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Carbohydrate Research 303 (1997) 353–358

CARBOHYDRATE RESEARCH

Note

# Deacetylation of chitin oligosaccharides of dp 2–4 by chitin deacetylase from *Colletotrichum lindemuthianum*

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Received 18 February 1997; accepted in revised form 2 June 1997

**Abstract**

Chitin oligosaccharides of degree of polymerization 2–4 were deacetylated by purified chitin deacetylase isolated from *Colletotrichum lindemuthianum* to give their corresponding breakdown products after purification by liquid chromatography. Data from FABMS analyses suggested that *N*,*N*′,*N*″,*N*‴-tetraacetylchitotetraose and *N*,*N*′,*N*″-triacetylchitotriose were converted into fully-deacetylated corresponding chitosan oligomers. Conversely, *N*,*N*′-diacetylchitobiose [$(GlcNAc)_2$] was deacetylated to give a product which showed an $[M + H]^+$ pseudomolecular ion at *m/z* 383, suggesting that either of the two acetyl groups were removed. Further data from $^1H$ NMR analyses confirmed that the reaction product was 2-acetamido-4-*O*-(2-amino-2-deoxy-β-D-glucopyranosyl)-2-deoxy-D-glucose [GlcN-GlcNAc]. The enzymatic method has three advantageous characteristics over chemical methods: (i) it does not cause unexpected degradation of the sugar chain, (ii) it is highly reproducible, and (iii) unique compounds such as GlcN-GlcNAc may be produced. © 1997 Elsevier Science Ltd.

*Keywords:* Chitin deacetylase; *Colletotrichum lindemuthianum*; Chitin oligomers

## 1. Introduction

Amino sugars are found in a variety of living organisms in all kingdoms. They are widely distributed not only in biopolymers (e.g., peptideglycan, chitin, chitosan and proteoglycan) but also in relatively low molecular weight sugar chains (e.g., glycoproteins, glycolipids, and free biosignals from leguminous bacteria) [1]. In general, amino sugars are incorporated into sugar chains in the *N*-acetylated form, but there are cases where they exist in the *N*-deacetylated form [2–4] and become further modified to the *N*-sulfated [5] or *N*-acylated [1] form. In these cases, *N*-deacetylation is a common step in the

Abbreviations: GlcN, 2-amino-2-deoxy-D-glucose; GlcNAc, 2-acetamido-2-deoxy-D-glucose; $(GlcN)_n$, β-(1 → 4)-linked *n*-mer of GlcN; $(GlcNAc)_n$, β-(1 → 4)-linked *n*-mer of GlcNAc; dp, degree of polymerization

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*PII* S0008-6215(97)00166-3

modification of sugar chains. Modified chains may acquire resistance to lysozyme [3], a regulatory role in cell growth and differentiation [4], or the physiological activity of heparan sulfate [5] or nodulation factors [1].

Deacetylation at *N*-acetyl amino sugar residues has been an important process in the fields of glycoscience and glycotechnology. The aims of *N*-deacetylation in these fields are as follows: (i) to determine the structure of the sugar chains following nitrous acid deamination [6], (ii) to investigate the change in biophysical activity using *N*-unsubstituted sugars [5,7], (iii) to synthesize substrates for the deacetylases using isotopically-labelled acetic anhydride [8,9] and (iv) to modify at *N*-amino groups of the amino sugars to synthesize artificial compounds [7]. Except for the mild deacetylation method of heparan sulfate using hydrazine and hydrazine sulfate [10], the chemical deacetylation method is not well established and causes unexpected side-reactions which reduce recovery. The establishment of methods for the deacetylation of *N*-acetyl amino sugars is, therefore, very important.

There are some reports on the characterization of purified chitin deacetylases [11–15], but these do not refer to the reaction products of chitin oligomers from the viewpoint of glycotechnology. Furthermore, chitin oligomers of low dp could not be deacetylated by some of the chitin deacetylases reported [11,12,15]. In this report, we have estimated the products of enzymatic deacetylation of chitin oligomers of degree of polymerization 2–4 by FABMS analyses, and for the dimeric compound, further characterization by $^1$H NMR is reported.

