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Chemical composition of *O*-(carboxymethyl)-chitins in relation to lysozyme degradation rates

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O-Carboxymethyl chitin (CM-chitin) is a water-soluble derivative of chitin, be carboxymethylated in 3- and 6-positions to varying extents.

CM-chitins with a total fraction of *O*-substitution from 0.65 to 1.5 were prepared, and characterised by high-field proton n.m.r. spectroscopy, allowing the determination of the fraction of *O*-substitution in positions 3 and 6, together with the fraction of *N*-acetylated units (F_A). The carboxymethylation in position 6 was found to be larger than in position 3 in all fractions, confirming that the reactivity at OH-6 is larger than at OH-3 in the carboxymethylation process. F_A of the CM-chitins varied from 0.85 to 0.95, showing no systematic variation with the total degree of carboxymethylation.

These well-characterised CM-chitins were degraded with hen egg white lysozyme. Initial degradation rates, *r*, were determined from plots of the viscosity decrease ($\Delta\ 1/[\eta]$) against time of degradation at pH 5.3 and ionic strength 0.1 (M). It was found that the CM-chitin-lysozyme system obeyed a Michaelis–Menten type of kinetics. The time course of degradation of CM-chitin with lysozyme was non-linear, suggesting different degradation rates of different sequences in CM-chitin. All *r*-values of CM-chitins were higher than the highest rate determined for a partially *N*-acetylated chitosan with F_A of 0.6. The *r*-values were found to increase with increasing F_A of the CM-chitins, while *r*-values decreased with increasing fraction of carboxymethylation. Furthermore, the results suggest that carboxymethylation in position 3 has a larger effect on reducing the rate than carboxymethylation in position 6. © 1997 Published by Elsevier Science Ltd

INTRODUCTION

Chitin is a $\beta(1\rightarrow4)$ linked structural polysaccharide which is composed of mainly 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc; A-unit). Chitin is found in the skeletal materials of crustaceans, molluscs, insects, fungi and yeasts and is one of the most abundant natural polymers with a large unexplored commercial potential.

Chitosan, partially *N*-deacetylated chitin, contains in addition to GlcNAc also 2-amino-2-deoxy-β-D-glucopyranose (GlcN; D-unit). Chitosan is soluble in dilute acid, as opposed to chitin which is known to be insoluble in aqueous solvents. One limitation of the use of most chitosans is that they are only soluble at pH-values below 6. For a number of applications, especially in the biomedical field, solubility at physiological pH-values is desirable. The synthesis of new soluble derivatives of chitin and chitosan by chemical modification has therefore been the subject of extensive research in the past decade.

O-Carboxymethylchitin (CM-chitin) (see Fig. 10a) is a negatively charged ether derivative of chitin, with a chemical structure resembling that of carboxymethyl-cellulose (CMC), and is in contrast to chitin and commercial chitosans soluble at neutral pH-values. The water solubility of CM-chitin becomes apparent when the fraction of substitution is more than 0.6 (Tokura *et al.*, 1983). CM-chitin has potential applications in industry, medicine and pharmacy, personal care, agriculture and biotechnology.

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Lysozyme (EC 3.2.1.17) hydrolyses not only the $\beta(1\rightarrow4)$ glycosidic linkage between *N*-acetyl-D-muramic acid and GlcNAc in the bacterial cell wall (Fig. 10b), but also the $\beta(1\rightarrow4)$ glycosidic linkage in chitin. However, the hydrolysis proceeds very slowly in highly crystalline chitins. The active binding site of lysozyme consists of six subsites named A, B, C, D, E and F, which can each bind one sugar ring. The cleavage takes place between the sugar units bound to subsite D and E. The binding between lysozyme and chitin-oligosaccharides has been extensively studied, showing an increasing binding constant from monomer to trimer (Imoto *et al.*, 1972). It has recently been shown that while a fully deacetylated chitosan has no detectable binding to lysozyme, a chitosan-containing monoacetylated sequences (i.e., an A-unit where both nearest neighbours are D-units) binds approx. 20-times more strongly than the dimer $(GlcNAc)_2$ to lysozyme, without any degradation taking place (Kristiansen *et al.*, 1996). Initial degradation rates of water-soluble chitosans (F_A from 0.1 to 0.6) with lysozyme from hen egg white and human milk has been determined (Nordtveit *et al.*, 1994, 1996). It was shown that the initial degradation rates increased very strongly with increasing F_A. Nishimura *et al.* (1986) found that the degradation of CM-chitins with lysozyme decreased when the fraction of carboxymethylation increased from 0.8 to 1.2, which was attributed to the increased carboxymethylation at position 3. The rate of hydrolysis seems to be influenced by the fraction of substitution, which depends on the reaction conditions (Tokura *et al.*, 1983), but very little quantitative data on the effect of different chemical composition on the degradation rate have been reported.

