Carbohydrate Research, 237 (1992) 333–338
Elsevier Science Publishers B.V., Amsterdam

Note

A ^{1}H NMR study of a fragment of partially *N*-deacetylated chitin produced by lysozyme degradation

Kenichi Ishiguro, Naoko Yoshie, Minoru Sakurai and Yoshio Inoue

Department of Biomolecular Engineering, Tokyo Institute of Technology, O-okayama, Meguro-ku, Tokyo 152 (Japan)

(Received March 25th, 1992; accepted in revised form July 6th, 1992)

Chitin, poly[2-acetamido-2-deoxy-(1 → 4)-β-D-glucopyranose], is obtained from the exoskelton of crustaceans, insects, fungi, and related organisms. Partially deacetylated chitin (DAC), prepared by *N*-deacetylation of chitin under heterogeneous or homogeneous conditions, is composed of 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc) and 2-amino-2-deoxy-β-D-glucopyranose (GlcN) units. Chitin, chitosan (highly deacetylated chitin), and DAC are easily degraded in nature by enzymes such as lysozyme, chitinase, and chitosanase. Until now, these compounds have been attractive for use in the industrial, biomedical, and pharmacological fields. The degree of *N*-deacetylation and the distribution of GlcNAc and GlcN units are considered to significantly affect the DAC properties such as lysozyme susceptibility[1] and solubility[2]. Therefore, it is important to analyze the microstructure of DAC.

Recently, high-resolution NMR spectroscopy has been used to characterize the sequence distribution of DAC. Vårum et al.[3] determined the degree of *N*-deacetylation and diad frequencies (nearest-neighbor unit probabilities) in DAC obtained under homogeneous and heterogeneous conditions by use of ^{1}H NMR spectroscopy. Furthermore, they determined diad and triad frequencies (both nearest-neighbor units probabilities) in DAC by ^{13}C NMR spectroscopy[4]. Fukamizo et al.[5] analyzed the structure of two partialy deacetylated chitotrioses, GlcN-GlcNAc-GlcNAc and GlcN-GlcN-GlcNAc by NMR spectroscopy.

In this study, we have tried to determine the terminal units of DAC oligomer by ^{1}H NMR spectroscopy as the first step to analyze the microstructure. The DAC oligomer was prepared by hydrolysis of *N*-deacetylated chitin (DAC polymer) with lysozyme. The average molecular weight (M_w) and the degree of *N*-deacetylation

Correspondence to: Dr. Y. Inoue, Department of Biomolecular Engineering, Tokyo Institute of Technology, O-okayama, Meguro-ku, Tokyo 152, Japan.

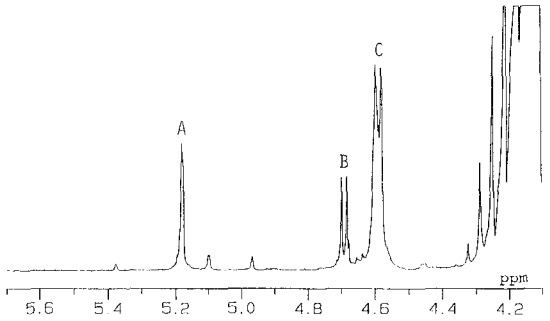

Fig. 1. ^{1}H NMR spectrum of the H-1 resonance region of $(GlcNAc)_3$ in D_2O at pD 4, 90°C.

of the DAC polymer were ~ 191 000 and ~ 66%, respectively. But after lysozyme degradation and purification with removal of the DAC polymers that were not sufficiently hydrolyzed, the average molecular weight (M_w) and the degree of *N*-deacetylation of the DAC oligomer were determined to be ~ 2000 and ~ 55%, respectively.

The spectra of chitin monomer (GlcNAc), trimer $(GlcNAc)_3$ and chitosan monomer (GlcN), trimer $(GlcN)_3$ were also measured in order to gain helpful information for the analysis of the DAC oligomer. The analysis of the NMR spectra of $(GlcNAc)^6$ and $(GlcN)_3$ (ref. 7) has been reported before. The assignments of the spectrum of $(GlcNAc)_3$, however, have not been reported except for the signals of the methyl protons[8]. Therefore, the assignments of other protons were determined herein via their COSY spectra.