## 2. Experimental

*Materials.*—Chitin oligomers were purchased from Seikagaku Kogyo Co., Japan, and chitosan oligomers were kindly provided by Prof. Usui of Shizuoka University, Japan. All other chemicals were of reagent grade.

*Purification of chitin deacetylase from Colletotrichum lindemuthianum.*—A Deuteromycete, *Colletotrichum lindemuthianum* (ATCC56676), was obtained from the American Type Culture Collection. Chitin deacetylase (E.C.3.5.1.41) secreted in the culture media was purified by the method of Tokuyasu et al. [15], and enzyme activity was measured according to the spectrophotometric method of Dische and Borenfreund [16]. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmole of GlcN residue (with free amino group) per minute when incubated with glycol chitin as the substrate.

*Deacetylation of chitin oligomers.*—The reaction mixture (400 μL) was composed of each chitin oligomer as the substrate (0.2%), 10 mM sodium tetraborate/HCl (pH 8.5), and purified enzyme solution [0.1 U for $(GlcNAc)_4$, 0.4 U for $(GlcNAc)_3$ and $(GlcNAc)_2$]. The deacetylation reaction was performed at 45 °C and monitored by HPLC.

*Monitoring of the deacetylation reaction by HPLC.*—Monitoring of the reaction was carried out by HPLC on a Dionex DX-300 chromatograph equipped with a pulsed amperometric detector (Dionex Co.). A prepacked column of Dionex CarboPac™ PA1 (4 × 250 mm) and a CarboPac™ PA1 guard column (4 × 50 mm) were used. The mobile phase was 30 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 $min^{-1}$, and the injection volume was 10 μL.

*Purification of reaction products for FABMS analyses.*—The enzyme was separated from the reaction mixture by centrifugation through Ultrafree C3-TGC filter (Millipore Co.). The resulting solution was dialyzed through an electric dialyzer (AC-120-02 cartridge on Micro Acylizer G0, Asahikasei Kogyo Co. Ltd.), concentrated under reduced pressure, and used for FABMS analysis.

*Analytical methods of FABMS.*—The FAB mass spectrum of each reaction product was obtained using a JEOL JMS-SX102A mass spectrometer. The positive ion mode was used for analyses, and glycerol was used as the matrix.

*Estimation of heterogenic dimeric sugar by $^1$H NMR analyses.*—The reaction mixture (1.2 mL) using $(GlcNAc)_2$ as the substrate was applied to a column containing Amberlite CG-120 cation exchange resin (2 mL; Orugano Co., Ltd.), and the column was washed with 6 mL of water and eluted with 8 mL of 0.5 N HCl. The free amino group from the reaction was detected by the method of Dische and Borenfreund [16]. Fractions (2 mL) were collected, and those containing the product (No. 4–7) were pooled, evaporated under reduced pressure, and dissolved in $D_2O$ (600 μL) for $^1$H NMR analyses. $^1$H NMR spectra were recorded on a Bruker DRX-600 spectrometer at 303 K, and all NMR data are reported in ppm ($\delta$) downfield from sodium 3-(trimethylsilyl)-1-propanesulfonate (internal standard).

## 3. Results and discussion

The time course of HPLC profiles during the enzymatic deacetylation of $(GlcNAc)_4$ is shown in Fig. 1. A broad peak for $(GlcNAc)_4$ at a retention time of approximately 17 min decreased gradually and disappeared after 240 min of reaction, and three new peaks appeared with retention times of approximately 13 min, 14 min and 17 min. The peak leaving the 14 min retention time appeared after 30 min and diminished after 120 min of reaction. The peak having the 13 min retention time increased linearly and formed a single peak after 240 min of reaction. This peak showed a very similar retention time to that of chitotetraose. Since we could not obtain a series of authentic, partially *N*-deacetylated chitin oligomers, it was unclear whether each peak was composed of a single compound. The shape of the peak at the 13 min retention time did not change after prolonged incubation, therefore, the whole solution was used for FAB-mass spectrometer analyses, following the removal of the enzyme and salts. John et al. [17] reported that recombinant chitooligosaccharide deacetylase from *Rhizobium meliloti* deacetylated only equimolar amounts of *N*-acetyl groups at the non-reducing end of the substrates. It remains to be investigated whether chitin deacetylase from *C. lindemuthianum* acts in an ordered fashion or randomly deacetylates the substrate.