The *O* and *N*-carboxymethylation of chitosan has previously been reported (Rinaudo *et al.*, 1992a, b; Le Dung *et al.*, 1994), and the fraction and distribution of substituents on each position were determined by n.m.r measurements. The carboxymethylation was reported to follow the order of reactivity: $OH\text{-}6 > OH\text{-}3 > NH_2$. Tokura *et al.* (1983) have studied the fraction and position of substitution of CM-chitin by ^{13}C-n.m.r. It was found that the resulting CM-chitin was mainly substituted in position 6. Gardner and Blackwell (1975) proposed that attack on OH-6 seems to be particularly favoured because OH-3 may be engaged in hydrogen bonding.

^1H-n.m.r. spectroscopy of hydrolysates of CM-cellulose has been used to determine the distribution of the carboxymethyl groups and hence the total fraction of substitution (Ho & Klosiewicz, 1980). It was shown that the reactivity of the hydroxyl groups in cellulose toward carboxymethylation varied in the order $OH\text{-}2 > OH\text{-}6 > OH\text{-}3$ (Ho & Klosiewicz, 1980; Parfondry & Perlin, 1977; Reuben & Conner, 1983). Except for early results, it appears that literature data agree that the reactivity of the hydroxyl group at C-2 toward carboxymethylation is slightly greater than at C-6.

We now report on the use of high-field proton n.m.r-spectroscopy to characterise CM-chitins. The fraction of *O*-carboxymethylation at positions 3 and 6, the total fraction of carboxymethylation and F_A was determined. In addition, ^{13}C-n.m.r-spectroscopy was used to characterise the CM-chitins. The initial degradation rates of CM-chitins with lysozyme were determined by the previously reported viscometric assay in an attempt to correlate the rates to the chemical composition of the CM-chitins.

MATERIALS AND METHODS

CM-chitins

CM-chitins 2–10 were prepared from alkali-chitin and monochloroacetic acid in 2-propanol at room temperature (Tokura *et al.*, 1983). CM-chitin 1 was provided by Nova Chem. Ltd., Halifax, Canada. The characterisation of the CM-chitins is given in Table 1.

Depolymerisation of CM-chitin for n.m.r. analysis

CM-chitin (30 mg) was dissolved in 4.5 ml deionised water, 0.2 M NaCl (1.5 ml) was then added, and pH was adjusted to 4 with HCl. The samples were depolymerised at 100°C for 4 h and then lyophilised. Each sample with a number-average degree of polymerization (DP_n) of approx. 50 was dissolved in D_2O and lyophilised (repeated three times in order to minimise the HOD signal).

^1H- and ^{13}C-n.m.r. spectroscopy

The samples were dissolved in D_2O in 5-mm tubes and the pD was adjusted with DCl or NaOD ($\sim$15 mg/ml, pD 2 for ^1H; $\sim$35 mg/ml, pD 4 for ^{13}C). 500 MHz ^1H-n.m.r. spectra were recorded at 20 and 90°C on a Bruker AM-500 spectrometer using 32 768 data points, 3.6 kHz spectral width, a 30° pulse and a pulse recycling time of 5 s. ^1H-decoupled 100 MHz ^{13}C-n.m.r. spectra were recorded at 20°C on a Jeol EX-400 spectrometer using 32 768 data points, 30 kHz spectral width, a 45° pulse and a pulse recycling time of 1.5 s. Chemical shifts (δ) are referred to external TMS relative to 3-(trimethyl)-silyl-propionic acid-d_4-sodium salt ($\delta = 2.4$ ppm for ^{13}C; $\delta = 0.00$ ppm for ^1H). The relative intensities of the resonances were determined by a computer program developed in our laboratory by Ole Einar Bakøy.

Viscosity assay for enzymatic hydrolysis of carboxymethylchitin

CM-chitin (30 mg) was dissolved in 6 ml deionised water, diluted with 6 ml 0.2 M KCl (unless for the

Table 1. Characterization of the CM-chitins with respect to fraction of acetylated units (F_A), carboxymethylation in position 6 (f_6) and position 3 (f_3), total carboxymethylation ($F=f_6+f_3$) and intrinsic viscosity ([η]).