Figs. 1 and 2 show the spectra of $(GlcNAc)_3$ and $(GlcN)_3$, respectively, measured in D_2O solutions at pD 4 and at 90°C. In the case of the terminal analysis of the (1 → 4)-linked oligosaccharide by ^{1}H NMR spectroscopy, the chemical shifts of the H-1 and H-4 signals are considered to be useful data, because their chemical shift values remarkably depend on whether they are involved in glycosidic linkage formation or whether they are on the terminal residues. The doublet signals A and B in Fig. 1 were well resolved and were assigned based on the COSY spectra to

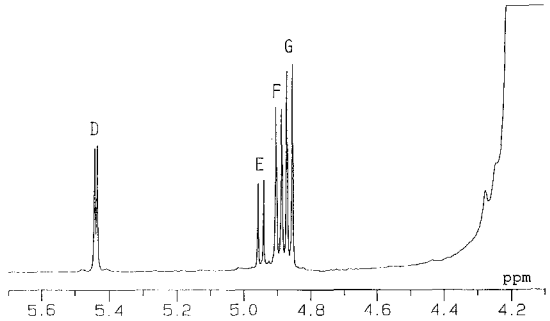

Fig. 2. ^{1}H NMR spectrum of the H-1 resonance region of $(GlcN)_3$ in D_2O at pD 4, 90°C.

TABLE I

H-1 chemical shifts (δ) of the reducing end unit for GlcNAc, $(GlcNAc)_3$, GlcN, and $(GlcN)_3$ in D_2O at pD 4 and 90°C

	δ (ppm)	
	H-1(α)	H-1(β)
GlcNAc	5.19	4.70
$(GlcNAc)_3$	5.18	4.69
GlcN	5.43	4.92
$(GlcN)_3$	5.43	4.94

H-1(α) and H-1(β) on the reducing end unit of $(GlcNAc)_3$, respectively. The resonances denoted by C of $(GlcNAc)_3$ arose from the H-1 protons on both internal and nonreducing end units. These were too overlapped to be individually assigned. Among the H-4 proton signals of $(GlcNAc)_3$, only the signal of the nonreducing end unit was assigned by comparison with the spectrum of the monomer, while the signals of the other units could not be assigned as they were severely overlapped. According to the assignment reported[7], the well-resolved doublet signals, D and E, in Fig. 2 were identified as H-1(α) and H-1(β), respectively, on the reducing end unit of $(GlcN)_3$. The doublet signals F and G were assigned to the H-1 protons on the internal and nonreducing end units of $(GlcN)_3$, respectively. As for the signals of the H-4 protons of $(GlcN)_3$, that of the nonreducing end only was assigned. The other H-4 protons could not be identified.

Table I shows the H-1 shift values of the reducing end unit of the monomers and trimers. It is noteworthy that the monomer and trimer have almost the same H-1 shift values for the reducing end. Table II shows the coupling constants of H-1(α) and H-1(β) on the reducing end unit of the monomers and trimers.

Fig. 3 shows the spectra of the DAC oligomer measured in D_2O solution at pD 4 and 90°C. In Fig. 3(b), the two groups of resonances P and Q were considered to be the H-1 protons on the internal GlcNAc and GlcN units, respectively, on the basis of their chemical shifts. A pair of weak but characteristic doublet signals are observed at 5.17 (X) and 4.69 ppm (Y). The relative intensity suggests they are due to H-1 on the terminal unit of the DAC oligomer. Moreover, the comparison of

TABLE II

Coupling constants (J) of H-1(α) and H-1(β) of the reducing end unit for GlcNAc, $(GlcNAc)_3$, GlcN, and $(GlcNAc)_3$ in D_2O at pD 4 and 90°C

	$J_{1,2}$ (Hz)	
	H-1(α)	H-1(β)
GlcNAc	3.3	8.1
$(GlcNAc)_3$	2.2	8.1
GlcN	3.7	8.4
$(GlcN)_3$	3.7	8.4

