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Determination of enzymatic hydrolysis specificity of partially *N*-acetylated chitosans

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Abstract

A new method for determining the specificity of hydrolysis of the linear binary heteropolysaccharide chitosan composed of (1 → 4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc; A-unit) and 2-amino-2-deoxy-β-D-glucopyranose (GlcN; D-unit) residues is described. The method is based on the assignments of the ^{13}C chemical shifts of the identity (A- or D-units) of the new reducing and non-reducing ends and the variation in their nearest neighbours, using low molecular weight chitosans with known random distribution of A- and D-units as substrate. A highly *N*-acetylated chitosan with fraction of acetylated units (F_A) of 0.68 and a number-average degree of polymerization (DP_n) of 30 was hydrolysed with hen egg-white lysozyme, showing that both the new reducing and non-reducing ends consisted exclusively of A-units, indicating a high specificity for A-units in subsites D_L and E_L on lysozyme. Our data suggests that the preceding unit of the reducing A-units is invariable, and based on earlier studies, most probably an A-unit, while the unit following the non-reducing A-units can be either an A- or a D-unit. A more detailed study of the specificity of lysozyme at subsite D_L was performed by hydrolyzing a more deacetylated chitosan ($F_A = 0.35$ and DP_n of 20) to a DP_n of 9, showing that even for this chitosan more than 90% of the new reducing ends were acetylated units. Thus, lysozyme depolymerizes partially *N*-acetylated chitosans by preferentially hydrolyzing sequences of acetylated units bound to site C_L, D_L and E_L of the active cleft, while there is no specificity between acetylated and deacetylated units to site F_L. In addition, a moderately *N*-acetylated chitosan with fraction of acetylated units (F_A) of 0.35 and a DP_n of 20 was hydrolysed with *Bacillus* sp. No. 7-M chitosanase, showing that both the new reducing and non-reducing ends consisted exclusively of D-units. Our data suggests that the nearest neigbour to the D-unit at the reducing end is invariable, and based on earlier studies, most probably a D-unit, while the unit following the non-reducing D-units can be either an A- or a D-unit. We conclude that the *Bacillus* chitosanase hydrolyzes partially *N*-acetylated chitosan by preferentially attacking sequences of three consecutive deacetylated units, hypothetical subsites C_C, D_C and E_C, where the cleavage occur between sugar units bound to subsites D_C and E_C. A hypothetical subsite F_C on the chitosanase show no specificity with respect to A- and D-units. The new NMR method described herein offers a time and labour-saving alternative to the procedure of extensive hydrolysis of the binary heteropolysaccharide chitosan and subsequent isolation and characterization of the oligosaccharides.

Keywords: Chitin; Chitosan; Lysozyme; Chitosanase; NMR; Sequence specificity; (*Bacillus* sp.)

1. Introduction

Depolymerization of heteropolysaccharides will in general often include some specificity in the reaction. This can be utilized for characterization of the undegraded polysaccharide after purification and structural characterization of the depolymerization products. One example is the stability towards acid hydrolysis of heteropolysaccharides which to a large extent depends on the monomer composition and the type of glycosidic linkage [1]. Enzymes that depolymerize heteropolysaccharides are another example. These will also show some specificity towards its sub-

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strate, the simplest example being endo- and exo-enzymes. Endo-enzymes that degrade linear binary heteropolysaccharide are in general expected to show some specificity in the binding and cleavage of the polymer chain, depending on the actual sequence recognized by the enzyme. By specificity, we refer to the identity of the sugar units adjacent to the glycosidic linkage which is hydrolysed. For chemically catalysed reactions — e.g., acid hydrolysis — the specificity includes mostly one or two sugar units, whereas for enzymatically catalysed reactions, it generally includes the number of sugar residues recognized by the active site of the enzyme. Some aspects of the specificity of alginate lyases depolymerizing alginates of varying content and sequence of (1 → 4)-linked α-L-guluronate and β-D-mannuronate have been investigated by determining the identity of the new reducing ends [2].

Partially *N*-acetylated chitosan is a linear copolymer of 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc; A-unit) and 2-amino-2-deoxy-β-D-glucopyranose (GlcN; D-unit) connected by (1 → 4) linkages. It has previously been shown that sequential arrangement of A- and D-residues in the polymer chain is governed by Bernoullian statistics [3,4].

Enzymatic degradation of the binary copolymer chitosan has been extensively studied. Lysozyme (EC 3.2.1.17) may in addition to its natural substrate (the bacterial cell wall polysaccharide composed of alternating residues of (1 → 4)-linked GlcNAc and *N*-acetyl muramic acid) also hydrolyze partially *N*-acetylated chitosans [5–8]. Recently, we presented a theoretical model [9] using data from hydrolysis of different chitin/chitosan oligomers to describe how the sequence of sugar residues affected the relative lysozyme hydrolysis rates of partially *N*-acetylated chitosans. The model could quantitatively explain the strong increase in lysozyme hydrolysis rates with increasing fraction of acetylated units (F_A) [8]. The six subsites on lysozyme recognizing a hexasaccharide are conventionally designated with the letters A_L to F_L, where the glycosidic linkage between the sugar residues bound to subsites D_L and E_L is hydrolysed. The subscript L is added to distinguish the subsites on lysozyme from the equivalent notation to be used here for chitosanase. By determining the identity of the new reducing end of a partially *N*-acetylated chitosan hydrolysed with lysozyme, it has been found that subsite D_L has a high specificity towards A-units [10]. Chitinases and chitosanases are widely distributed in nature, and show different specificities towards A- and D-units in partially *N*-acetylated chitosans [11–14]. A chitosanase from *Bacillus* sp. No. 7-M has been reported to be highly specific for D-units when hydrolyzing partially *N*-acetylated chitosan [12]). Although the number of subsites in the active site of this chitosanase is unknown, we have designated the possible subsites interacting with sugar units A_C to F_C, where the glycosidic linkage between the sugar residues bound to subsites D_C and E_C is hydrolysed. This is done merely by analogy with lysozyme, as the number of subsites on the chitosanase are unknown.