The HPLC profile of the deacetylation process when $(GlcNAc)_3$ was used as the substrate was similar to Fig. 1, finally giving a single peak upon prolonged reaction. As for $(GlcNAc)_2$, no intermediate peak was detected by HPLC, however, a new peak increased in accordance with the decrease of the $(GlcNAc)_2$ peak.

These end products were purified, concentrated, and analysed on the FAB-mass spectrometer. The products derived from $(GlcNAc)_4$ and $(GlcNAc)_3$ showed $[M + H]^+$ pseudomolecular ions at *m/z* 663 and 502, respectively (Fig. 2a, b), indicating that all of the acetyl groups in the substrate molecule had been removed to produce the corresponding deacetylated chitosan oligomer. The product derived from $(GlcNAc)_2$ gave an $[M + H]^+$ pseudomolecular ion at *m/z* 383, suggesting that one of the two acetyl groups in the substrate molecule had been removed (Fig. 3). To investigate which of the two acetyl groups was removed, the main product was purified by chromatography on Amberlite CG-120, evaporated, and dissolved in $D_2O$ for $^1H$ NMR analyses. The purity of the sample was checked by Dionex

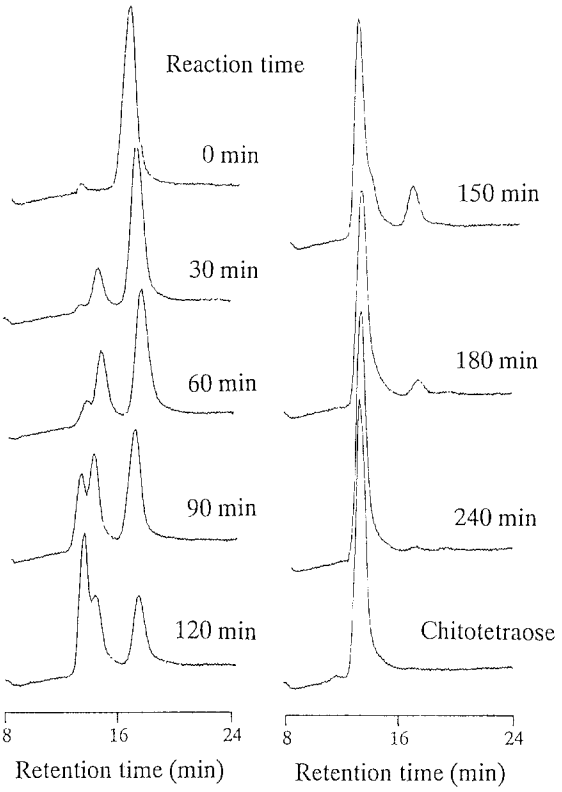


Fig. 1. Time course of the HPLC profiles obtained during the enzymic deacetylation of $(GlcNAc)_4$.

HPLC and compared with the peak observed from the reaction mixture.

The product afforded an equilibrium mixture of $\alpha$- and $\beta$-anomers at the reducing end in $D_2O$, which were separately observed in the $^1H$ NMR spectrum. Although some $^1H$ signals overlapped each other and the HDO signal interfered with assignment, a gradient double quantum filtered correlation spectrum (DQF-COSY) permitted the assignment of all signals. In addition to GlcN-GlcNAc, we assigned the $^1H$ NMR signals of $(GlcNAc)_2$ for comparison, and these are summarized in Table 1. Acetyl signals of GlcN-GlcNAc were found as two singlets ($\delta$ 2.053, $\delta$ 2.055 ppm) corresponding to two anomers, so that the compound contains a single acetyl group. This result was consistent with the FABMS data mentioned above. Comparing the H-2′ signal of GlcN-GlcNAc and $(GlcNAc)_2$, the former ($\alpha$: 3.161, $\beta$: 3.153) showed an upfield shift of 0.6 ppm relative to the latter ($\alpha$: 3.764, $\beta$: 3.754). These chemical shifts corresponded to $-CHNH_2$ and $-CHNHCOCH_3$ substitution patterns, respectively, so that the upfield shift was caused by the removal of the acetyl group. Hence, the results showed that the enzyme preferentially removed an acetyl group from the 2′-position of

