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Mode of action of chitin deacetylase from *Mucor rouxii* on partially *N*-acetylated chitosans

Aggeliki Martinou[a], Vassilis Bouriotis[a,b], Bjørn T. Stokke[c], Kjell M. Vårum[d,*]

[a] *Enzyme Technology Division, Institute of Molecular Biology and Biotechnology, PO Box 1515, Heraklion 711 10, Crete, Greece*

[b] *Department of Biology, Division of Applied Biology and Biotechnology, University of Crete, PO Box 1470, Heraklion 711 10, Crete, Greece*

[c] *Norwegian Biopolymer Laboratory (NOBIPOL), Department of Physics, The Norwegian University of Science and Technology, 7034 Trondheim, Norway*

[d] *Norwegian Biopolymer Laboratory (NOBIPOL), Department of Biotechnology, The Norwegian University of Science and Technology, 7034 Trondheim, Norway*

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Abstract

The mode of action of chitin deacetylase (CDA) from *Mucor rouxii* on fully water–soluble partially *N*-acetylated chitosans was investigated. The Michaelis–Menten constants of three high-molecular-weight chitosans with initial fraction of acetylated units (F_A) of 0.08, 0.35, and 0.62 were determined to 2.1 ± 0.4, 1.7 ± 0.7, and 2.1 ± 0.3 mg/mL, respectively. The relative rate of enzymatic deacetylation increased linearly with increasing F_A on the chitosans, indicating that CDA does not preferentially attack any sequences in the chitosan molecules. A water-soluble and highly *N*-acetylated chitosan with F_A of 0.681, having a Bernoullian distribution of acetylated (**A**) and deacetylated (**D**) units, and a number-average degree of polymerization (dp_n) of 30, was selected as substrate for CDA for more detailed studies of the mode of action. The chitosan was enzymatically deacetylated to decreasing F_A-values (F_A of 0.582, 0.400, and 0.188), and the nearest neighbour frequencies (F_{AA}, F_{AD}, F_{DA} and F_{DD}) were determined by NMR spectroscopy, showing that the transition frequencies F_{AD} and F_{DA} were lower than expected from a random (Bernoullian) distribution in the further enzymatically deacetylated chitosans, while F_{AA} and F_{DD} were higher compared to a random distribution. The experimental results were compared with model data, assuming an endo-type mechanism with no preferential attack at any sequences in the chitosan chain. The comparison suggested that CDA hydrolysed acetyl-groups according to a multiple attack mechanism, with a degree of multiple attack of at least three. No deacetylation could be detected at the non-reducing end of the enzymatically deacetylated chitosans.

Keywords: Chitosan; Chitin deacetylase; *Mucor rouxii*

* Corresponding author.

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1. Introduction

Chitin is a water-insoluble linear polysaccharide of (1→4)-linked 2-acetamido-2-deoxy-β-D-glucose units (GlcNAc, **A**-unit). Chitin is the natural substrate of chitin deacetylase (CDA), an enzyme hydrolyzing in-chain acetamido groups on chitin [1], thereby converting **A**-units to **D**-units (2-amino-2-deoxy-β-D-glucose (GlcN)). The products of CDA are chitosans, a family of water-soluble linear binary copolymers of **A**- and **D**-units, where the fraction of **A**-units (F_A) can vary from 0 to 0.7 [2–4].

Enzymatic action patterns for enzymes that modify in-chain units on a linear binary heteropolysaccharide may be divided into three main types designated *multiple-chain*, *multiple attack*, and *single-chain* mechanisms. While the multiple-chain mechanism with no preferred attack will result in a binary heteropolysaccharide with a Bernoullian (random) distribution of the units, the multiple attack and the single-chain mechanism will generate block-copolymer structures. The latter types of enzymatic action patterns have been reported to occur by epimerisation of alginate [5] and de-esterification of pectins [6].

Using ^{1}H and ^{13}C NMR spectroscopy, we have recently reported methods [7,8] to determine the nearest-neighbour (diad) frequencies F_{AA}, F_{AD}, F_{DA}, and F_{DD} of chitosans. Such data specify the average fraction of places along the chain where an **A**-unit is followed by an **A**, an **A** followed by a **D**, and so on. It was found that water-soluble chitosans prepared by both homogeneous and heterogeneous thermochemical deacetylation gave values for the diad and triad frequencies that were consistent with a random arrangement of **D**- and **A**-units [7,8]. The identification of the different sequences in chitosans has been used to model the change in the diad and triad frequencies in lysozyme-depolymerized chitosan [4,9].