The specificity of chitosan-degrading enzymes has traditionally been studied by extensive enzymatic degradation of the polysaccharide and subsequent isolation and characterization of the oligomers. This is a labour-intensive procedure which is complicated by the large number of possible oligosaccharides resulting from the degradation of the binary heteropolysaccharide (i.e., 4 disaccharides, 8 trisaccharides, 16 tetrasaccharides, etc.) and not always conclusive because of the possibility for transglycosylation. We now report on a new method for determining the specificity of enzymatic hydrolysis of partially *N*-acetylated chitosans. By the use of high-field carbon NMR-spectroscopy the identity of the new reducing and non-reducing ends and the variation in their nearest neighbours can be determined. By this method the site-specificity of four subsites on the chitosan-depolymerizing enzyme may be determined. The method is demonstrated by investigating the specificity of hen egg-white lysozyme and a *Bacillus* chitosanase towards well-characterized partially *N*-acetylated chitosans.

2. Materials and methods

2.1. Chitosans

Chitin was isolated from shrimp shells. Two chitosan samples with different degrees of *N*-acetylation were prepared by homogeneous *N*-deacetylation of chitin [15]. Both chitosans were depolymerized by nitrous acid and reduced with $NaBH_4$ as previously described [16], to obtain lower molecular weight chitosans suitable for direct analysis by NMR spectroscopy and enzymatic depolymerization studies. The degrees of *N*-acetylation were determined by high-field proton NMR spectroscopy [3] to be 68% ($F_A = 0.68$) and 35% ($F_A = 0.35$), and the intrinsic viscosities, [η], to 44 ml/g and 68 ml/g, respectively [17].

2.2. Lysozyme degradation of chitosan

The chitosan sample with $F_A = 0.68$ and [η] = 44 ml/g was used for lysozyme depolymerization studies. The procedure employed for the lysozyme degradation of chitosan has been described previously [9]. In addition, the chitosan sample used for chitosanase degradation (see below) was depolymerized with lysozyme.

2.3. Chitosanase degradation of chitosan

The chitosan sample with $F_A = 0.35$ and [η] = 68 ml/g was used for chitosanase depolymerization studies. A solution of chitosan (30 mg) in distilled water (6 ml) was prepared and solid NaCl was added to an ionic strength of

0.05 M, and the pH was adjusted to 5.5. Chitosanase from *Bacillus* sp. No. 7-M was purified as described earlier [12]. The chitosan solution with chitosanase (10 U) was incubated at 37°C for 5 h. The reaction was stopped by boiling for 4 min and the pH subsequently adjusted to 4.5. The chitosan-chitosanase solution was finally lyophilized.

2.4. Nuclear magnetic resonance spectroscopy

The number average degree of polymerization, DP_n, of the enzyme degraded chitosans was determined by high-field proton NMR spectroscopy using a Jeol EX-400 or Bruker dpx 300 spectrometer by determining the reducing end resonances of H-1 compared to the internal H-1 resonances [10]. The proton NMR spectrum of the lysozyme-depolymerized chitosan with $F_A = 0.35$ were recorded at 25°C, as the solution precipitated upon heating. The enzyme degraded chitosan samples were analysed by ^{13}C-NMR spectroscopy using a Bruker AM-500 (125.76 MHz) as previously described [4]. The chemical shifts are given relative to internal sodium 3-(trimethylsilyl)-propionate-d_4 (0.00 ppm). The interpretations of the spectra were based on the reported assignments of carbon-resonances in the trimer A-A-A $(GlcNAc)_3$ [18], the hexamer D-D-D-D-D-D $((GlcN)_6$ and higher oligomers of D-units [19] and our previous assignments of ^{13}C-NMR spectra [4]. While absolute values of ^{13}C chemical shifts can be difficult to compare, as the shift values are given relative to different standards and at different temperatures, relative shift-differences between carbons can be directly compared. The resonances for the non-reducing- and reducing ends were assigned based on the similarity of difference in chemical shifts of the resonances in central and terminal units of the $(GlcNAc)_3$ and $(GlcN)_6$ and that observed for the ends introduced by enzymatic degradation. Some of the resonances for the non-reducing- and reducing ends from the chitosanase degraded chitosan were not resolved, and the sample was therefore in an additional experiment