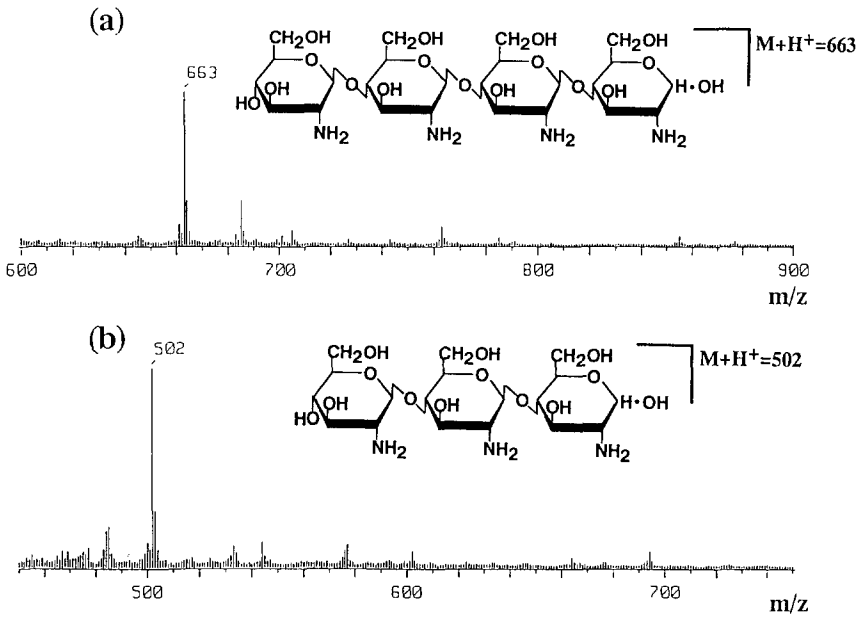


Fig. 2. FAB mass spectra of the reaction product after treatment of $(GlcNAc)_4$ (a) or $(GlcNAc)_3$ (b) with chitin deacetylase.

$(GlcNAc)_2$ to give GlcN-GlcNAc. The ratio of two anomers ($\alpha/\beta = 6/4$) and the purity of the sample (more than 97%) were also determined by NMR signal integration.

This is the first report on the reaction products from low molecular chitin oligomers by chitin deacetylase and provides basic information for research fields such as mycology, plant pathology, enzymology, and glycotechnology.

The significance of chitin deacetylase secretion during the infection of cucumbers by the pathogen, *Colletotrichum lagenarium*, was discussed in terms of the defense system against plant chitinases and the degradation system of elicitor-active chitin oligomers into the deacetylated form [18]. *C. lindemuthianum* is a pathogen of beans and also secrets chitin deacetylase [19]. During interaction with the host plant, it is possible that chitin oligomers are produced by the action of plant chitinases on the cell wall of the fungus. The enzymatic deacetylation of these oligomers may also occur *in vivo* as investigated in vitro. The influence of deacetylated oligomers on the interaction between plants and this plant pathogen remains to be investigated.

Chitin deacetylases have been purified and characterized from some Zygomycetes [11,12] and Deuteromycetes [13–15,18], and discussed from the viewpoints of cell wall chitosan formation [11,12], autolysis [13] and interaction with the host plant [14]. Most chitin deacetylases reported can deacetylate $(GlcNAc)_5$, and one report suggests that $(GlcNAc)_5$ can be thoroughly deacetylated into $(GlcN)_5$, as de-