Chitin deacetylase from *Mucor rouxii* has been purified to homogeneity and further characterized by employing both conventional and immunoaffinity chromatography [10,11]. The enzyme is an acidic glycoprotein with a molecular weight of ~75 kDa and a carbohydrate content of 30%. Further biochemical characterization revealed that the enzyme has a very narrow specificity for chitinous substrates [12].

In this report the mode of action of purified CDA from *M. rouxii* using partially *N*-acetylated chitosans as substrates has been determined. The results suggest that CDA from *M. rouxii* does not preferentially attack any sequences in the chitosan chains, and that the acetyl groups are removed according to a multiple attack mechanism.

2. Experimental

Chitin was isolated from shrimp shells [13]. The chitosan samples were prepared by homogeneous *N*-deacetylation of chitin [14], or by heterogeneous *N*-deacetylation and extraction of the acid-soluble fraction [15,16]. The characterisation of the chitosans used for the CDA kinetics experiments are given in Table 1, where the fraction of acetylated units (F_A) and the diad frequencies were determined by ^{1}H NMR spectroscopy [7] and the intrinsic viscosities ([η]) as previously described [17].

The chitosan sample used as substrate in the NMR experiments was depolymerized by nitrous acid and reduced with $NaBH_4$ [4,18], affording a lower-molecular-weight chitosan suitable for direct analysis by NMR spectroscopy before and after enzymatic deacetylation. The characterisation of the chitosan sample used as substrate for CDA in the NMR experiments was performed by ^{13}C NMR spectroscopy [8]. The F_A of the substrate was determined to 0.681, and the number-average degree of polymerization (dp_n) to 30, as determined from the intensity of the non-reducing end C-3 and C-5 resonances compared to the internal C-3 and C-5 resonances [4].

Chitin deacetylase from *M. rouxii* was purified to homogeneity by immunoaffinity chromatography

Table 1
Characterisation of chitosan fractions used in the enzyme kinetics study. The experimental diad frequencies of each chitosan are compared with the calculated diad frequencies of a chitosan with random (Bernoullian) distribution of **A**- and **D**-units

Sample	F_A	F_{AA}	$F_{AD}=F_{DA}$	F_{DD}	[η] (mL/g)
Sample 1	0.08	0.02	0.06	0.86	1580
Sample 1 (random)	0.08	0.01	0.07	0.85	
Sample 2	0.15	0.04	0.12	0.73	740
Sample 2 (random)	0.15	0.02	0.13	0.72	
Sample 3	0.35	0.11	0.22	0.44	760
Sample 3 (random)	0.35	0.12	0.23	0.42	
Sample 4	0.51	0.30	0.22	0.26	450
Sample 4 (random)	0.51	0.26	0.25	0.24	
Sample 5	0.62	0.41	0.21	0.17	820
Sample 5 (random)	0.62	0.38	0.24	0.14	

as previously described [11]. Initial rates of deacetylation as a function of the chitosan concentration were determined for the chitosans with F_A of 0.08, 0.35, and 0.62 (see Table 1). The reactions were performed in 25 mM glutamate buffer (pH 4.5) at 50 °C using 0.17 to 4 mg/mL of the chitosan substrates and 10 mU/mL of CDA in a final volume of 0.3 mL. The enzymatic deacetylation reaction was stopped by decreasing the pH by adding 50 μL 0.1 M HCl and subsequent heat inactivation (10 min at 100 °C). The initial rates of deacetylation were obtained from the time course of the increase in concentrations of free acetate, which were enzymatically determined [12]. K_M and V_{MAX} values were determined from Lineweaver–Burk plots and Eadie–Hofstee plots. Standard deviations of K_M and V_{MAX} values were determined from the Eadie–Hofstee plots.

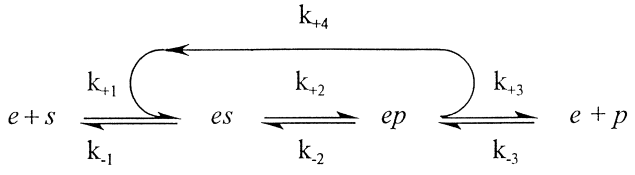
Initial rates of deacetylation as a function of F_A were determined at a substrate concentration of 4 mg/mL and with 10 mU/mL of CDA, ensuring substrate saturation.