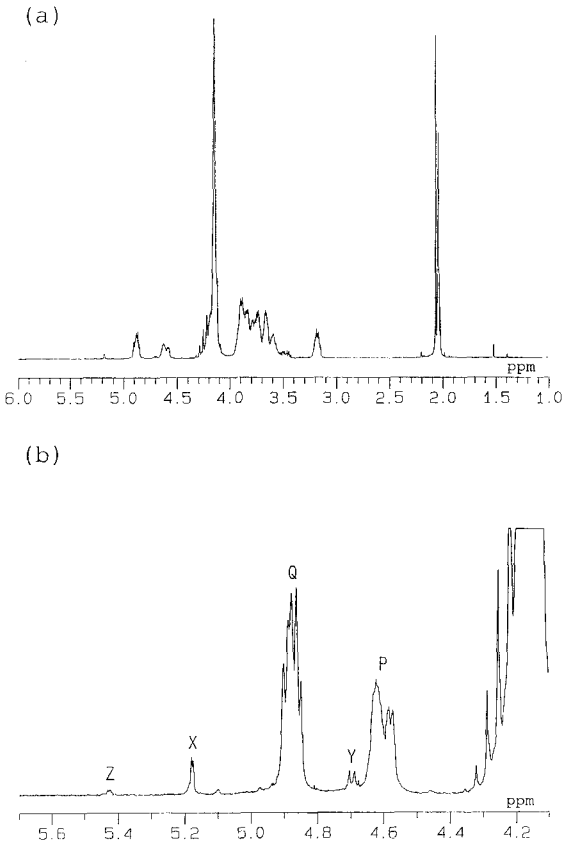

Fig. 3. (a) ^{1}H NMR spectrum of DAC oligomer in D_2O at pD 4, 90°C. (b) Expansion of the H-1 resonance region of (a).

their shift values with those in Table I clearly reveals that the signals X and Y correspond to H-1(α) and H-1(β) of the reducing GlcNAc end unit. The coupling constants of the signals X and Y which are 2.2 and 8.1 Hz, respectively, confirm this assignment. These values correspond to those of H-1(α) and H-1(β) on the reducing end unit of $(GlcNAc)_3$ as shown in Table II. In Fig. 3, the very weak signal Z could be found at 5.42 ppm. This shift value reveals that a small amount of DAC oligomer produced by lysozyme hydrolysis has a GlcN unit as a reducing end. The coupling constant of the signal Z, 3.6 Hz, corresponding to that of the H-1(α) on the reducing terminal unit of $(GlcN)_3$, also, agrees with this idea. Provided that the signal Z is due to H-1(α), a paired weak signal due to H-1(β) should be found around 4.94 ppm (see Table I); however, in practice, the large resonance Q makes it impossible to find such a signal. On the assumption that the tautomeric equilibrium ratios α anomer : β anomer of the reducing GlcNAc and GlcN end units are equivalent, the areas of the signals X and Z indicated that the relative rate of the reducing GlcN end units of the DAC oligomer is $\sim$ 15–19%.

Furthermore, the areas of the signals due to the H-1 protons of the GlcNAc units, compared with those of the GlcN units, indicates that the degree of *N*-deacetylation of the DAC oligomer is ~ 53%. This value obtained from the NMR spectrum is almost coincident with that obtained from the IR spectrum (~ 55%).

The degree of polymerization of the DAC polymer as a substrate was more than 1000, and that of the DAC oligomer as a hydrolysate was ~ 11. Thus the amount of reducing end units originating from the beginning substrate could be neglected. It is concluded that 81–85% of the DAC oligomer molecules obtained by lysozyme degradation have a reducing GlcNAc end unit, although the degree of *N*-deacetylation is 55%. The DAC oligomer of 15–19% may have a reducing GlcN end unit. In view of the action of lysozyme on partially deacetylated chitin, Amano and Ito[9] have proposed the importance of the *N*-acetyl groups of the *N*-acetylglucosamine units located on the subsites C and E of lysozyme in lysozyme-catalyzed hydrolysis. Here, it can be said that the subsite D of lysozyme prefers a GlcNAc unit to a GlcN unit in the hydrolysis of partially deacetylated chitin with lysozyme. The nonreducing end units could not be identified in this study because of the severe overlap of their resonances with other proton resonances. Further studies are in progress.