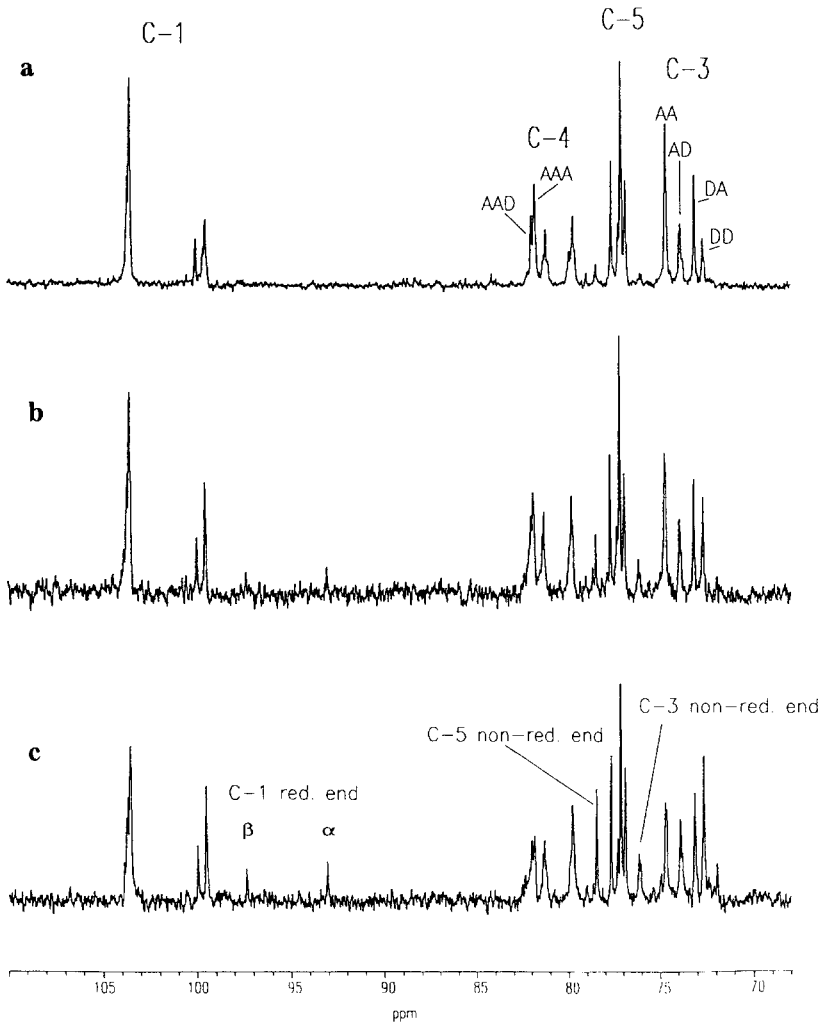

Fig. 1. ^{13}C proton-decoupled NMR spectrum (125 MHz) of the chitosan substrate with $F_A = 0.68$ and $DP_n = 30$ (a) and the same chitosan lysozyme-hydrolysed to $DP_n = 20$ (b) and to $DP_n = 7$ (c) in D_2O at pD 4 and 90°C.

reduced conventionally with $NaBH_4$, converting the reducing ends to 2,5-anhydro-D-mannitol before analysis by ^{13}C-NMR.

2.5. Simulation of lysozyme depolymerization of chitosan

The fraction of deacetylated and acetylated units introduced as reducing ends (denoted -D and -A, respectively) by the lysozyme hydrolysis was modelled using the previous subsite model for lysozyme action of chitosans [9]. The model was extended to also compute the chemical composition and the diads of the new reducing and non-reducing ends. The ensembles representing the substrates were obtained by simulating the nitrous acid depolymerization of high molecular weight monodisperse ensembles with the chemical composition determined by NMR (F_A = 0.68 and F_A = 0.35 for the two samples studied). The ratio between the rate constants for nitrous acid depolymerization of D–D and D–A linkages, k_{DD} and k_{DA}, were set to k_{DD}/k_{DA} = 1.0 according to previous determination of this ratio [20]. The number and weight average degree of polymerization, DP_n and DP_w, for the ensemble with F_A = 0.35 used for simulation of lysozyme action was DP_n = 20.2 and DP_w = 38.5, and DP_n = 31.7 and DP_w = 61.7 for the ensemble used for F_A = 0.68. Parameter sets II and IV of the previous simulation [9], as well as an extension of these two sets were used in the calculations.

3. Results and discussion

3.1. Lysozyme-depolymerized chitosan

A highly *N*-acetylated chitosan (F_A = 0.68) with a number-average degree of polymerization (DP_n) of 30 was selected as substrate for lysozyme. Fig. 1 show part of the 125 MHz proton decoupled ^{13}C-NMR spectrum of the chitosan substrate and the same chitosan with increasing degrees of lysozyme depolymerization. From the proton NMR spectrum of the lysozyme-degraded chitosan (data not shown), it was found that the new reducing ends were exclusively from A-units. Our results confirm that subsite D_L of the active cleft of lysozyme has a much higher affinity for A-units than for D-units for hydrolysis to occur. However, from the ^{13}C-NMR spectra in Fig. 1, we can get more detailed sequence information about both the identity of the new reducing and non-reducing ends, and their nearest neighbours.

The complexity of the spectrum given in Fig. 1 arises due to sequence-dependent shifts of the carbons (Fig. 1a), and due to the formation of the new reducing and non-reducing ends, which is seen by the appearance of new resonances in Fig. 1b and c. Table 1 summarizes the assignment of the different resonances of C-1, which further include comparison to reported carbon-resonances of the fully acetylated trimer (A-A-A; [18]) and the fully deacetylated hexamer (D-D-D-D-D-D; [19]). The data show that the α- and β-anomer resonances of C-1 of the chitosan are found at 92.9 ppm and 97.4 ppm respectively, which is a upfield shift of 10.7 ppm and 6.2 ppm from the AA diad (103.6 ppm), in agreement with the 10.80 and 6.43 ppm upfield shift of the α- and β-anomer of C-1 of A-A-A compared to the middle unit [18]. This is given as the relative difference in Table 1. The absence of sequence splitting in the resonances of the α- and β-anomer of C-1 suggests that the preceding unit of the reducing A-units is independent of the composition of the chitosan. If this neighbouring unit is an A, it means that also subsite C_L must bind an A-unit for hydrolysis to occur, in accordance with data from hydrolysis of oligomers [5]. However, the possibility that the C-1 anomers are insensitive to the neigbouring unit can not be excluded. Note also the absence of reducing end resonances from D-units in the area for these resonances [19], in accordance with the proton NMR-spectra. The non-reducing end of C-1 is not resolved from the diad resonances, in accordance with the small shift of 0.19 ppm of the non-reducing end of A-A-A compared to the middle unit [18].

The assignments of the diad frequencies and the reducing/non-reducing ends were likewise performed on C-4 (Table 2), C-5 (Table 3) and C-3 (Table 4). This was carried out in order to get information on the specificities of the individual subsites on lysozyme towards A- and D-units. Assignments of the resonances of C-2 and C-6

Table 1
Shift assignments and relative shift differences of carbon 1 for enzyme depolymerized chitosan compared to the fully acetylated trimer (A-A-A; [18]) and the fully deacetylated hexamer (D-D-D-D-D-D; [19])

Reference oligomers	A-A-A (middle unit)	A-A-A (reducing unit)	Rel. diff. (middle-reducing)	D-D-D-D-D-D (internal units)	D-D-D-D-D-D (reducing unit)	Rel. diff. (internal-reducing)
	102.51	91.71 (α) 96.08 (β)	10.80 (α) 6.43 (β)	98.26	89.72 (α) 93.41 (β)	8.54 (α) 4.85 (β)
Enzyme-depolymerized chitosan	-A-A-	-A-A	Rel. diff.	-D-D-	-D-D	Rel. diff.
	103.6	92.9 (α) 97.4 (β)	10.7 (α) 6.2 (β)	100.0	91.3 (α) 95.0 (β)	8.7 (α) 5.0 (β)

Table 2
Shift assignments and relative shift differences of carbon 4 for enzyme depolymerized chitosan compared to the fully acetylated trimer (A-A-A; [18]) and the fully deacetylated hexamer (D-D-D-D-D-D; [19])

Reference oligomer	A-A-A (middle unit)	A-A-A (reducing unit)	Rel. diff. (middle-reducing)	D-D-D-D-D-D (internal units)	D-D-D-D-D-D (reducing unit)	Rel. diff. (internal-reducing)
	80.55	80.99 (α) 80.55 (β)	−0.44 (α) 0.00 (β)	77.25	77.31 (α) 77.51 (β)	−0.06 (α) −0.26 (β)
Enzyme-depolymerized chitosan	-A-A-	-A-A	Rel. diff.	-D-D-	-D-D	Rel. diff.
	82.0	82.4 (α) – (β)	−0.4 (α) – (β)	79.8	– (α) – (β)	– (α) – (β)

were prohibited by overlapping resonances for end and internal residues.

