM (final) buffer A, and the purified chitin deacetylase [3.7 units for $(GlcNAc)_3$ and 0.19 unit for the others]. The reaction mixture was incubated at 40 °C for 20 min for $(GlcNAc)_3$ and for 10 min for the others, and the reaction was stopped by the addition of 0.5 mL of 33% acetic

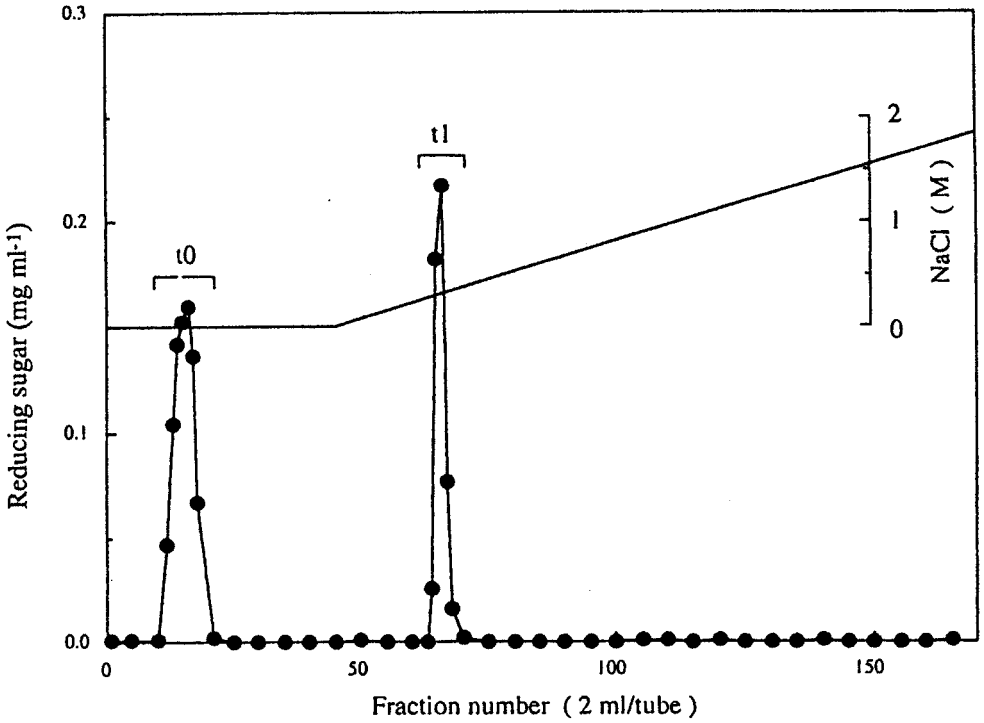

FIGURE 2: Chromatogram of hydrolysates of $(GlcNAc)_3$ by the chitin deacetylase on CM-Sephadex C-25. Fractions indicated by a bar (t1) were combined. Symbols: (●) reducing sugar; (—) gradient of NaCl.