EXPERIMENTAL

The samples of GlcNAc, $(GlcNAc)_3$, GlcN, and $(GlcN)_3$ were purchased from Seikagaku Kogyo Co., Ltd. DAC oligomer obtained by lysozyme hydrolysis was kindly supplied by Dr. Sashiwa, Tottori University. It was prepared as follows: deacetylated chitin, of which the molecular weight and the degree of *N*-deacetylation were ~ 191 000 and ~ 66%, respectively, (10 g) was dissolved in 0.1 M acetate buffer (1000 mL), and the hydrolysis was initiated by adding 0.7 g of lysozyme to the deacetylated chitin solution at 37°C. After 6 days, the hydrolysis was stopped by addition of trichloroacetic acid and the precipitate was removed by centrifugation. The pH of the supernatant was then adjusted to pH 8–9 with 10% aq NH_3 solution, yielding a precipitate, which was the higher molecular weight fraction of the hydrolysate. The molecular weight of the precipitate was ~ 25 000. After removal of the precipitate by centrifugation, the supernatant was precipitated by pouring it into 2000 mL of acetone. The precipitate, which was DAC oligomer hydrolysed with lysozyme, was filtered, washed with acetone, and dried in vacuo.

For the NMR sample, the powdered chitin- and chitosan-monomer, trimer, and DAC oligomer were dissolved in D_2O. The pD of each sample was adjusted to pD 4 by addition of DCl and NaOD. The pD values were designated by the pH-meter readings without correction for the isotope effect. The ^1H NMR spectra were recorded with a JEOL GX-500 spectrometer at 90°C. The chemical shifts were referenced to sodium 3-(trimethylsilyl)-1-propanesulfonate (DSS).

The degree of *N*-deacetylation of the DAC oligomer was estimated from the elemental analysis and by the IR method of Sannan et al.[10]. The molecular weight of the DAC oligomer was measured by GPC with a pullulan standard on a Shimadzu LC-6A apparatus (column: Asahipak GS-220H, GS-310H, GS-510H; eluent: 0.1 M acetate buffer (pH 4.5); flow rate: 1 mL/min; column temp.: 50°C).

ACKNOWLEDGMENTS

The authors thank Professor S. Tokura of Hokkaido University for helpful discussions, and Dr. H. Sashiwa of Tottori University for kindly supplying the DAC oligomer samples and helpful discussions.

REFERENCES

1 H. Sashiwa, H. Saimoto, Y. Shigemasa, R. Ogawa and S. Tokura, *Int. J. Biol. Macromol.*, 12 (1990) 295–296.
2 S. Aiba, *Int. J. Biol. Macromol.*, 11 (1989) 249–252.
3 K.M. Vårum, M.W. Anthonsen, H. Grasdalen, and O. Smidsrød, *Carbohydr. Res.*, 211 (1991) 17–23.
4 K.M. Vårum, M.W. Anthonsen, H. Grasdalen, and O. Smidsrød, *Carbohydr. Res.*, 217 (1991) 19–27.
5 T. Fukamizo, A. Ohtakara, M. Mitsutomi, and S. Goto, *Agric. Biol. Chem.*, 55 (1991) 2653–2655.
6 S.J. Perkins, L.N. Johnson, and D.C. Phillips, *Carbohydr. Res.*, 59 (1977) 19–34.
7 A. Domard, C. Gey, and F. Taravel, *Int. J. Biol. Macromol.*, 13 (1991) 105–109.
8 F.W. Dahlquist and M.A. Raftery, *Biochemistry*, 8 (1969) 713–725.
9 K. Amano and E. Ito, *Eur. J. Biochem.*, 85 (1978) 97–104.
10 T. Sannan, K. Kurita, K. Ogura, and Y. Iwakura, *Polymer*, 19 (1978) 458–459.