Carbon 4 (see Fig. 1a and Table 2) are separated into three major resonances which have been assigned to the diads AA, AD and DA/DD [4]. The resonance at ≈ 82 ppm is the AA-diad, which is split into two resonance lines, assigned to the triads AAA (at the lowest ppm-value) and AAD. It is evident that the AAA-triad decreases in intensity relative to AAD as the DP_n of the chitosan sample decreases due to lysozyme hydrolysis. The α-anomer of the trimer A-A-A are shifted downfield (to higher ppm-value) by 0.44 ppm compared to the middle unit [18], and a small resonance line can be seen downfield from the AAD-diad. The β-anomer resonance of C-4 does not shift [18], and is probably found within the internal resonances of C-4 at 82 ppm. The non-reducing end of C-4 of the trimer A-A-A is shifted upfield by 9.51 ppm compared to C-4 of the middle unit [18], and the same shift for the non-reducing end of the chitosan would shift the resonance into the DD-diad of C3 at 72.6 ppm.

The assignments of the different resonances of C-5 are given in Table 3. In addition to the internal units, the non-reducing end units of C-5 could also be assigned for both A-units and D-units. The increase of non-reducing ends of exclusively A-units with increasing lysozyme degradation are evident in Fig. 1 at 78.5 ppm, meaning that productive modes for hydrolysis exclusively present A-units to subsite E_L. A minor resonance of non-reducing end resonances from D-units can be identified at 79.1 ppm in Fig. 1 [19]. However, this signal is also present in the sample which was not degraded by lysozyme, and does not increase with increasing lysozyme degradation. It should be noted that the original reducing ends of the chitosan sample consisting of 2,5-anhydro-D-mannose units have been reduced to 2,5-anhydro-D-mannitol. The non-reducing ends of the chitosan used as substrate for lysozyme, consist of both A- and D-units, as seen in Fig. 1a.

Table 4 gives the assignments of the resonances of C-3. The resonances of this carbon are separated into four relatively well-resolved peaks corresponding to the four diads (Fig. 1a), which have been assigned previously [4]. All the reducing and non-reducing end signals are resolved from the internal units (Table 4). However, the non-reducing end of an A-unit from C-4 and the α-anomer of C-5 of the reducing end are shifted into the -D-D- diad, which is evident from the apparent increase in this diad with increasing lysozyme degradation (Fig. 1). The assignments of the different C-3 resonances were straightforward from the reference oligomers (Table 4). Note the absence of sequence splitting in the resonances of the α- and β-anomer of C-3, as for C-1, which again suggests that the preceding unit of the reducing A-units may not be dependent on the composition of the chitosan. The increase of the resonance at 76.1 ppm from C-3 of exclusively A-units at the non-re-

Table 3
Shift assignments and relative shift differences of carbon 5 for enzyme depolymerized chitosan compared to the fully acetylated trimer (A-A-A; [18]) and the fully deacetylated hexamer (D-D-D-D-D-D; [19])

Reference oligomer	A-A-A (middle unit)	A-A-A (reducing unit)	Rel. diff (middle-reducing)	A-A-A (non-reducing unit)	Rel. diff. (middle-nonreducing)	D-D-D-D-D-D (internal units)	D-D-D-D-D-D (reducing unit)	Rel. diff. (internal-reducing)	D-D-D-D-D-D (non-reducing unit)	Rel. diff. (internal-non-reducing)
	75.75	71.28 (α) 75.84 (β)	4.47 (α) −0.09 (β)	77.17	−1.42	75.79	70.78 (α) 75.38 (β)	4.81 (α) 0.21 (β)	77.20	−1.61
Enzyme-depolymerized chitosan	-A-A-	-A-A	Rel. diff. (internal-reducing)	A-A-	Rel. diff. (internal-nonreducing)	-D-D-	-D-D	Rel. diff. (internal-reducing)	D-D-	Rel. diff. (internal-non-reducing)
	77.2	– (α) – (β)	– –	78.5	−1.3	77.3	– (α) – (β)	– (α) – (β)	78.9	−1.6

Table 4
Shift assignments and relative shift differences of carbon 3 for enzyme depolymerized chitosan compared to the fully acetylated trimer (A-A-A; [18]) and the fully deacetylated hexamer (D-D-D-D-D-D; [19])

Reference oligomer	A-A-A (middle unit)	A-A-A (reducing unit)	Rel. diff. (middle-reducing)	A-A-A (non-reducing unit)	Rel. diff. (middle-non-reducing)	D-D-D-D-D-D (internal units)	D-D-D-D-D-D (reducing unit)	Rel. diff. (internal-reducing)	D-D-D-D-D-D (non-reducing unit)	Rel. diff. (internal-non-reducing)
	73.44	70.54 (α) 73.73 (β)	2.90 (α) −0.29 (β)	74.74	−1.30	70.88	68.70 (α) 71.05 (β)	2.18 (α) −0.17 (β)	72.57	−1.69
Enzyme-depolymerized chitosan	-A-A-	-A-A	Rel. diff. (internal-nonreducing)	A-A-	Rel. diff. (internal-reducing)	-D-D-	-D-D	Rel. diff. (internal-reducing)	D-D-	Rel. diff. (internal-non-reducing
	74.7	71.9 (α) 75.0 (β)	2.8 (α) −0.3 (β)	76.1	-1.4	72.6	70.3 (α) 72.7 (β)	2.3 (α) −0.10 (β)	74.4	−1.8