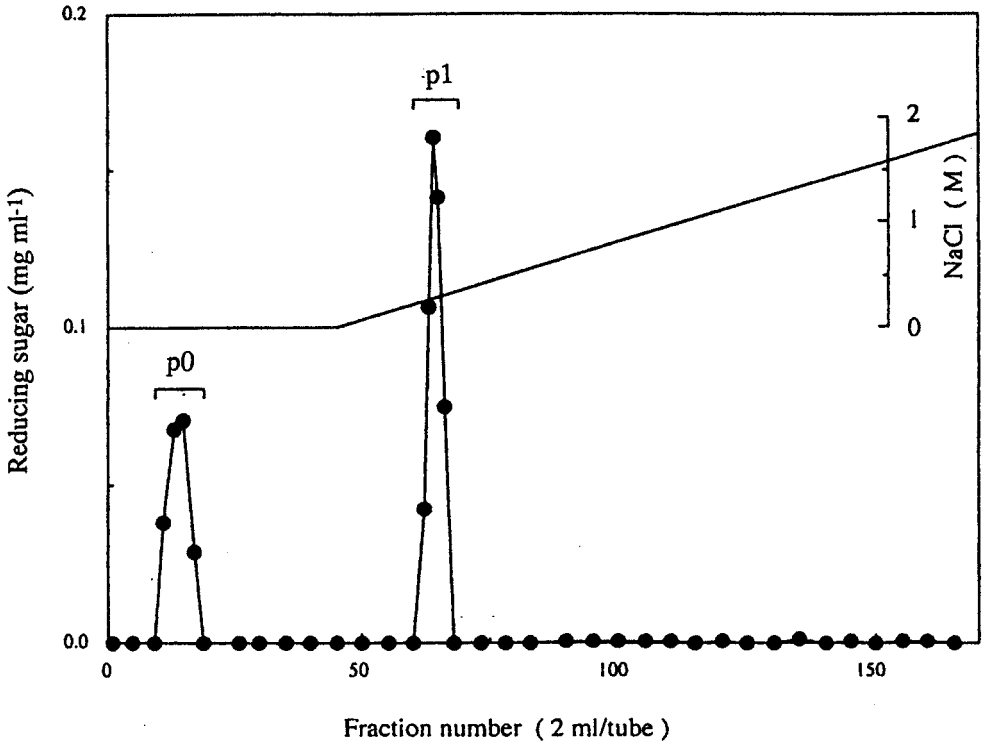

FIGURE 3: Chromatogram of hydrolysates of $(GlcNAc)_5$ by the chitin deacetylase on CM-Sephadex C-25. Fractions indicated by a bar (p1) were combined. Symbols: (●) reducing sugar; (—) gradient of NaCl.

acid followed by boiling for 3 min. The reaction products were desalted, and monodeacetylated compounds were separated by cation-exchange column chromatography on CM-Sephadex C-25 as mentioned above. The fractions indicated in Figures 2−4 (t1, p1, and h1, respectively) were desalted with a Micro Acilyzer S1 and then lyophilized for further structural analysis.

Structural Analysis of Deacetylation Products: (a) Digestion of the Product by Exoglycanases. The lyophilized sample was dissolved (with a final concentration of 0.4%) in 20 mM sodium phosphate buffer (pH 6.5) with β-GlcNAcase (1 unit mL^{-1}, final). The reaction mixture was incubated at 37 °C for 20 h, and the reaction was terminated by boiling for 3 min. To half of the reaction mixture was added β-GlcNase

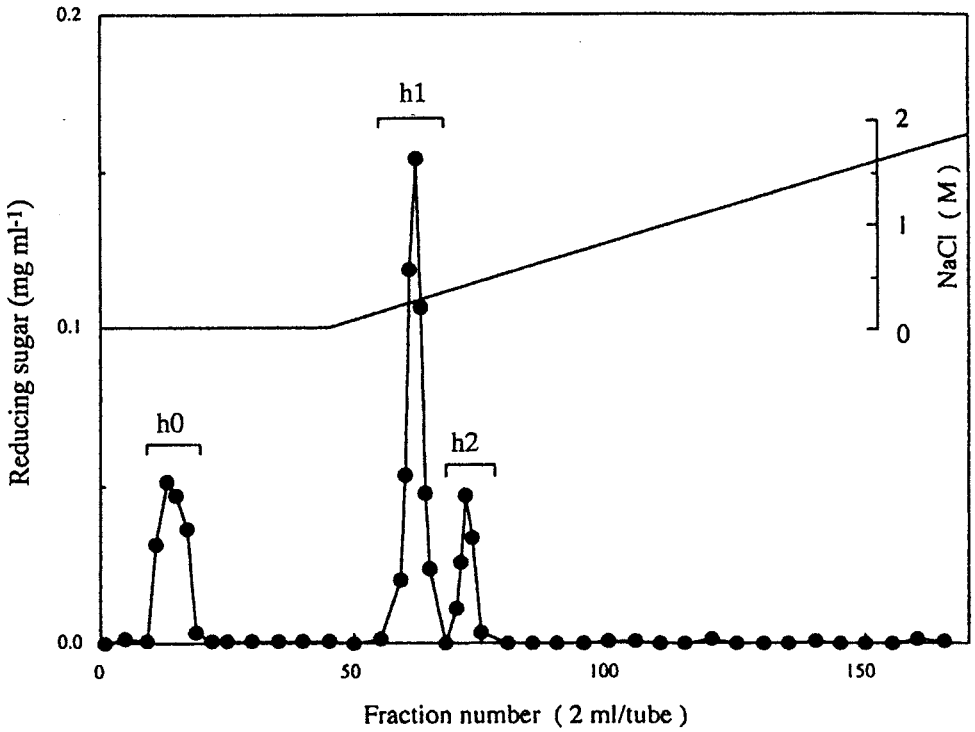

FIGURE 4: Chromatogram of hydrolysates of $(GlcNAc)_6$ by the chitin deacetylase on CM-Sephadex C-25. Fractions indicated by a bar (h1) were combined. Symbols: (●) reducing sugar; (—) gradient of NaCl.

solution (final concentration of the enzyme 0.44 unit mL^{-1}). The β-GlcNase digestion was performed by incubation at 37 °C for 24 h, and the reaction was terminated by boiling for 15 min. The sample digested with β-GlcNAcase or β-GlcNAcase/β-GlcNase was estimated by both normal-phase column chromatography and MALDI-TOF MS analysis as mentioned below.