	F_A	f_6	f_3	F	[η](ml/g)
CM-chitin 1	0.95	1.01	0.47	1.48	1092
CM-chitin 2	0.86	0.6	0.17	0.77	360
CM-chitin 3	0.93	0.67	0.19	0.86	415
CM-chitin 4	0.95	0.67	0.19	0.86	570
CM-chitin 5	0.90	0.82	0.24	1.06	560
CM-chitin 6	0.90	0.77	0.21	0.98	610
CM-chitin 7	0.91	0.72	0.32	1.04	602
CM-chitin 8	0.85	0.48	0.17	0.65	469
CM-chitin 9	0.93	0.94	0.35	1.29	70
CM-chitin 10	0.89	1.1	0.34	1.44	277

variation in ionic strength) and pH was adjusted to 5.3 with HCl. Lysozyme from hen egg white (Sigma L 6876) was dissolved in deionised water and diluted to a concentration of 0.75 mg/ml. The viscosity assay and the determination of initial degradation rates were performed at 20°C as described previously (Nordtveit *et al.*, 1994).

RESULTS AND DISCUSSION

Identification of resonances in the spectra

The 500-MHz ^{1}H-n.m.r. spectrum of CM-chitin 9 is shown in Fig. 1, and seven resonances (Fig. 1a–g) were identified (Table 2). The spectra were obtained at two different temperatures (20 and 90°C) in order to shift the solvent signal (HOD) from resonances b, c and d. The assignment of the resonances of H-1**D** (a), H-1**A** (b), H-2**A**, in addition to the H-3/6 protons (e), H-2**D** (f) and the acetyl-protons (g) were based on the reported spectrum of partially *N*-acetylated chitosans (Vårum *et al.*, 1991a) and the ^{1}H-n.m.r. chemical shift data for D-glucose and its mono-carboxymethylated derivatives (Kragten *et al.*, 1992). The carboxymethylation had little influence on the chemical shifts of the ring protons, at mostly 0.15 ppm. Hence, the chemical shift of carboxymethyl protons in a monosubstituted residue is not expected to be dramatically different from the chemical shift of the same protons substituted in a disubstituted residue.

The resonances of the 3- and 6-substituted carboxymethyl-protons (-O-CH$_2$-COOD) of CM-chitin occur in the spectral region between 4.1 and 4.6 ppm. This assignment is based on the expected upfield shift of the protons upon increasing the pD from 2 to 5 (Fig. 2), the absence of proton resonances in this region for partially *N*-acetylated chitosans (Vårum *et al.*, 1991a) and the reported chemical shifts (4.1–4.5 ppm) of the methylene protons of CMC (Ho & Klosiewicz, 1980).

Resonance (c) (Fig. 1) is assigned to one of the two chemically non-equivalent 3-substituted methylene protons, whereas resonance (d) is assigned to the other 3-substituted methylene proton (probably as the shoulder on the downfield side) in addition to the two 6-substituted methylene protons. In the ^{1}H-n.m.r. spectrum taken at pD 5 (Fig. 2) all four different methylene protons display individual resonances. The higher intensity of resonance (d) compared with resonance (c) is consistent with the expected higher reactivity of the hydroxyl group at carbon 6 compared with the hydroxyl group at carbon 3. The identification of the 6-substituted methylene protons was also confirmed by a 2D-heterocorrelated n.m.r. experiment (data not shown). In addition, the large coupling constant of 16.5 Hz of resonance (c) is in accordance with a geminal coupling constant.

Possible shielding effects of an *O*-carboxymethyl group on the ^{13}C nuclei of CM-chitin were examined by reference to 2-, 3- and 6-*O*-carboxymethyl derivatives of D-glucose (Perlin *et al.*, 1970). From these results, along with the ring carbon resonances of unsubstituted, partially *N*-acetylated chitosans (Vårum *et al.*, 1991b), most of the resonances were solved. The introduction of an *O*-carboxymethyl group will usually promote strong deshielding, i.e., large downfield shifts (7–11 ppm), for the ^{13}C-nucleus in α-position to the substituent and smaller shielding, i.e., upfield shifts (1–2 ppm), for the ^{13}C-nucleus in β-position to the substituent. Hence, signals that exhibit such large, downfield shifts relative to those of partially deacetylated chitins, are used to assign the substituent groups in the polymer. Figure 3 shows the 100 MHz proton decoupled ^{13}C-n.m.r. spectrum of CM-chitin 3. The resonance of C-6 shifted downfield (from 63 to 72 ppm) upon carboxymethylation, while the resonance of C-5 shifted upfield (from 77.5 to 76 ppm). The carboxymethylation of the hydroxyl group in 3-position shifted the resonance of C-3 downfield from 75 to 79 ppm and the resonance of C-2 upfield from 57 to 58 ppm. We have assigned the resonance at 74 ppm to be the –CH$_2$– groups both from C-3 and C-6. ^{13}C-n.m.r spectra of *O*, *N*-carboxymethyl chitosan has been interpreted by Rinaudo *et al.* (1992a, b). These authors, however, do not take the shifts associated