ducing end is evident from Fig. 1, as also found for C-5. However, this resonance at 76.1 ppm is split into two lines, indicating that this carbon is sensitive to the nearest neighbour and that this neighbour can be either an A-unit or a D-unit. The relative intensities of the two resonance lines at 76.1 ppm were found to be identical to the relative amount of A and D-units in the chitosan. The two resonances were identified as -A-A- (at the higher ppm-value) and -A-D- (at the lower ppm-value) in a separate experiment where a partially *N*-acetylated chitosan with F_A = 0.51 were degraded with lysozyme where the two resonances were identical in intensity (data not shown). Since the A- and D-units are randomly distributed along the chain and the neighbouring unit to the non-reducing end is located in subsite F_L on lysozyme during the hydrolysis, it is concluded that there is no selectivity for A- or D-unit in subsite F_L of lysozyme for degradation of partially *N*-acetylated chitosans.

Extensive lysozyme degradation of a partially *N*-acetylated chitosan ($F_A = 0.32$) and subsequent isolation and characterization of the oligomers have been reported [5]. They also reported the quantitative yield of each oligosaccharide, and found that fully acetylated monomer, dimer and trimer were the dominating oligomers. However, significant amount of the dimer A-D (6.1%) were also found, together with the tetramers A-D-A-D (3.2%) and A-A-A-D (1.7%) [5]. All these oligomers contain a D-unit at the reducing end, implying that subsite D_L on lysozyme can also bind a deacetylated unit, which appear to be at variance with the present finding. However, it should be noted that the chitosan used by Amano and Ito was much more deacetylated ($F_A = 0.32$) compared to our chitosan ($F_A = 0.68$). This fact considerably change the

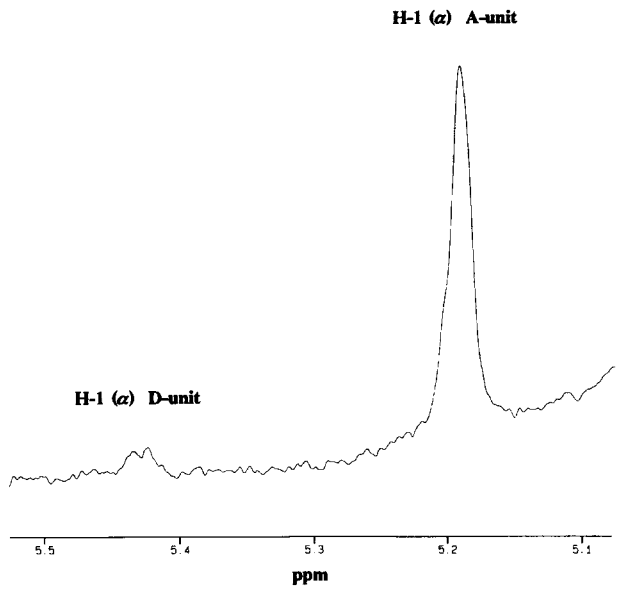

Fig. 2. ^{1}H-NMR spectrum (300 MHz) of the α-anomeric region of the reducing end units of H-1 of a chitosan with $F_A = 0.35$ ($DP_n = 20$) depolymerized with lysozyme to a DP_n of 9 in D_2O at pD 4 and 25°C.

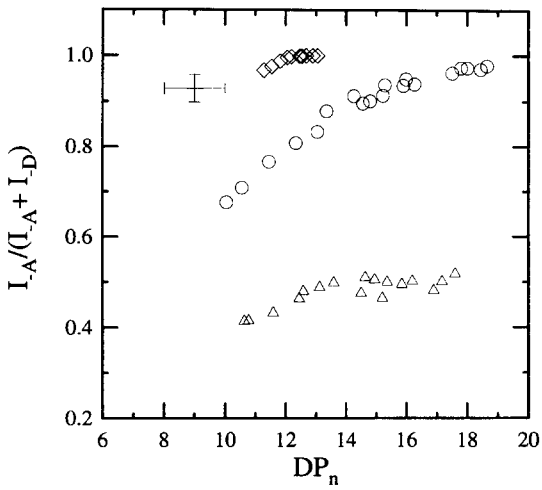

Fig. 3. Experimentally determined (+) and simulated fraction of acetylated residues on the reducing end, $I_{-A}/(I_{-D}+I_{-A})$ versus number average degree of polymerization DP_n, starting from an ensemble with $F_A = 0.35$, $DP_n = 20.2$ and $DP_w = 38.5$ for parameter set II (△), set IV (◇) [9] and set IVb (○). Parameter set IVb is identical to parameter set IV except for increasing the selectivity of subsite D_L to the same as for subsite E_L. I_{-A} and I_{-D} is the amount of acetylated and deacetylated units at the new reducing ends, respectively.

relative concentrations of hexamers presented to lysozyme. It should also be noted that Amano and Ito [5] fractionated their chitosan after extensive degradation by lysozyme and that only the low-molecular weight fraction was characterized, while in the present work we have characterized the whole fraction of the lysozyme-hydrolysed chitosan. In order to more directly compare our results with previous results [5] with respect to the specificity at subsite D_L, a chitosan with $F_A = 0.35$ and a DP_n of 20 was hydrolysed with lysozyme. The identities of the new reducing ends were analysed by proton-NMR spectroscopy [10]. Fig. 2 shows part of the proton-NMR spectrum of the chitosan which were lysozyme-degraded to a DP_n of 9. The α-anomer of the new reducing A-units occur at 5.19 ppm while the α-anomer of the new reducing D-units occur at 5.43 ppm, confirming the dominance of A-residues on the new reducing ends also for the chitosan with $F_A = 0.35$.