Units of chitin deacetylase activity were estimated using 166 nmol hexa-*N*–acetylchitohexaose [$(GlcNAc)_6$] in a total volume of 500 μL 25 mM glutamate buffer (pH 4.5). The incubation time was 15 min at 50 °C, and the reaction was terminated by heating to 100 °C prior to acetate determination [10]. One unit of chitin deacetylase activity was defined as the amount of enzyme required to produce 1 μmol of acetate per min when incubated with $(GlcNAc)_6$ as described above [12].

Enzymatic deacetylation reactions of the chitosan substrate with F_A of 0.681 and dp_n 30 (Table 2) were performed in 25 mM glutamate buffer, pH 4.5, at 50 °C using 3 mg/mL of the chitosan substrate. The reactions were performed in dialysis membrane tubes (molecular weight cut-off of 10 k Da) in order to remove the acetate released, known to be a CDA inhibitor [12]. The chitosan substrate was incubated with increasing amounts of purified chitin deacetylase (30–300 U) to obtain chitosans with suitable F_A-values. Reactions were stopped by heat inactivation (100 °C, 15 min), and the samples were extensively dialysed in deionized water before lyophilization. The dialysis did not remove significant amounts of the chitosans. This was verified by checking the dp_n-values of the chitosan substrate and the enzymatically deacetylated chitosan products.

The ^{13}C NMR experiments were performed on the chitosan samples as previously described [8]. As the chitosan substrate was of a low molecular weight (dp_n 30), no further depolymerization was necessary before obtaining the spectra.

The action pattern of CDA on the chitosan substrate was modelled using the following general scheme for a prossesive enzyme:



(1)

where e, s and p are the enzyme, substrate and product, respectively, k_{+n} and k_{-n} $(n = 1, 3)$ are the forward and reverse rate constants, and k_{+4} is the forward rate constant for the prossesivity of the enzyme. The reverse prossesivity was not included. It was assumed that CDA was an irreversible enzyme and that the reversible product inhibition was not significant, i.e., $k_{-2} = 0$ and $k_{-3} = 0$ under the present experimental conditions. Because the polymeric substrate is changing during the deacetylation, we implemented the (pseudo)-steady

Table 2
Composition (F_A) and diad frequencies of the substrate chitosan and three enzymatically deacetylated chitosan products (a, b and c) determined by ^{13}C NMR spectroscopy

	F_A	F_{AA}	F_{AD}	F_{DA}	F_{DD}
Substrate	0.681 ± 0.027	0.426 ± 0.033	0.212 ± 0.010	0.230 ± 0.006	0.118 ± 0.025
Random	0.681	0.464	0.217	0.217	0.102
Chitosan a	0.582 ± 0.010	0.361 ± 0.012	0.211 ± 0.011	0.217 ± 0.010	0.208 ± 0.010
Random	0.582	0.339	0.243	0.243	0.175
Chitosan b	0.400 ± 0.017	0.193 ± 0.007	0.206 ± 0.016	0.207 ± 0.016	0.394 ± 0.032
Random	0.400	0.16	0.240	0.240	0.360
Chitosan c	0.188 ± 0.013	0.099 ± 0.004	0.085 ± 0.014	0.089 ± 0.01	0.728 ± 0.020
Random	0.188	0.035	0.153	0.153	0.659

state approximation of the intermediates in the algorithm:

$$\frac{\partial[es]}{\partial t} = k_{+1}[e][s] + k_{+4}[ep] - k_{-1}[es] - k_{+2}[es] = 0 \quad (2a)$$

$$\frac{\partial[ep]}{\partial t} = k_{+2}[es] - k_{+3}[ep] - k_{+4}[ep] = 0 \quad (2b)$$

