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Kinetics of de-*N*-acetylation of the chitin disaccharide in aqueous sodium hydroxide solution

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ABSTRACT

Chitosan is prepared from chitin, a process which is carried out at highly alkaline conditions, and that can be performed either on chitin in solution (homogeneous deacetylation) or heterogeneously with the chitin as a solid throughout the reaction. We report here a study of the de-*N*-acetylation reaction of the chitin dimer (GlcNAc–GlcNAc) in solution. The reaction was followed by ^{1}H NMR spectroscopy in deuterated aqueous sodium hydroxide solution as a function of time, sodium-hydroxide concentration and temperature. The ^{1}H NMR spectrum of GlcNAc–GlcNAc in 2.77 M deuterated aqueous sodium hydroxide solution was assigned. The interpretation of the ^{1}H NMR spectra allowed us to determine the rates of de-*N*-acetylation of the reducing and non-reducing ends, showing that the reaction rate at the reducing end is twice the rate at the non-reducing end. The total deacetylation reaction rate was determined as a function of the hydroxide ion concentration, showing for the first time that this de-*N*-acetylation reaction is second order with respect to hydroxide ion concentration. No significant difference in the deacetylation rates in deuterated water compared to water was observed. The activation energy for the reaction (26–54 °C) was determined to 114.4 and 98.6 kJ/mol at 2.77 and 5.5 M in deuterated aqueous sodium hydroxide solution, respectively.

1. Introduction

Generic chitosans are prepared from chitin, and this process is currently performed by chemical de-*N*-acetylation in concentrated sodium-hydroxide at high temperatures in a heterogeneous process where the chitin/chitosan remains in the solid state during the process. However, the same process may also be performed in a homogeneous process with the chitin solubilized in the alkaline solution.[1] The homogeneous de-*N*-acetylation of chitin has been studied as a function of temperature, and the activation energy was determined to 22 kcal/mol (92 kJ/mol). The same authors assumed that the reaction was first order with respect to hydroxide ion (at 10% aqueous NaOH-concentration), and found that the reaction was pseudo-first order with respect to acetamido groups. However, their attempt to study the de-*N*-acetylation of the monomer GlcNAc was unsuccessful, which they attributed to decomposition.[2]

There is considerable interest in the heterogeneous deacetylation process, as this is a cost-effective process for producing chitosans from chitin and the functionality of chitosans has been found to depend on their degree of acetylation.[3] Studies of this process are more complicated due to the insolubility and crystalline nature of chitin. However, activation energies in the range from 40 to 45 kJ/mol have been reported,[4] which is less half the reported value in the homogeneous process mentioned above. It is, however, more difficult to interpret results from the heterogeneous processes, as the local concentrations of for example, hydroxyl ions at the solved exposed surface where the reaction occurs may be quite different from known concentration in solution.

In order to study the de-*N*-acetylation reaction in more detail, we decided to investigate the kinetics of the deacetylation reaction by directly following changes in time-resolved ^{1}H NMR spectra (in alkaline solutions) with the chitin dimer as a model substance. The results reveal that one of the Lobry de Bruyn-Alberda van Ekenstein (LdB-AvE) reactions occurs, that is, the epimerisation reaction as demonstrated by the disappearance of the H-2 proton as it was replaced by deuterium in the NMR spectra. Moreover, we demonstrate that the de-*N*-acetylation reaction is in second order with respect to the (deuterated) hydroxide ion, and that the reaction rate at the reducing end is twice the rate at the non-reducing end.

2. Experimental

2.1. NMR of the chitin dimer in deuterated aqueous sodium hydroxide solution

Chitin dimer (AA) was purchased from Seikagaku, Japan. (99.5%) D_2O and (99.5% D) NaOD 40% w/w were purchased from CDN isotopes, Canada. Stock solution was prepared by dissolving 30 mg/mL chitin dimer in D_2O and aliquant 100 μL into eppendorf tube and stored at −35 °C. Prior to dissolving the oligomers, the NaOD was saturated with nitrogen gas to remove oxygen in order to prevent side-reactions.

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All NMR experiments were recorded on BRUKER Avance DPX 400 or 300 spectrometer equipped with 5 mm QNP probe and 5 mm z-gradient DUL (C/H) probe, respectively. The NMR data were processed and analysed with Bruker XwinNMR ver 3.5 and TopSpin 3.0 software.

Different chitin dimer samples were prepared where ~3 mg (AA and DD) were dissolved in 2.77 M NaOD (in D_2O) with sodium 3-(trimethylsilyl) propionate-d_4 (TSP) as internal standard. Homonuclear 1D, 2D correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY) and ^{13}C heteronuclear single quantum coherence (HSQC) experiment were recorded at 25 °C and used to assign the chemical shifts.

For time-resolved NMR, pseudo 2D spectrum (first dimension is ^1H spectrum and second dimension is time spaced), the samples were prepared by mixing the chitin dimer (3 mg) with D_2O and NaOD 40% w/w to a total volume of 500 μL with the desired NaOD concentration. Then the sample was immediately inserted into the preheated NMR instrument. The recorded spectrum is a pseudo-two-dimensional type experiment recording a 1D ^1H NMR spectrum every 10 min. The recorded 1D carbon ^1H spectrum contains 16 K data points, has a spectral width of 12 ppm, 16 scans with a 30 degree flip angle, relaxation delay of 1 s (total recording time of 49 s). In order to check the isotope effect a sample was prepared and a time-resolved spectrum was recorded (32 scans, with presaturation of the water resonance, total recording time of 89 s) as described above with 10% D_2O/90% H_2O instead of 99.9% D_2O.