We simulated the lysozyme hydrolysis of chitosan by the subsite model for lysozyme depolymerization of chitosans [9], by modelling the fraction of D- and A-units introduced as new reducing ends by lysozyme hydrolysis. Fig. 3 shows the simulated fraction of the acetylated residues on the reducing end, given as $I_{-A}/(I_{-D}+I_{-A})$ versus DP_n for a chitosan with $F_A = 0.35$ for three parameter sets. The simulation based on parameter set II [9], which was most directly related to the experimental data on relative hydrolysis rates of specified oligomers reported by Amano and Ito [5], show that about 40 to 50% of the new reducing ends to be acetylated for $F_A = 0.35$ and $DP_n = 20.2$ (initially). Even the simulation based on parameter set IV, which have a higher preference for A-units compared to D-units in subsite D_L, shows that about 70%

of the new reducing ends to be acetylated at a DP_n of 9 (Fig. 3). However, by increasing the selectivity for A-units relative to D-units of subsite D_L to the same as for subsite E_L (parameter set IVb in Fig. 3), it was possible to model a lysozyme depolymerization of the chitosan with $F_A = 0.35$ where the new reducing ends consisted almost exclusively of acetylated units with decreasing DP_n.

Comparing the experimental value of $I_{-A}/(I_{-D} + I_{-A})$ of 0.93 (Fig. 2) with the model data in Fig. 3 shows that the applied subsite model can only explain the experimental value if the selectivity for acetylated residues to subsite D_L is larger than the oligomer hydrolysis data reported by Amano and Ito indicate [5]. Thus, it is difficult to reconsile the relative hydrolysis rates on purified partially *N*-acetylated oligomers reported by Amano and Ito with the chemical composition of the new reducing ends introduced by lysozyme hydrolyzing chitosan. One reason for this may be the competition between different sequences as presented on the polymeric substrates, whereas only one type of substrate was presented at the time for the oligomers. Another possibility for this apparent discrepancy may reside in the assumed negligible transglycosylation pathway in the modelling combined with different importance of this pathway in the two sets of experiments.

3.2. *Bacillus chitosanase-depolymerized chitosan*

Whereas a highly *N*-acetylated chitosan was chosen as substrate for lysozyme, a moderately *N*-acetylated chitosan ($F_A = 0.35$) was selected as substrate for the *Bacillus* chitosanase. From the proton-NMR spectrum of the chitosanase-degraded chitosan (data not shown), it was found that the new reducing ends were exclusively from D-units. This is in agreement with the characterization of isolated oligomers from *Bacillus* chitosanase degraded chitosan, which were found to consist exclusively of deacetylated units at the reducing ends [12]. The ^{13}C-NMR spectra provide additional information about substrate specificity.

Part of the 125 MHz proton decoupled ^{13}C-NMR spectrum of the chitosan substrate and the same chitosan

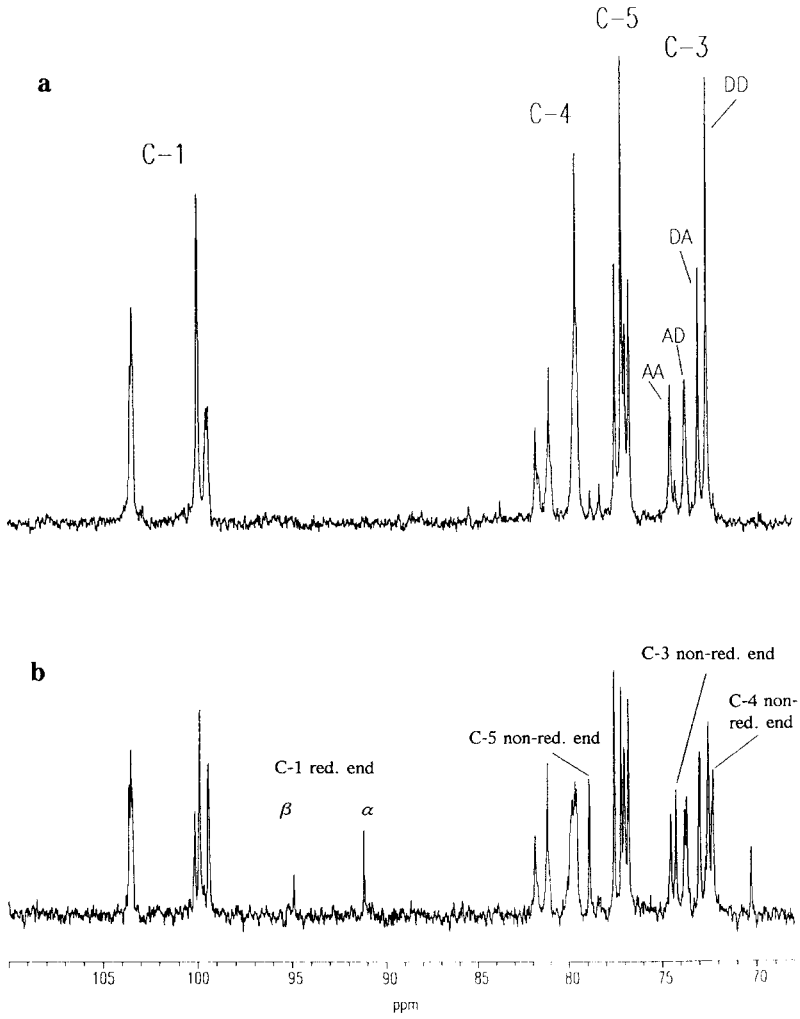

Fig. 4. ^{13}C proton-decoupled NMR spectrum (125 MHz) of the chitosan substrate with $F_A = 0.35$ and $DP_n = 20$ (a) and the same chitosan hydrolysed with *Bacillus* chitosanase to $DP_n = 10$ (b) in D_2O at pD 4 and 90°C.

depolymerized with *Bacillus* chitosanase is shown in Fig. 4a and b, respectively. The assignments of the different resonances of C-1 of *Bacillus*-depolymerized chitosan are given in Table 1. The α- and β-anomer resonances of the new reducing ends are seen at 91.3 and 95.0 ppm, respectively. This is an upfield shift of 8.7 and 5.0 ppm from the DD diad, in close agreement with the upfield shifts of 8.54 and 4.85 ppm of α- and β-anomer of C-1 of the fully deacetylated hexamer (D-D-D-D-D-D) compared to the middle unit [19]. The absence of sequence splitting in the resonances of the α- and β-anomer of C-1 suggests, as for the chitosan sample depolymerized by lysozyme, a high specificity of the *Bacillus* chitosanase for the preceding unit of the reducing D-unit. If this neighbouring unit is a D, it means that the sugar-residue bound to the hypothetical subsite C_C on the *Bacillus* chitosanase, must bind a D-unit for hydrolysis to occur. This is in accordance with data from chitosanase hydrolysis of partially *N*-acetylated chitosan [12], where the oligomers containing both A- and D-units were exclusively terminated with two deacetylated units at the reducing end. However, as for lysozyme depolymerization, we cannot exclude the possibility that the C-1 anomers are insensitive to the neigbouring unit. Note also the absence of reducing end resonances from A-units in the area for these resonances [18], in accordance with the proton NMR-spectra. The non-reducing end of C-1 is not resolved from the diad resonances, in accordance with the small shift of 0.25 ppm of the non-reducing end of the fully deacetylated hexamer compared to the internal units [19].