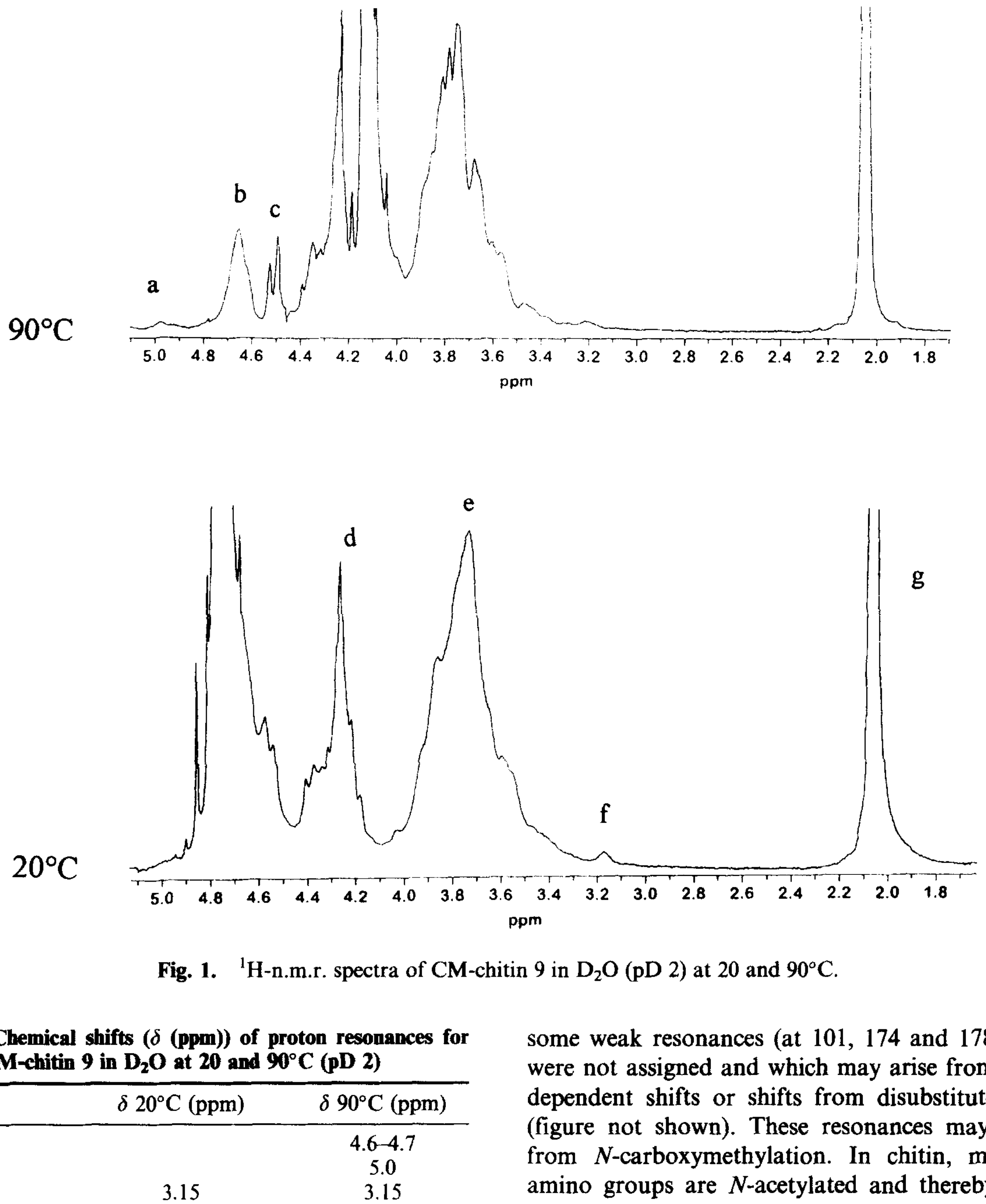

Fig. 1. ^{1}H-n.m.r. spectra of CM-chitin 9 in D_2O (pD 2) at 20 and 90°C.