Combined with the equation for the conservation of total enzyme, this is implemented as follows: the chitosan substrate was represented as an ensemble of 500 chitosan chains with $dp_n = 30$, and the *N*-acetyl groups were introduced according to the experimentally determined Bernoullian distribution to a total F_A of 0.681. For the given substrate and enzyme concentration, the equilibrium condition for the *es* complex was calculated, and positions in the ensemble were selected at random and new residues reselected whenever a **D**-unit was selected, to allow binding of the required number of enzymes at the equilibrium condition. This corresponds to an endo-type mechanism with no preferential attack at any sequences, but excluding **D**-units. The time needed to establish the equilibrium condition for the *es* complexes was considered negligible compared to the deacetylation reaction time, i.e., that the enzymatic reaction is not diffusion limited. Following the deacetylation of the residues encountered, the simulations allowed the enzymes either to relocate to neighbouring residues in a prosessive manner or to be released from the polymeric substrate and attack other positions according to the new equilibrium concentration of the *es* complex, and the procedure stated above. The ratio between the rate constants for the relocation and the release of the substrate, k_{+4}/k_{+3}, specifies the prosessivity, or degree of multiple attack of the enzyme, which was used as an adjustable parameter. A high degree of multiple attack, e.g., the termination of deacetylation when either encountering a chain end or a **D**-unit followed by a new attack by the enzyme, corresponds to the single-chain mechanism [19,20]. Likewise, the limiting case of a multichain attack mechanism, e.g., deacetylation of one unit per attack followed by random attack elsewhere in the chitosan ensemble, is also embodied in the model when the degree of multiple attack is low [19]. Changes in the sequence distributions of the chitosan during the simulated enzymatic deacetylation were determined for comparison to the experimental data. The average length of acetylated sequences in the substrate used in the experimental part is clearly an upper limit to the degree of multiple attack that can be expected.

3. Results and discussion

Kinetics of CDA deacetylating chitosans.—The initial rates of the enzymatic deacetylation reaction as a function of the substrate concentration and chemical composition of the chitosans (F_A from 0.08 to 0.62; see Table 1) were determined by following the increase in acetate concentration with time in the reaction mixture. The Michaelis–Menten constants (K_M) for three chitosans (F_A-values of 0.08, 0.35, and 0.62) were determined using Lineweaver–Burk plots (Fig. 1) and Eadie–Hofstee plots. K_M-values of the chitosans with F_A of 0.08, 0.35, and 0.62 were determined to 2.1 ± 0.4, 1.7 ± 0.7 and 2.1 ± 0.3 mg/mL, respectively. Thus, substrate saturation of CDA was achieved at about the same weight concentrations of chitosan for the three substrates. Assuming that the rate of dissociation of the enzyme–substrate complex to enzyme and substrate is low compared to the rate of formation of enzyme and product, the approximately equal K_M-values indicate that the affinity of CDA to the three chitosans are also equal.

The relative rate of enzymatic deacetylation of the chitosans at substrate saturation (relative to a

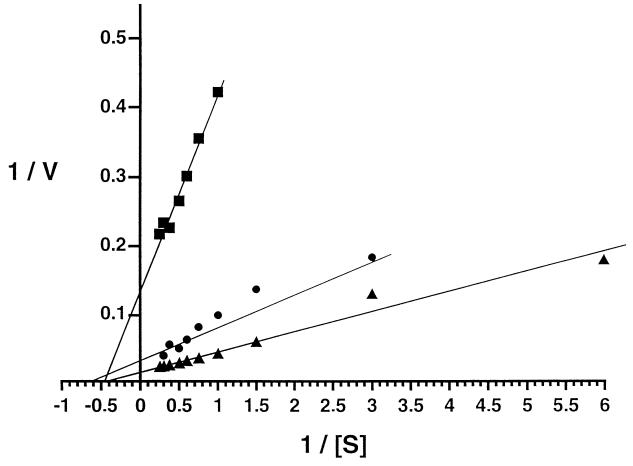


Fig. 1. Lineweaver–Burk plots for CDA with three substrate chitosans. ■: $F_A = 0.08$; ●: $F_A = 0.35$; and ▲: $F_A = 0.62$. Units are in mL/mg of chitosan (abcissa) and min/μM (ordinate).

rate of 1 of $F_A = 0.08$) as a function of the chemical composition of the chitosans (F_A) is given in Fig. 2, showing a linear increase in enzymatic deacetylation rate with increasing F_A-values. As the distribution of **A**- and **D**-units in water-soluble partially *N*–acetylated chitosans is random, both the equal K_M-values and the linear increase in deacetylation rates with increasing F_A-values indicate that CDA does not preferentially attack any sequence in the chitosan molecule. Such preferential attacks have been shown for pectin esterase [6,21].