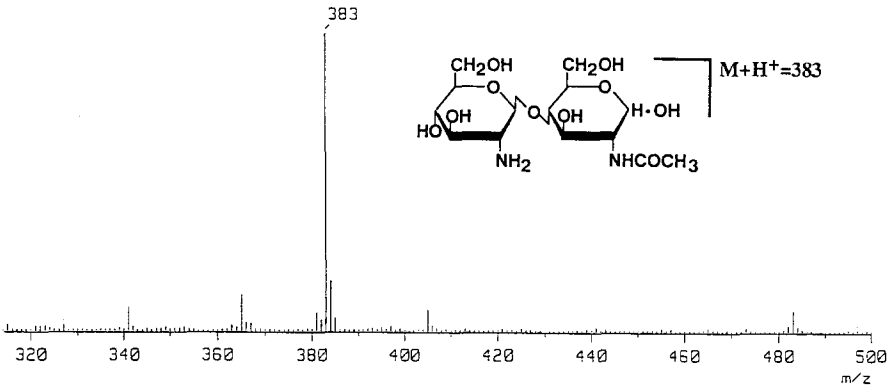


Fig. 3. FAB mass spectrum of the reaction product after treatment of $(GlcNAc)_2$ with chitin deacetylase.

termined by FABMS analyses [15]. The substrate specificity on chitin oligomers of dp 2–4 differs between species. Chitin deacetylase from (i) *Mucor rouxii* [11] and *C. lindemuthianum* (obtained from Deutsche Sammlung von Mikroorganismen, no. 63144) [14] cannot degrade oligomers whose dp were less than three, (ii) *Absidia coerulea* [12] cannot degrade $(GlcNAc)_2$ and (iii) *Aspergillus nidulans* [13] and *C. lindemuthianum* ATCC 56676 [15] can deacetylate $(GlcNAc)_2$ and $(GlcNA)_3$. Tsigos and Bouriotis reported on the purification and characterization of chitin deacetylase from *C. lindemuthianum* (DSM No. 63144) [14], but some of the reported properties including substrate specificity are different from those reported by Tokuyasu et al. [15]. There is also a large difference in the molecular weight of the two deacetylases; the former, a highly glycosylated protein, has a molecular mass of approximately 150 kDa, compared to approximately 31.5 kDa for the latter. Whether the functions of these two enzymes are the same or different remains to be investigated.

From the enzymological point of view, it is of great interest that $(GlcNAc)_2$ was converted not into $(GlcN)_2$ but into the hetero-disaccharide, GlcN-GlcNAc, while $(GlcNAc)_4$ and $(GlcNAc)_3$ were converted into the thoroughly-deacetylated products $(GlcN)_4$ and $(GlcN)_3$, respectively. The mechanism of substrate recognition by this enzyme should be