Table 2. Chemical shifts (δ (ppm)) of proton resonances for CM-chitin 9 in D_2O at 20 and 90°C (pD 2)

Proton	δ 20°C (ppm)	δ 90°C (ppm)
H-1A		4.6–4.7
H-1D		5.0
H-2D	3.15	3.15
H-3′ (1 proton)		4.5
H-6′ (2 protons) +H-3′ (1 proton)	4.1–4.45	
Acetyl-H (3 protons)	2.05	2.05
H-2A+H-3/6 (6 protons)	3.4–4.1	

′ Refers to protons of a carboxymethylated unit.

with the substitution into consideration. From the ^{13}C-n.m.r. spectrum we can deduce that the carboxymethylation at C-6 is higher than at C-3 in reasonable agreement with ^{1}H-n.m.r. data.

In the ^{13}C-n.m.r. spectra of the CM-chitins with the highest fraction of carboxymethylation we observed some weak resonances (at 101, 174 and 178 ppm) that were not assigned and which may arise from sequence-dependent shifts or shifts from disubstituted residues (figure not shown). These resonances may also arise from *N*-carboxymethylation. In chitin, most of the amino groups are *N*-acetylated and thereby protected from possible carboxymethylation, in contrast to chitosan, which has a higher fraction of amino groups which may be carboxymethylated along with the hydroxyl groups (Nud'ga *et al.*, 1973; Hayes, 1986). As the F_A-values are higher than 0.85, the fraction of *N*-carboxymethylation should be very limited. In a ^{1}H-n.m.r. spectrum of *N*-carboxymethylchitosan (Rinaudo *et al.*, 1992a), the *N*-carboxymethyl-protons give characteristic resonances between 3 and 3.5 ppm, different from the H-2D signal at 3.15 ppm. These resonances were absent in our spectra.

Determination of the fraction of substitution

From the assignment of the proton resonances, it was possible to determine the fraction of substitution in

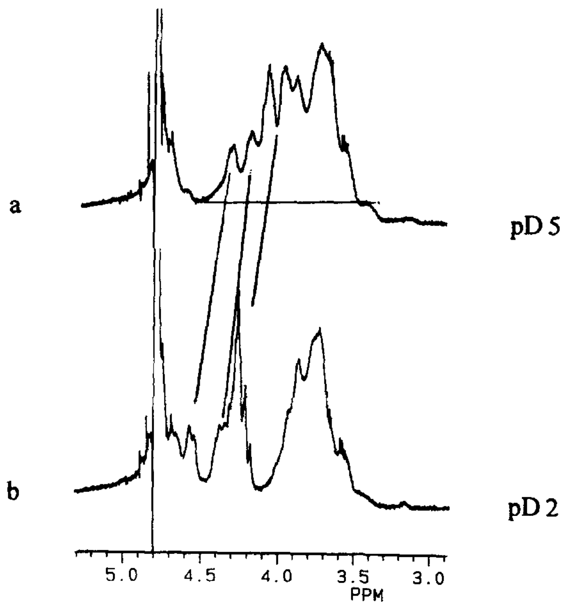

Fig. 2. ^{1}H-n.m.r. spectra of CM-chitin 1 in D_2O at pD 2 and 5 (20°C).

both 3- and 6-positions. The ^{1}H-n.m.r. spectra were recorded at two different temperatures, and the intensities were normalised by using the acetyl peak. The fraction of carboxymethylation in 3-position, f_3, is given as the area of *one* carboxymethyl proton from the 3-position divided by the total area of H-1:

$$f_3 = I_c/(I_b + I_f)$$

The value of H-1_{total} can also be obtained by summing $I_a + I_b$. The fraction of carboxymethylation in 6-position, f_6, is given as the area of *one* carboxymethyl proton from the 6-position divided by the total area of H-1:

$$f_6 = 1/2(I_d - I_c)/(I_b + I_f).$$

The total fraction of *O*-carboxymethylation, *F*, is then $f_3 + f_6$. Note that this number can vary between 0 and 2.

Figure 4 shows the fraction of 3- and 6-carboxymethylation as a function of total fraction of carboxymethylation, as interpreted from the ^{1}H-n.m.r. data. The carboxymethylation at position 6 increases with a slight negative curvature as the total fraction of carboxymethylation increases, while the carboxymethylation at position 3 increases with a positive curvature with increasing total carboxymethylation. The carboxymethylation at position 6 is always larger than at position 3. Hence, the reactivity at OH-6 is larger than at OH-3. This result is in agreement with what is reported for CMC (Ho & Klosiewicz, 1980), where the reactivity of the hydroxyl groups varied in the order OH-2 > OH-6 > OH-3. Our result is also in agreement with the results from carboxymethylated chitosan, where the order of reactivity of the three possible positions was: OH-6 > OH-3 > NH_2 (Rinaudo *et al.*, 1992a, b). Tokura *et al.* (1983) found no carboxymethylation in position 3 for CM-chitins with fraction of 6-substitution between 0.6 and 0.8. However, their ^{13}C-n.m.r. spectra were obtained with undegraded CM-chitin at low magnetic field.