Sequence analysis of chitosan substrate ($F_A = 0.681$ and $dp_n = 30$) and CDA-deacetylated chitosan products.—A chitosan with a fraction of *N*-acetylated units of 0.681 and a dp_n of 30 was selected as substrate for the chitin deacetylase for more detailed sequence studies. The sequential structure of the chitosan substrate corresponded closely to a Bernoullian distribution of the **A**- and **D**-units (Table 2). Fig. 3 shows part of the 125 MHz ^{1}H- decoupled ^{13}C NMR spectra of the chitosan substrate and the chitosan products with increasing degrees of enzymatic deacetylation (F_A of 0.582, 0.400, and 0.188). Table 2 gives the chemical composition (F_A) and the nearest–neighbour (diad) frequencies for the substrate and the three further enzymatically *N*–deacetylated chitosans (sample a, b and c) determined from ^{13}C NMR spectroscopy, together with the calculated diad frequencies for a Bernoullian distribution. The data show that the transition frequencies (F_{AD} and F_{DA}) are lower than expected from a random distribution in the further enzymatically deacetylated chitosans, while F_{AA} and F_{DD} are higher. Such a change in the nearest-neighbour frequencies associated with enzymatic deacetylation of chitosans are qualitatively in agreement with an enzyme operating according to a multiple-attack mechanism.

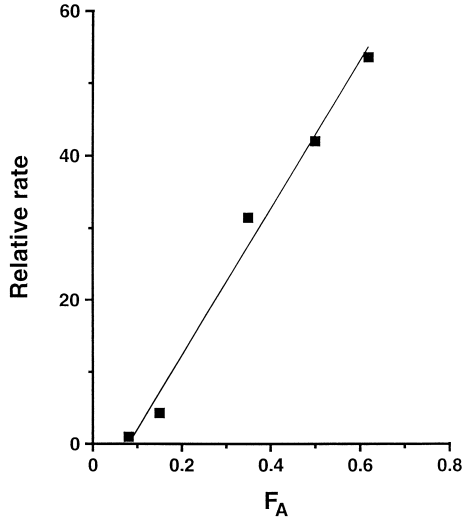


Fig. 2. Initial rate of deacetylation (relative to a rate of 1 of $F_A = 0.08$) versus F_A for CDA.

Comparison of experimental sequence data with model data.—The experimental results were compared with model data, assuming an endo-type mechanism with no preferential attack at any chitosan sequences, in accordance with the data from the enzyme kinetics. Fig. 4 shows the experimentally determined diad fractions (F_{AA}, F_{AD}, F_{DA} and F_{DD}) versus the fraction of *N*–acetylated units (F_A) for the chitosan substrate and the three enzymatically deacetylated products. In the same figure is also shown the simulated diad frequencies assuming that the CDA action pattern is either single-chain, multiple attack with degree of multiple attack equal to three, or multiple-chain attack for the substrate with $F_A = 0.681$ and $dp_n = 30$. The comparison of the experimentally determined diad frequencies with the model data shows that the diad frequencies of the further enzymatic deacetylated samples deviate from a Bernouillian distribution, and hence, that the enzymatic mechanism can not be described in terms of multiple-chain attack. The comparison of experimental diad frequencies with the model data (Fig. 4) suggests that the chitin deacetylase hydrolyses acetyl groups according to a multiple attack mechanism, with a degree of multiple attack of at least three. It is difficult to quantitatively determine the degree of multiple attack because the block-length distribution in the chitosan substrate (the number-average block length (**A**-units) of the chitosan substrate is 3.2) represents an experimental constraint in the present sample with respect to the degree of multiple attack. This limitation can in principle be relaxed by using substrates with higher F_A–values, but decreasing solubility of more highly acetylated substrates in aqueous solvents represents a practical limitation for the CDA–chitosan system. Attempts to use an amorphous chitin as substrate were unsuccessful, as no acid-soluble chitosan could be isolated and characterised.