Table 1
$^1$H NMR [a] data for GlcN-GlcNAc · HCl and $(GlcNAc)_2$

| Proton | GlcN-GlcNAc · HCl | | | $(GlcNAc)_2$ | | |
|---|---|---|---|---|---|---|
| | δ (ppm) | Multiplicity [b] | J (Hz) | δ (ppm) | Multiplicity [b] | J (Hz) |
| (α-amoner) | | | | | | |
| H-1 | 5.210 | d | 2.2 | 5.199 | d | 2.7 |
| H-2 | 3.933 | m | | 3.89 | m | |
| H-3 | 3.933 | m | | 3.90 | m | |
| H-4 | 3.886 | m | | 3.635 | dd | 10.9, 7.3 |
| H-5 | 4.027 | ddd | 9.7, 4.1, 2.5 | 3.892 | ddd | 10.9, 4.7, 2.2 |
| H-6 | 3.845 | dd | 12.5, 2.5 | 3.797 | dd | 12.1, 2.2 |
| | 3.794 | dd | 12.5, 4.1 | 3.679 | dd | 12.1, 4.7 |
| H-1′ | 4.894 | d | 8.3 | 4.604 | d | 8.5 |
| H-2′ | 3.161 | dd | 10.7, 8.3 | 3.764 | dd | 10.4, 8.5 |
| H-3′ | 3.712 | dd | 10.7, 8.5 | 3.583 | dd | 10.4, 8.5 |
| H-4′ | 3.519 | dd | 10.1, 8.5 | 3.482 | dd | 9.8, 8.5 |
| H-5′ | 3.561 | ddd | 10.1, 5.1, 2.4 | 3.517 | ddd | 9.8, 5.5, 2.1 |
| H-6′ | 3.938 | dd | 12.3, 2.4 | 3.928 | dd | 12.2, 2.1 |
| | 3.782 | dd | 12.3, 5.1 | 3.758 | dd | 12.2, 5.5 |
| Ac | 2.055 | s | | 2.076 | s | |
| | | | | 2.046 | s | |
| (β-anomer) | | | | | | |
| H-1 | 4.743 | d | 8.3 | 4.706 | d | 8.5 |
| H-2 | 3.716 | dd | 10.2, 8.3 | 3.70 | m | |
| H-3 | 3.762 | dd | 10.2, 8.7 | 3.69 | m | |
| H-4 | 3.876 | dd | 8.7, 10.0 | 3.623 | dd | 10.2, 8.3 |
| H-5 | 3.654 | ddd | 10.0, 4.5, 2.5 | 3.524 | ddd | 10.2, 5.5, 2.1 |
| H-6 | 3.899 | dd | 12.6, 2.5 | 3.839 | dd | 12.1, 2.1 |
| | 3.777 | dd | 12.6, 4.5 | 3.657 | dd | 12.1, 5.5 |
| H-1′ | 4.894 | d | 8.3 | 4.595 | d | 8.5 |
| H-2′ | 3.153 | dd | 10.7, 8.3 | 3.754 | dd | 10.4, 8.5 |
| H-3′ | 3.707 | dd | 10.7, 8.5 | 3.575 | dd | 10.4, 8.5 |
| H-4′ | 3.512 | dd | 10.1, 8.5 | 3.474 | dd | 9.7, 8.5 |
| H-5′ | 3.553 | ddd | 10.1, 5.1, 2.4 | 3.509 | ddd | 9.7, 5.5, 2.1 |
| H-6′ | 3.938 | dd | 12.3, 2.4 | 3.925 | dd | 12.2, 2.1 |
| | 3.778 | dd | 12.3, 5.1 | 3.756 | dd | 12.2, 5.5 |
| Ac | 2.053 | s | | 2.074 | s | |
| | | | | 2.044 | s | |

[a] (600.13 MHz, $D_2O$, 303 K).
[b] d, doublet; m, multiplet.

investigated not only to understand its significance but also to aid in the modification of substrate specificity, efficiency or stability of the enzyme.

As mentioned in the introduction, the deacetylation of the *N*-acetyl groups in sugar chains is very important to the field of glycotechnology. Nelson et al [7]. reported that the synthetic sialyl Lewis a ($sLe^a$) analog in which the GlcNAc residue was converted into GlcN showed a dramatic change in physiological activity. Chemical deacetylation methods, however, are not well established because conditions used for the chemical deacetylation cause unexpected side reactions such as the degradation of sugar chains or hydrazidation at carboxyl groups in sugar chains [20]. In this report, we offer an alternative method to deacetylate sugar chains. The method potentially possesses three advantages: (i) it does not cause unexpected degradation of sugar chain structures, (ii) it is highly reproducible, and (iii) unique compounds such as GlcN-GlcNAc may be produced. This enzymatic method, however, has a disadvantage in its limited substrate specificity. The data about the reaction products from deacetylation reported herein are of great importance for application in glycotechnology and for some special demands; e.g., the hetero-disaccharide, GlcN-GlcNAc, obtained by chitin deacetylase from *C. lindemuthiaum* would be a valuable material of organic syntheses or as the substrate of glycosidases.

## Acknowledgements

This work was partly supported by a grant (Leading Research Utilizing Potential of Regional Science and Technology) from the Science and Technology Agency of the Japanese Government. We are thankful to Ms. C. Aoyagi, Ms. K. Kano and Ms. M. Arakida for excellent technical assistance during this work. Thanks are also due to Dr. S. Hamamatsu for useful discussions and Dr. J.M. Pauric for a critical reading of the manuscript.

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