The assignments of the resonances of C-4 are given in Table 2. For C-4, the α- and the β-anomer of a D-unit at the reducing end of the fully deacetylated hexamer are only marginally shifted compared to the internal units [19], and are not resolved from the diad frequencies in our spectra. C-4 of the non-reducing end of the fully deacetylated hexamer (D-D-D-D-D-D) is shifted upfield by 6.78 ppm compared to C-4 of the internal units [19]. The same shift for the non-reducing end of the C-4 chitosan would shift the resonance to ≈ 73 ppm. However, it can be seen from Fig. 4b that the intensity of the resonance of the diad -D-D- of C-3 (at 72.6 ppm) is increased, and we assume that this is due to the non-reducing end of C-4.

As can be seen from Table 3, the non-reducing ends of C-5 of both A- and D-units in the chitosan could be assigned. The increase of non-reducing ends of exclusively D-units with increasing chitosanase degradation are evident in Fig. 4 at 78.9 ppm, meaning that the sugar-residue bound to the hypothetical subsite E_C on the *Bacillus* chitosanase must bind a D-unit for hydrolysis to occur.

Carbon 3 (see Fig. 4a and Table 4) are separated into four major resonances which have been assigned to the diads AA, AD, DA and DD [4]. The DD-diad appear at highest field (72.6 ppm). It is seen that this diad decrease in intensity relative to the other diads with chitosanase hydrolysis (Fig. 4b). The α-anomer of C-3 of a D-unit at 70.3 ppm (Fig. 4 and Table 4) is well resolved from the internal diads, and the absence of sequence splitting in this resonance, as for C-1, again suggests that preceding unit of the reducing D-units may not be dependent on the composition of the chitosan. The non-reducing end of C-3 of D-units appear at 74.4 ppm (Fig. 4 and Table 4). This non-reducing resonance line occur as a single line, in contrast to the non-reducing end of C-3 after lysozyme degradation. The non-reducing end of C-4 of D-units appear at 72.4 ppm. However, the interpretation of the last resonance is complicated by the partly overlapping resonance of the α-anomer of C-5. It may be tempting to conclude that the nearest neighbour residue to the non-reducing end is invariable. However, Izume and coworkers [12] reported that in addition to the fully deacetylated dimer, trimer and tetramer, the pentamer D-A-D-D-D and the hexamer D-A-D-D-D-D were detected among the oligosaccharides from chitosanase-degraded chitosan. Although the yield of the different oligosaccharides was not quantitated, the presence of the diad -D-A- at the non-reducing end indicate that the enzyme may not be specific for D-units as the nearest neighbour to the new non-reducing ends. In order to resolve partly overlapping resonance lines, both the quality of the sample and the shimming of the NMR-spectrometer is crucial. We reduced the chitosanase degraded chitosan by $NaBH_4$, which simplified the ^{13}C-NMR spectrum since C-1 of the reducing end is converted into the corresponding alcohol, implying no α/β-anomers. By this reduction, we were able to resolve the non-reducing end of C-4 which was previously not resolved from the α-anomer of C-5. Moreover, the resonance of C-4 at the non-reducing end was split into two resonance lines, and the intensities of the two resonance lines were found to be identical to the amount of A- and D-units in the chitosan. The two resonances were identified as D-D- (at the higher ppm-value) and D-A- (at the lower ppm-value) in a separate experiment where a partially *N*-acetylated chitosan with $F_A = 0.15$ were degraded with chitosanase where the D-D-resonance was increased compared to the D-A-resonance in proportion to the increase in D-units compared to A-units in the chitosan. Small sequential chemical shift differences as observed on the non-reducing ends are difficult to predict, and we have not attempted to explain why the nearest-neighbour sequential splitting could only be seen at the non-reducing end of C-4 after $NaBH_4$-reduction. Analogous to the conclusion on lysozyme on the selectivity of subsite F_L, we can conclude that there is no selectivity for A- or D-unit in the hypothetical chitosanase subsite F_C. A straightforward explanation for this is the absence of a subsite F_C, but broad specificity could be equally possible.

Extensive *Bacillus* degradation of a partially *N*-acetylated chitosan ($F_A = 0.24$) and subsequent isolation and characterization of the oligomers have been reported [12]. However, the quantitative yield of each oligosaccharide were not determined. It was found that fully deacety-

lated dimer, trimer and tetramer in addition to the hetero-oligosaccharides D-D-A-D-D, D-A-D-D-D, D-D-A-D-D-D and D-A-D-D-D-D. All these oligosaccharides contain two or more D-units at the reducing end, consistent with our results of exclusively D-units at the reducing end with no sequence splitting of the reducing end carbons. The non-reducing ends of the oligosaccharides isolated by Izume and coworkers [12] contained a D-unit, while the following unit could be either a deacetylated or a acetylated unit, consistent with our finding of exclusively D-units at the non-reducing end with a splitting of the non-reducing C-4 into two resonance lines. Izume and coworkers [12] further reported that the hexasaccharide D-A-D-D-D-D was not depolymerized by the *Bacillus* chitosanase. This may seem at variance with our data as this hexasaccharide could be expeted to be hydrolysed to D-A-D-D and D-D. The reason for the resistance to hydrolysis of the oligomer D-A-D-D-D-D may be that it binds in a non-productive binding mode to the chitosanase, or the hyphothetical subsites A_C and B_C contributing to the overall hydrolysis specificity. A detailed analysis along the lines carried out for lysozyme action on chitosan [9] could potentially solve this question, but sufficient data applying this approach to chitosan is not available. This may also explain why the oligosaccharides D-A-A-D-D, D-A-A-D-D-D, D-D-A-A-D-D and D-A-D-A-D-D were not found among the oligomers identified previously [12]. It should be noted that the reaction products from *Bacillus* chitosanase hydrolysis of chitosans may depend on their F_A, as discussed previously herein for lysozyme.