Polarity of CDA.—Grasdalen et al. [6] showed that the rate of de-esterification of a low-molecular-weight pectin by tomato pectin esterase was about three times faster at the reducing end than at the non-reducing end, and concluded that the

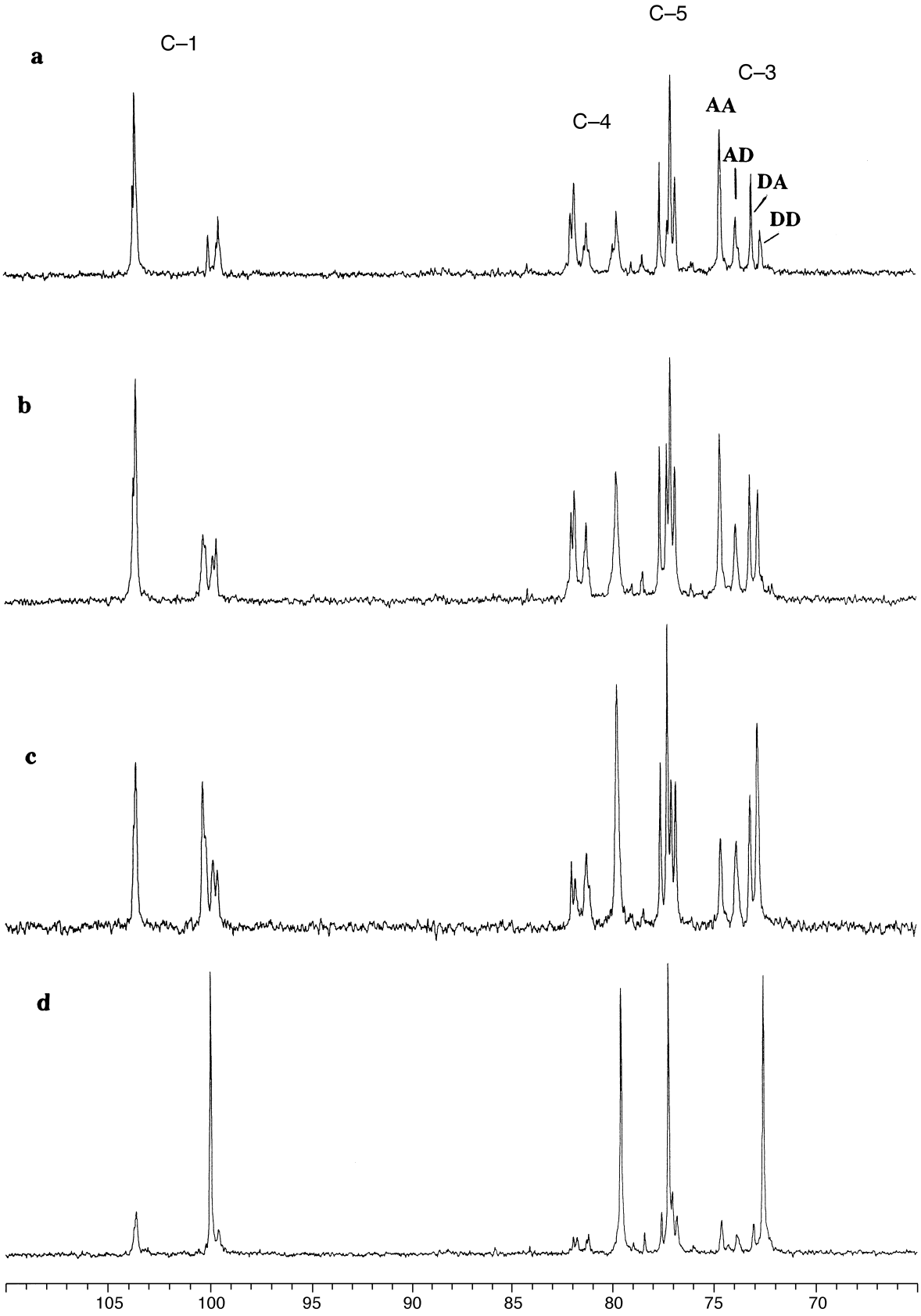


Fig. 3. Part of the ^{13}C NMR spectra (125 MHz) of a solution of the chitosan substrate with (a) $F_A = 0.681$ and (b) the chitosans produced by incubating the chitosan substrate with increasing amounts of chitin deacetylase from *M. rouxii*: 6 mU of enzyme for 45 min; (c) 11 mU for 45 min; and (d) 70 mU for 2 h.