Lysozyme degradation of CM-chitin

The effect of substrate concentration on the initial rate of lysozyme degradation of CM-chitin 1 (*r*) as given in Fig. 5 shows Michaelis–Menten-type kinetics. Substrate saturation occurs at a CM-chitin concentration of approx. 0.23 g/dl, as previously determined for partially *N*-acetylated chitosans (Nordtveit *et al.*, 1994). The decrease in rates above saturation may be explained by an underestimation of the rate of chain splitting by the viscometric assay, when the reduced viscosity is increasingly higher than the intrinsic viscosity as discussed previously (Nordtveit *et al.*, 1994). It is also possible that the increased ionic strength associated with the increased CM-chitin concentration may reduce the rate (see below).

When plotting initial degradation rates (*r*) against lysozyme concentration (Fig. 6, curve 1), we find a non-linear relationship despite the assumption of substrate saturation at 0.23%. This effect may be due to long-range electrostatic interactions between the negatively charged CM-chitin chain and the positively charged lysozyme according to the following rationale: the local concentration of lysozyme around the CM-chitin chains, C_{LP}, will always be higher than in the bulk of the

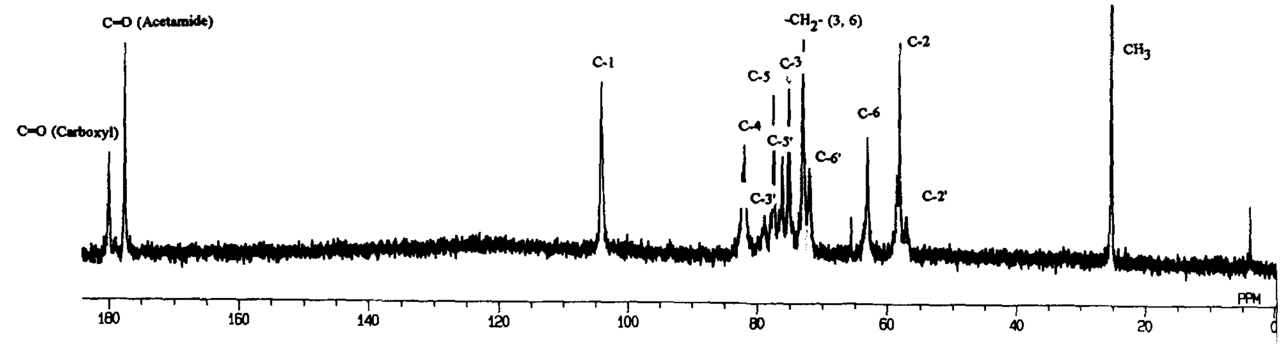

Fig. 3. ^{13}C proton decoupled n.m.r. spectra (100 MHz) of a solution of CM-chitin 3 (at pD 4 and 20°C).

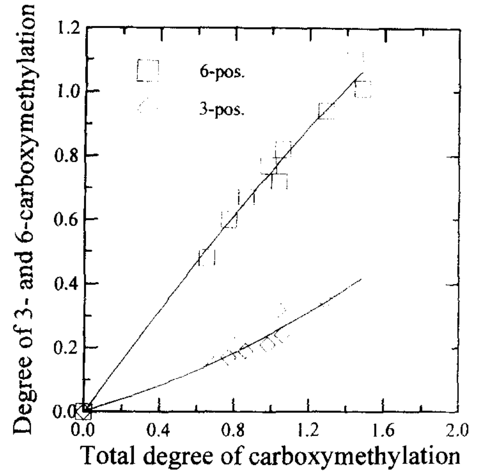

Fig. 4. The fraction of substitution in 3- and 6-position, f_3 and f_6, plotted against the total fraction of carboxymethylation, F.