Our results are summarized in Fig. 5, where the 16 different tetrade sequences in chitosan are shown and positioned in the active site of the enzyme in such a way that the tetrasaccharide is hydrolysed in the middle (indicated by an arrow). The specificity of hen egg-white lysozyme and *Bacillus* chitosanase are indicated by the preferential hydrolysis of only 2 of the 16 tetrade sequences. For lysozyme, the existence of two additional subsites (A_L and B_L) has been identified, and previous results suggest that there may also be some specificity in site A_L and/or B_L with respect to the initial degradation rates of lysozyme towards partially *N*-acetylated chitosan [8].

The NMR-method described herein offers a time and labour-saving alternative to the extensive enzymatic degradation and subsequent isolation and characterization of the oligosaccharides for determination of sequence specificities of depolymerization of the heteropolysaccharide chitosan. The method should also be well suited for determining the specificity of chitinase/chitosanase which hydrolyse the glycosidic linkage between an A-unit and a D-unit or between a D-unit and an A-unit, and may also be applied for studying the specificity of hydrolysis of other binary heteropolysaccharides.

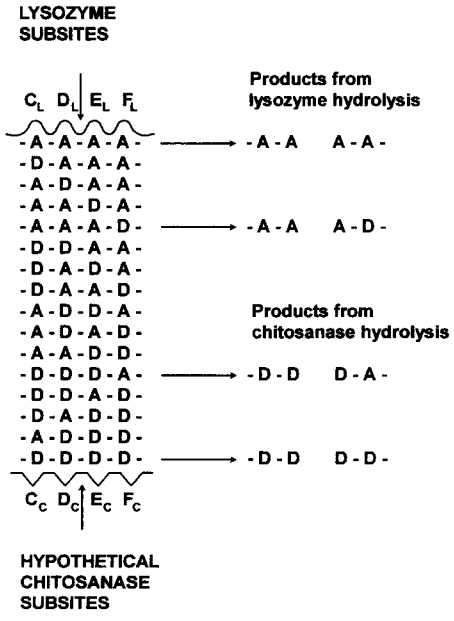

Fig. 5. Schematic illustration of the specificity of hydrolysis of lysozyme and *Bacillus* chitosanase towards the 16 different tetrade sequences in chitosan. Other sequences than indicated by the arrows on the figure may also be cleaved, but at a much lower rate. The tetrade sequences are positioned in the active site of the enzyme in such a way that the tetrades are hydrolysed at the arrow.

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References

[1] BeMiller, J.N. (1967) Adv. Carbohydr. Chem. 22, 25–108.
[2] Haugen, F., Kortner, F. and Larsen, B. (1990) Carbohydr. Res. 198, 101–109.
[3] Vårum, K.M., Anthonsen, M.W., Grasdalen, H. and Smidsrød, O. (1991) Carbohydr. Res. 211, 17–23.
[4] Vårum, K.M., Anthonsen, M.W., Grasdalen, H. and Smidsrød, O. (1991) Carbohydr. Res. 217, 19–27.
[5] Amano, K. and Ito, E. (1978) Eur. J. Biochem. 85, 97–104.
[6] Hirano, S., Tsushida, H. and Nagao, N. (1989) Biomaterials, 10, 574–576.
[7] Sashiwa, H., Saimoto, H., Shigemasa, Y., Ogawa, R. and Tokura, S. (1990) Int. J. Biol. Macromol. 12, 295–296.
[8] Nordtveit, R.J., Vårum, K.M. and Smidsrød, O. (1994) Carbohydr. Polym. 23, 253–260.
[9] Stokke, B.T., Vårum, K.M., Holme, H.K., Hjerde, R.J.N. and Smidsrød, O. (1995) Can. J. Chem. 73, 1972–1981.
[10] Ishiguro, K., Yoshie, N., Sakurai, M. and Inoue, Y. (1992) Carbohydr. Res. 237, 333–338.
[11] Ohtakara A., Matsunaga H. and Mitsutomi M. (1990) Agric. Biol. Chem. 54, 3191–3199.
[12] Izume, M., Nagae S., Kawagishi, H., Mitsutomi, M. and Ohtakara, A. (1992) Biosci. Biotechnol. Biochem. 56, 448–453.

[13] Fenton, D.M. and Eveleigh, D.E. (1981) J. Gen. Microbiol. 126, 151–165.
[14] Mitsutomi, M., Kidoh, H., Tomita, H. and Watanabe, T. (1995) Biosci. Biotechnol. Biochem. 59, 529–531.
[15] Sannan, T., Kurita, K. and Iwakura, Y. (1976) Macromol. Chem. 177, 3589–3600.
[16] Vårum, K.M., Ottøy, M.H. and Smidsrød, O. (1994) Carbohydr. Polym. 25, 65–70.
[17] Draget, K.I., Vårum, K.M., Moen, E., Gynnild, H. and Smidsrød, O. (1992) Biomaterials 13, 635–638.
[18] Boyd, J., Porteous, R. and Soffe, N. (1985) Carbohydr. Res. 139, 35–46.
[19] Domard, A. and Cartier, N. (1989) Int. J. Biol. Macromol. 11, 297–302.
[20] Vårum, K.M., Holme, H.K., Izume, M., Stokke, B.T. and Smidsrød, O. (1996) In: Proceedings of the 1st International Conference of the European Chitin Society (Brest, France, Sept. 11–13, 1995) (Domard, A., Jeuniaux, C., Muzzarelli, R.A.A. and Roberts, G.A.F., eds.), pp. 173–183, Jacques André Publ., Lyon.