2.2. Determination of de-*N*-acetylation rate constants

The de-*N*-acetylation of the chitin dimer was determined by monitoring the intensity of the acetate signal in relation to the total intensity for both acetyl and acetate signals in the NMR spectra. The fraction of deacetylated dimer AD or DA (F_D) was determined and plotted as a function of time, and the initial rate of de-*N*-acetylation was determined as the slope for the initial linear part of the curve.

The natural logarithm of the fraction of deacetylated units (F_D) plotted as a function of time resulted in an initial linear relation. The rate constants of the de-*N*-acetylation reaction were determined as the slope of the linear region in these plots.

Molar fractions of each dimer (AD and DA) in the reaction mixture were calculated from the ratio of the β-anomer proton of each dimer to the sum of β-anomer protons of the dimers. Rate constant of de-*N*-acetylation at reducing end (k_{DA}) and at non-reducing end (k_{AD}) was determined as the slopes of the plots of the natural logarithm of the DA and AD molar fractions against time at the initial stage of the reaction, respectively.

2.3. Determination of activation energies

Arrhenius plots were created from rate constants obtained for de-*N*-acetylation at two different concentrations of NaOD (2.77, 5.5 M) at three different temperatures (26, 45, 54 °C). Activation energies were calculated as the slopes of these plots.

3. Results and discussion

3.1. ^1H NMR spectra of chitin dimer in NaOD/D_2O

Figure 1 shows the ^1H spectra of the chitin dimer GlcNAc–GlcNAc (panel A) together with the monomer GlcNAc (panel B) and the fully de-*N*-acetylated dimer GlcN–GlcN (panel C) in deuterated aqueous sodium hydroxide solution at 2.77 M and 25 °C. The large resonance of the solvent signal appears at 5.2 ppm (at 25 °C).

The chitin dimer and monomer spectra in deuterated aqueous sodium hydroxide solution are remarkably similar to the same spectra in deuterated water,[5,6] considering that the hydroxyl groups at this high pH-value are (partially) ionized. The assignments of the non-reducing end anomeric proton at 4.5 ppm are evident from the comparison of the two spectra. Coupling constants are also similar to those in deuterated water,[5,6] indicating that the conformation of the chitin dimer is the same as in water, that is, with a diequatorial glycosidic linkage with the sugar rings in the $^4C_1$ conformation. The acetyl-protons appear as three resonances, that is, the non-reducing end acetyl and two reducing end acetyl resonances (α- and β-anomer). Note also that the acetate protons appear well separated from the acetyl-protons, making it easy to monitor the de-*N*-acetylation reaction.

The chitosan dimer (GlcN–GlcN) spectrum in deuterated aqueous sodium hydroxide solution is also similar to the spectrum of chitosan dimer in deuterated water.[5] However, the chemical shift of H-2 proton is shifted upfield about 0.5 ppm as compared to the shift for H-2 proton when the amino-group is protonated.[5] Also, the anomeric protons are shifted upfield at the high pD-values of this solvent. The assignments of the H-1/H-2 resonances are straightforward from the spectra (Fig. 1), and coupling constants are again similar to those in deuterated water. Resonances were also assigned using the 2D COSY, TOCSY, ^{13}C HSQC spectra of GlcNAc–GlcNAc (data not shown), and the assignments are summarized in Table 1.

Having assigned the resonances in the spectra in Figure 1, we went on to examine the de-*N*-acetylation of the chitin dimer. Figure 2 shows the spectra of the chitin dimer (GlcNAc–GlcNAc) at 4.13 M and 45 °C after 10 and 1000 min (bottom part). In the upper part of Figure 2 is shown a stacked plot of the anomer region and the acetyl/H-2 (deacetylated) region. As the de-*N*-acetylation of the chitin dimer proceeds, new resonances appear in the spectrum. In the acetyl region, the acetyl-resonances (at ~2–2.1 ppm) decrease with concomitant increase in the resonances from the acetate protons (at ~1.9 ppm). By integrating the acetyl and acetate resonances as a function of time, the total rate of de-*N*-acetylation could be determined with a high accuracy. From the previous assignments of the resonances of the chitin dimer (AA), the chitin monomer (A), and the chitosan dimer (DD) (see Fig. 1 and Table 1), we can also identify the resonances of the two partially deacetylated dimers GlcNAc-GlcN (AD) and GlcN-GlcNAc (DA) in the anomer region of the spectrum. However, we noticed that the anomeric resonance of the reducing ends of the various dimers did not appear as doublets but as singlets, and also that the resonance of the H-2 (deacetylated) gradually decreased with time.

In alkaline solution of carbohydrates, the Lobry de Bruyn-Alberda van Ekenstein (LdB-AvE) reactions may occur.[7] LdB-AvE involves two reactions, epimerization and aldose-ketose interconversion. Since carbon 2 in chitin/chitosan oligosaccharides is not connected to a hydroxyl group but an amino or acetamido group, we need only to consider the epimerisation reaction (Scheme 1). From the ^1H NMR spectra of the monomer D-glucosamine obtained at different time in 2.77 M NaOD (in D_2O), it was determined that the H-2 resonance gradually decreased with time and that the anomeric resonance gradually changed from a doublet to a singlet. The change of the β-anomeric resonance to a singlet was also observed with the *N*-acetylated monomer. This is due to the ene-diol equilibrium reaction with the loss of stereochemistry at carbon 2, which will gradually substitute H-2 with deuterium from the solvent (deuterated water), see Scheme 1. As deuterium will not be detected in the ^1H NMR spectra and since the quadrupole moment of deuterium will result in a collapse of the coupling fine structure for the neighbouring protons,[8] this explains the change in the glucosamine spectrum with time. It should also be noted that this epimerization reaction is fast in comparison to the de-*N*-acetylation reaction at the present conditions. Thus, the