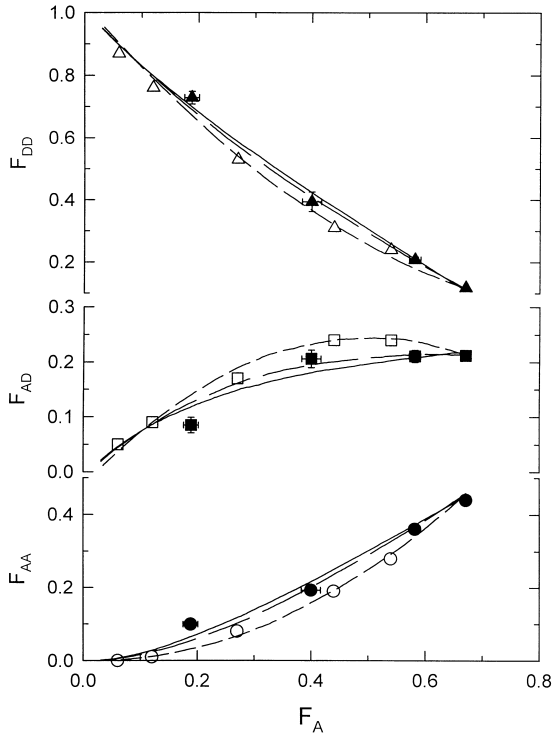


Fig. 4. Experimentally determined diad fractions F_{AA} (○, ●), F_{AD} (□, ■) and F_{DD} (△, ▲) of the chitosan substrate with $F_A = 0.681$ (filled symbols) and chitosans which were *N*-deacetylated under homogeneous conditions (open symbols, data from ref. [8]) together with samples that were further enzymatically deacetylated with the chitosan with $F_A = 0.681$ as substrate. The calculated diad fractions (originating from the substrate) for chitosan deacetylase acting on the chitosan with F_A of 0.681 as substrate assuming single-chain attack (——), multiple attack with degree of multiple attack equals three (— — —), and multiple-chain attack (– – –).

polarity of the enzyme was preferentially towards the reducing end. With respect to the polarity of CDA, it is not possible to compare the rate of deacetylation at the reducing and non-reducing end, since the chitosan substrate was prepared by nitrous acid depolymerization and subsequently reduced, thereby creating a 2,5-anhydro-D-mannitol unit at the "reducing end". However, the non-reducing end ^{13}C signals of **A**- and **D**-units of C-5 are well separated from each other and from the other resonances in the ^{13}C NMR spectrum, and are found at 78.5 ppm (**A**-unit) and 78.9 ppm (**D**-unit) [4]. Note that the fractional content of **A**-units at the non-reducing end of the substrate is about the same as in the chitosan chain [22]. No apparent increase in the non-reducing end **D**-units relative to **A**-units upon enzymatic deacetylation from the substrate with $F_A = 0.681$ [Fig. 3(a)] to the chitosan with $F_A = 0.188$ [Fig. 3(d)] was observed. This result may be explained by (i) a polarity of CDA towards the reducing end, or (ii) the inability of CDA to deacetylate the residue at the non-reducing end independently of its polarity.

The mode of action of the chitin deacetylase from *M. rouxii* suggests that the cell-wall chitosan from this fungus may be a block polymer. However, the naturally occurring chitosan isolated from *M. rouxii* is highly deacetylated, with F_A around 0.1 ([23]; Vårum and Martinou, unpublished results), and it is difficult to analyze the distribution of acetyl groups in chitosans with such low F_A-values with sufficient precision. It should, however, be noted that both F_A and the distribution of **A**- and **D**-units in fungal cell wall chitosans are important for both their biodegradability by chitinases/chitosanases and the identity of the resulting oligosaccharides with respect to chain length and acetylation pattern, as different enzymes may selectively cleave one or more of the four different glycosidic linkages (**A–A**, **A–D**, **D–A** and **D–D**) in partially *N*-acetylated chitosans [4,24,25].

Present applications of chitosan are based on relatively highly deacetylated chitosans (F_A less than 0.2) which are produced by thermochemical alkaline deacetylation of chitin. We have here demonstrated that by further enzymatic deacetylation of a water-soluble chitosan, chitosans with a distribution of **A**- and **D**-units deviating from the random (Bernoullian) distribution may be produced. A future challenge in this field would be to prepare partially *N*–acetylated chitosans by enzymatic deacetylation with chitin as raw material, a process which so far has only been achieved *in vivo* by the chitosan-containing fungi.

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