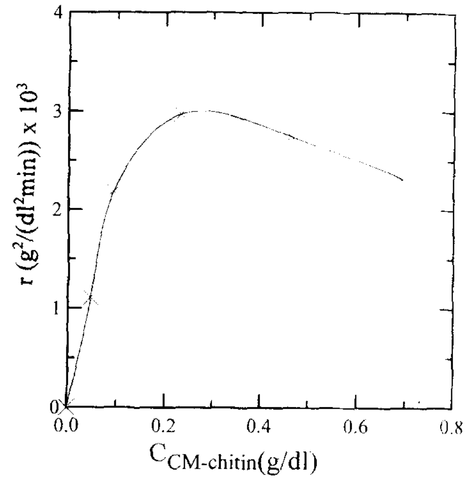

Fig. 5. Inital lysozyme degradation rates, r, of CM-chitin 1 (at pH 5.3 and 20°C) as a function of CM-chitin concentration. Concentration of lysozyme was 0.058 mg/ml.

solution, C_{LB}. The ratio C_{LP}/C_{LB} will always decrease with increasing total concentration of lysozyme, C_{L0}, and result in a decreasing ratio C_{LP}/C_{L0} and a negative curvature in a plot of r against C_{L0} as observed in curve 1. Addition of salt will decrease the ratio C_{LP}/C_{LB} and reduce r if such electrostatic interactions operate. In order to investigate this, salt concentration was increased from 0.1 to 0.5 M in curve 2 (Fig. 6). A reduction in r was observed, in agreement with the above discussion. The increased salt concentration which results in a more uniform concentration of lysozyme in the solution will also be expected to make C_{LP}/C_{L0} less dependent on C_{L0}, and give a more linear curve, as observed in Fig. 6. The substrate concentration was also increased well above saturation (curve 3) without obtaining full linearity (but a reduction in r as discussed in connection with Fig. 5). From the data in Fig. 5 and Fig. 6 we conclude that the enzyme is saturated at a concentration of CM-chitin of 0.23%, and that the apparent deviations from Michaelis–Menten-type kinetics are caused by an approximation in the viscosity assay and charge effects.

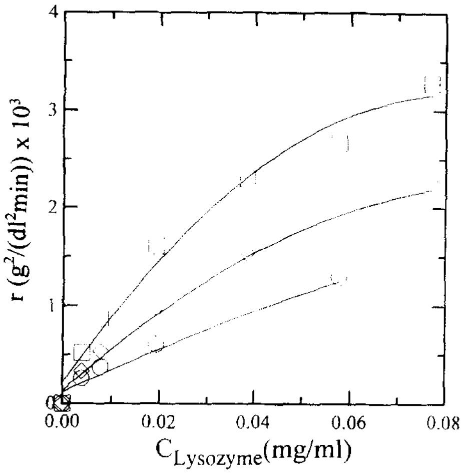

Fig. 6. Initial lysozyme degradation rates, r, on CM-chitin 1 (pH 5.3 and 20°C) as a function of lysozyme concentration. Curve 1: □, $C_{\text{CM-chitin}} = 0.23$ g/dl and 0.1 M KCl; Curve 2: O, $C_{\text{CM-chitin}} = 0.23$ g/dl and 0.5 M KCl; Curve 3: ◇, $C_{\text{CM-chitin}} = 0.32$ g/dl and 0.1 M KCl.

The time course of degradation of four selected CM-chitins with lysozyme is non-linear, as seen from Fig. 7. The reason for the non-linearity may be different degradation rates of different sequences in CM-chitin, as is found for the partially N-acetylated chitosan–lysozyme system (Nordtveit *et al.*, 1994).

CM-chitins with fraction of substitution from 0.65 to 1.48 ($F_A > 0.85$ as determined by proton n.m.r. spectroscopy) were degraded with lysozyme, and the degradation rates varied with a factor of almost 7, from 0.025 to 0.17 (g^2/(dl^{2}min)). When comparing the absolute degradation rates of the CM-chitins with

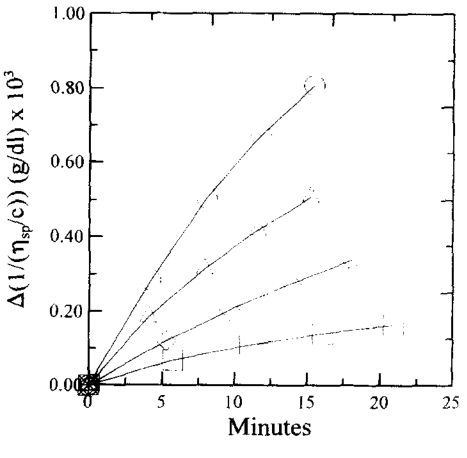

Fig. 7. Time course of degradation of CM-chitins (0.23 g/dl) with different fraction of carboxymethylation by lysozyme (0.058 mg/ml). □, CM-chitin 1; ◇, CM-chitin 8; O, CM-chitin 4; △, CM-chitin 6.