(b) Normal-Phase Column Chromatography. The sample was run on an HPC column with a flow rate of 1.0 mL min^{-1} and a mobile phase of 70% acetonitrile in water. The elution of the sample was detected by monitoring the refractive index (830-RI, Japan Spectroscopic Co. Ltd., Tokyo, Japan) and/or the absorbance at 210 nm (875-UV, Japan Spectroscopic Co. Ltd.).

(c) MALDI-TOF MS Analysis. The analysis of the oligosaccharides was performed by a Voyager Elite matrix-assisted laser desorption ionization time-of-flight mass spectrometer (PerSeptive Biosystems, Framingham, MA). The acceleration voltage was 20 kV and 2,5-dihydroxybenzoic acid was used as the matrix.

Deacetylation of Substrates at a Low Concentration. The reaction mixture (200 μL) contained 100 μM substrate, the chitin deacetylase (100 microunits), and 20 mM (final) buffer A. The mixture was incubated at 30 °C for 5, 10, 15, 30, 60, 90, and 120 min. The reaction was terminated by the addition of 200 μL of 33% acetic acid, and the velocity of the deacetylation reaction was estimated by the quantification of GlcN residues, as described (*17*). Kinetic parameters were determined with 300 microunits of the chitin deacetylase after the data were fitted by linear regression. The absorption coefficients of the purified enzymes were predicted according to the following equation: A_{280nm} = [5800(no. of tryptophan residues) + 1390(no. of tyrosine residues)]/(molecular mass of the protein).

RESULTS

Structural Analysis of Partially N-Deacetylated Chitooligosaccharides. Deacetylation products from a chitin deacetylase from *C. lindemuthianum* were separated by CM-Sephadex C-25 column chromatography, and fractions indicated in Figures 1−4 were estimated by HPC column separation before and after exoglycosidase digestion of them, as well as by MALDI-TOF MS analysis (Table 1). In our preliminary experiments, we found a unique property of the HPC column in the separation of partially *N*-deacetylated chitooligosaccharides; the retention times of partially deacetylated chitooligosaccharides with GlcN at their reducing ends shift later than those of chitooligosaccharides with corresponding degrees of polymerization, while those of partially deacetylated chitooligosaccharides with GlcNAc at their reducing ends are the same as those of chitooligosaccharides. The property was deduced from the following compounds with the retention times on column chromatography shown in parentheses; $(GlcNAc)_2$ (6.7 min); GlcNGlcNAc (6.7 min); GlcNAcGlcN (8.6 min); $GlcN_2$ (8.5 min); $(GlcNAc)_3$ (9.1 min); GlcNGlcNAcGlcNAc (9.3 min); GlcNGlcNGlcNAc (9.3 min); GlcNAcGlcNAcGlcN (11.7 min); GlcNAcGlcNGlcN (12.3 min); $GlcN_3$ (12.0 min); $(GlcNAc)_4$ (12.7 min); GlcNAcGlcNGlcNAcGlcNAc (13.2 min); GlcNGlcNGlcNAcGlcNAc (13.1 min); GlcNGlcNGlcNGlcNAc (13.4 min); GlcNAcGlcNAcGlcNGlcN (17.1 min); GlcNAcGlcNGlcNGlcN (18.1 min); and $GlcN_4$ (17.9 min). We applied the shift in the retention times for the estimation of the samples shown in Table 1. According to the analysis data, the structures of all the reaction products could be determined.

Deacetylation of Chitooligosaccharides. The chitin deacetylase from *C. lindemuthianum* is known as an enzyme that

Table 1: Structural Analysis of Partially *N*-Deacetylated Chitooligosaccharides

sample[a]	no. of GlcN residues deduced by MALDI-TOF MS analysis	shift of the retention time of the product on HPC column	estimation of the product after exoglycosidase digestion[b] (1) β-GlcNAcase digestion	(2) further β-GlcNase digestion	determined structure (ratio)[c]
a1, b1, c1	1	−	2 mer [1]	GlcN, GlcNAc	GlcNAcGlcNAcGlcNGlcNAc
c2, d2	2	−	3 mer [2]		GlcNAcGlcNGlcNGlcNAc] [60/40 (c2)]
		−	4 mer [2]	3 mer [1]	GlcNGlcNAcGlcNGlcNAc
d3, e3	3	+	3 mer [3]	GlcN	GlcNAcGlcNGlcNGlcN] [20/80 (d3)]
		−			GlcNGlcNGlcNGlcNAc
e4	4				$GlcN_4$
t1	1	+	GlcN, GlcNAc		GlcNAcGlcNAcGlcN]
		−	2 mer [1]		GlcNAcGlcNGlcNAc] (28/50/22)
		−	3 mer [1]		GlcNGlcNAcGlcNAc
p1	1	−	2 mer [1]		GlcNAcGlcNAcGlcNAcGlcNGlcNAc] (42/58)
		−	3 mer [1]	2 mer [0]	GlcNAcGlcNAcGlcNGlcNAcGlcNAc
h1	1	−	2 mer [1]		GlcNAcGlcNAcGlcNAcGlcNAcGlcNGlcNAc]
		−	3 mer [1]	2 mer [0]	GlcNAcGlcNAcGlcNAcGlcNGlcNAcGlcNAc] (28/28/43)
		−	4 mer [1]	3 mer [0]	GlcNAcGlcNAcGlcNGlcNAcGlcNAcGlcNAc

[a] See Figures 1−4. [b] The degree of polymerization of the product was estimated by analysis with an HPC column. The number of GlcN residues deduced by MALDI-TOF MS analysis is indicated in brackets. [c] The ratio of each product was estimated according to the peak area on the chromatographic profile on an HPC column.

can deacetylate $(GlcNAc)_4$ and $(GlcNAc)_5$ much faster than $(GlcNAc)_3$ and $(GlcNAc)_2$ (*17*) and that can fully deacetylate $(GlcNAc)_4$ into $GlcN_4$ (*23*). For the purpose of investigating the deacetylation mode by the enzyme, the order of the deacetylation was determined with $(GlcNAc)_4$ as the substrate. Figure 1 shows the chromatographic profiles of the deacetylation products of $(GlcNAc)_4$ on CM-Sephadex C-25, and combined fractions termed a1, b1, c1, c2, d3, e3, and e4 containing deacetylated products were analyzed (Table 1). After a 10 min reaction, a monodeacetylated product, GlcNAcGlcNAcGlcNGlcNAc, was exclusively produced, which indicates that four GlcNAc residues in $(GlcNAc)_4$ are rigidly recognized in the enzyme. Then we tentatively deduced the subsites for the four GlcNAc residues as −2, −1, 0, and +1, respectively, from the nonreducing end to the reducing end, and the *N*-acetyl group in the GlcNAc residue settled at subsite 0 is exclusively deacetylated from $(GlcNAc)_4$. Figure 1 also implies that no processivity for the deacetylation can be found and that $(GlcNAc)_4$ is a more preferable substrate than GlcNAcGlcNAcGlcNGlcNAc. The time course of the deacetylation reaction indicates that some pathways for the conversion of $(GlcNAc)_4$ into $GlcN_4$ exist (Figure 5).

Initial Deacetylation Products of $(GlcNAc)_3$, $(GlcNAc)_5$, and $(GlcNAc)_6$. Figures 2−4 show the chromatographic profiles on CM-Sephadex C-25 of the initial deacetylation products of $(GlcNAc)_3$, $(GlcNAc)_5$, and $(GlcNAc)_6$, respectively. The initial products from $(GlcNAc)_3$ were a mixture of three isomers (Table 1), suggesting that no rigid recognition of the trimer by the enzyme exists. As for $(GlcNAc)_5$, the initial products were as predicted according to the data of $(GlcNAc)_4$; two products can be produced by the recognition of the substrate by the four deduced subsites of the enzyme as underlined, respectively; GlcNAcGlcNAcGlcNAcGlcNAcGlcNAc and GlcNAcGlcNAcGlcNAcGlcNAcGlcNAc. No processivity was found in the course of the deacetylation of $(GlcNAc)_5$. The difference in the ratio of the two products was of little significance (42:58, Table 1), which supports the hypothesis that the number of subsites for recognition of the substrates is four. The initial products of $(GlcNAc)_6$ were also as predicted, which also supports the hypothesis. No processivity was found, although some of the deduced dideacetylated products were produced. Thus it is strongly suggested that the chitin deacetylase possesses four subsites as deduced above and exhibits a multiple-chain mechanism for the deacetylation of the substrates.

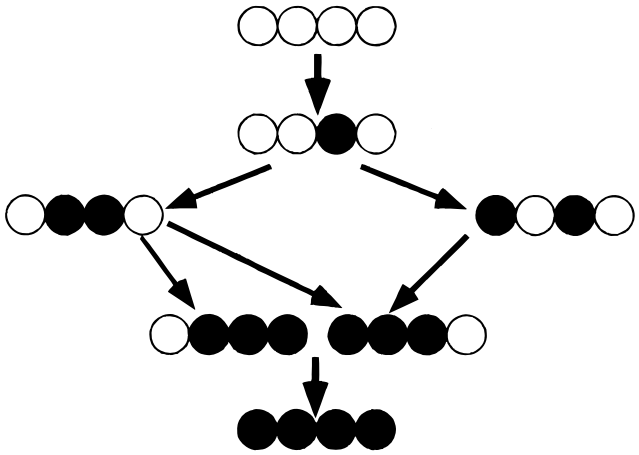

FIGURE 5: Pathways for the conversion of $(GlcNAc)_4$ into $GlcN_4$ by the chitin deacetylase. Symbols: (○) GlcNAc residue; (●) GlcN residue.

Deacetylation of Substrates at a Low Concentration. The deacetylation rates of the substrates at 100 μM [about twice the concentration of K_m values for $(GlcNAc)_4$ and $(GlcNAc)_5$] were analyzed (Figure 6), and the initial deacetylation rates for $(GlcNAc)_4$ and $(GlcNAc)_5$ were comparable. Under these conditions, $(GlcNAc)_3$ was not significantly deacetylated, indicating that no detectable deacetylation can be observed when less than three subsites are occupied by the substrates. We found that the positioning of a GlcN residue instead of a GlcNAc residue at subsite −1 or +1 does not significantly affect the deacetylation rate nor the kinetic parameters (Table 2). On the other hand, the positioning of a GlcN residue at subsite −2 significantly decreases the deacetylation rate, as well as the k_{cat}/K_m value.

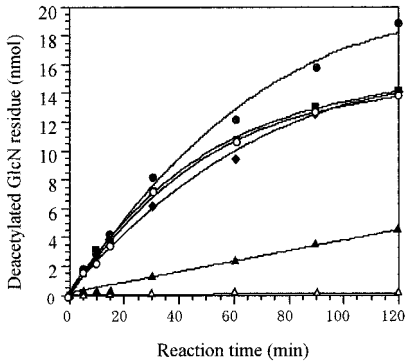

FIGURE 6: Time course of deacetylation of $(GlcNAc)_n$ and monodeacetylated $(GlcNAc)_4$ derivatives by the chitin deacetylase. The initial concentration of each substrate is 100 μM. Symbols for substrates: (●) $(GlcNAc)_5$; (■) $(GlcNAc)_4$; (▲) GlcNGlcNAcGlcNAcGlcNAc; (◆) GlcNAcGlcNGlcNAcGlcNAc; (○) GlcNAcGlcNAcGlcNAcGlcN; (△) $(GlcNAc)_3$.

Table 2: Kinetic Parameters of the Chitin Deacetylase for Oligomeric Substrates

substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)
$(GlcNAc)_5$	69.9	53.1	0.760
$(GlcNAc)_4$	81.6	53.1	0.651
GlcNGlcNAcGlcNAcGlcNAc	307	22.7	0.0739
GlcNAcGlcNGlcNAcGlcNAc	81.4	57.5	0.706
GlcNAcGlcNAcGlcNAcGlcN	87.0	58.7	0.675

DISCUSSION

Judging from the structures of the initial deacetylation products of $(GlcNAc)_n$, we estimated the mode of action of the chitin deacetylase from *C. lindemuthianum* as a multiple-chain mechanism, contrasting with the results of the chitin deacetylase from *M. rouxii*, which deacetylates substrates with the mode of a multiple attack mechanism from the nonreducing ends (*22*). The difference in the reaction modes between these two deacetylases can be also observed in the deacetylation products of $(GlcNAc)_4$ by each enzyme; the former gives six major products derived from one initial deacetylation product, GlcNAcGlcNAcGlcNGlcNAc (Figure 5), with some pathways toward $GlcN_4$, while the latter gives only four products, all of which are processively deacetylated from the nonreducing end (*22*). Although the amino acid sequences of both enzymes have already been determined with some homologous parts between each other (*30*), the three-dimensional structures of both enzymes have not yet been elucidated, which can be clues for analysis of the difference in the reaction modes. Recently we established a novel method to obtain a highly active recombinant chitin deacetylase from *C. lindemuthianum* in the culture medium of *Escherichia coli* cells, which will accelerate the analysis of the structure as well as the mechanism of catalysis (*31*).

In this report we have deduced four subsites, -2 to $+1$, involved in the recognition of substrates by the chitin deacetylase from *C. lindemuthianum*, and no evidence has been found that more than four subsites exist in the enzyme according to the analysis data of the deacetylation products of $(GlcNAc)_5$ and $(GlcNAc)_6$. The subsite concept in glycosylhydrolases was originally proposed for putative sugar-binding sites in the catalytic domains, and it has promoted a better understanding of the reaction mechanisms (*32*−*34*). Davies et al. (*35*) proposed a unified nomenclature for sugar-binding subsites in glycosylhydrolases; the subsites are labeled from $-n$ (at the nonreducing end) to $+n$ (at the reducing end), and cleavage occurs between the -1 and $+1$ sites (*35*). In this report we have modified this nomenclature for its application to the chitin deacetylase by adding an additional subsite, 0. The *N*-acetyl group of the sugar residue at subsite 0 is removed by the enzyme. A small difference can be seen in the ratio of the initial deacetylation products with $(GlcNAc)_5$ and $(GlcNAc)_6$ as substrates (Table 1). There is a small preference for cleavage of the third *N*-acetyl group from the nonreducing end over any other *N*-acetyl group. One possibility for the preference is the direction of access of the substrates; whether the substrates are apt to access the enzyme from their nonreducing ends or their reducing ends may affect the ratio. According to the data of the initial deacetylation products of $(GlcNAc)_3$, it is suggested that the binding of the substrate at two subsites (0, $+1$) or three [(-2, -1, 0) and (-1, 0, $+1$)] is enough for the enzyme to work for catalysis. Previously we have found that the enzyme cannot deacetylate GlcNAc and exclusively produces GlcNGlcNAc from $(GlcNAc)_2$ (*23*), indicating that neither binding at only one subsite at position 0 nor at the two subsites (-1, 0) is enough for the catalysis, while binding at the two subsites (0, $+1$) is enough. The deduction of the subsites suggests that the settling of the parts of substrates at either subsite -2 or $+1$ is essential for the deacetylation reaction.

At a low concentration (100 μM), GlcNGlcNAcGlcNAcGlcNAc could be relatively slowly deacetylated compared to other monodeacetylated $(GlcNAc)_4$ derivatives, which have similar rates to $(GlcNAc)_4$ as the substrate. According to the data, we estimated that only subsite -2 strongly recognizes an *N*-acetyl group in a D-glucosamine residue. This is the first report referring to the preference of amino sugar residues at each subsite in the enzyme. In this study, we elucidated two crucial properties of the chitin deacetylase; the reaction mode of the multiple chain mechanism and the preference of the GlcNAc residue at subsite -2, which significantly affects the distribution of the deacetylated parts in the deacetylation products of chitin. It is postulated that the position of the first attack of the enzyme on the polysaccharide chain restricts the position of the second attack (because a GlcNAc residue at the second neighboring site in the direction of the reducing end side from the first deacetylated GlcN residue is less preferable for the further deacetylation by the enzyme), which could promote the production of a deacetylation product with a well-dispersed distribution of free amino groups.

From the viewpoint of the plant pathology, chitin deacetylases from Deuteromycetes are known to be secreted during the infection process. It was also reported that less chitin in the cell walls of *C. lindemuthianum* during the initial infection process could be detected by use of a lectin wheat germ agglutinin as a cytochemical probe (*36*), implying that chitin deacetylases may be involved in the deacetylation of chitin during the infection. The difference in mechanism of chitin deacetylases from *C. lindemuthianum* and *M. rouxii* may be related to the former being a plant pathogen and the latter a saprobe with chitosan as major wall component. The role of the chitin deacetylase in the fungus/plant interaction remains to be elucidated.

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