Table 3. Lysozyme degradation rates (*r*) and relative rates of degradation of the partially *N*-acetylated chitosans and the CM-chitin (with degradation rate of chitosan with $F_A = 0.12$ equal to 1)

Substrates	r (g^2/(dl^2 min))^a	Relative degradation rate
Chitosan $F_A = 0.12$	$0.05 \cdot 10^{-3}$	1
Chitosan $F_A = 0.42$	$2.31 \cdot 10^{-3}$	46.2
Chitosan $F_A = 0.60$	$18.68 \cdot 10^{-3}$	373.6
CM-chitin 1	$27.6 \cdot 10^{-3}$	552
CM-chitin 2	$57.6 \cdot 10^{-3}$	1150
CM-chitin 3	$148.9 \cdot 10^{-3}$	2980
CM-chitin 4	$170.8 \cdot 10^{-3}$	3420
CM-chitin 5	$115.1 \cdot 10^{-3}$	2300
CM-chitin 6	$108.5 \cdot 10^{-3}$	2170
CM-chitin 7	$66.2 \cdot 10^{-3}$	1320
CM-chitin 8	$55.0 \cdot 10^{-3}$	1100
CM-chitin 9	$38.6 \cdot 10^{-3}$	772
CM-chitin 10	$25.3 \cdot 10^{-3}$	506

Concentration of substrate: 0.231%. Concentration of lysozyme: 0.58 mg/ml.

[a] In two earlier papers (Nordtveit *et al.*, 1994, 1996), absolute values of degradation rates based on similar viscometric assay as used here are given, which are one thousand times too high. This does not affect any of the conclusions in the two papers, which are all based upon relative degradation rates.

water-soluble chitosans of different F_A-values (Table 3), we observe that all the CM-chitins were degraded with a rate higher than the chitosan with the highest fraction of acetylation ($F_A = 0.60$). A log–log plot of the lysozyme degradation rates of partially *N*-acetylated chitosans and CM-chitins vs F_A is given in Fig. 8. The results for partially *N*-acetylated chitosans with an average slope of 3.6 have previously been analysed in detail (Stokke *et al.*, 1995) and have been proposed to be a result of the requirements of consecutive sequences of 3–4 acetyl groups in the binding site of lysozyme to obtain maximal initial degradation rates. The plot for CM-chitins shows that the rates of degradation are in the range of what is expected when comparing with the rates of partially *N*-acetylated chitosans. A higher slope than for chitosans is suggested, meaning an even higher dependency for acetylated groups in the binding site of lysozyme. Since the variation in F_A in area (a) in Fig. 9 (see below) is limited and the fraction of carboxymethylation varies among the samples, further analysis of the effect of acetyl groups is not possible.

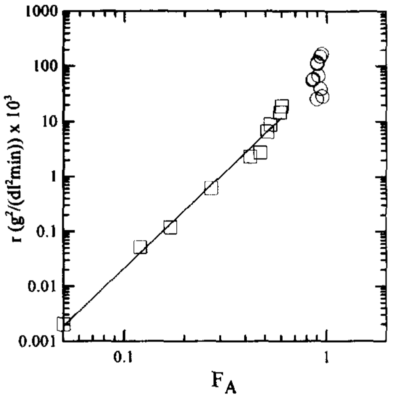

Fig. 8. Double logarithmic plot of initial degradation rates of lysozyme (0.58 mg/ml) on chitosans (□) and CM-chitins (○) vs the fraction of acetylated units (F_A) of the substrates. Concentration of substrates was 0.23 g/dl.

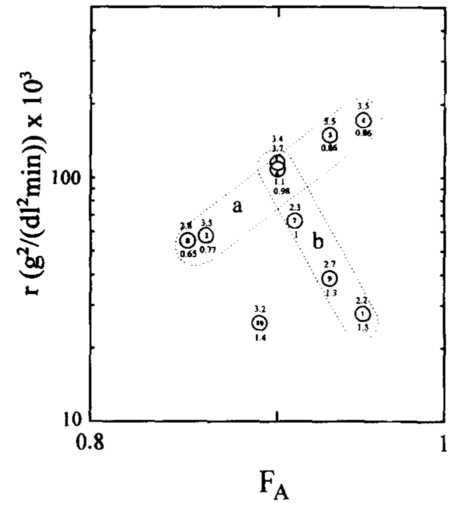

Fig. 9. Double logarithmic plot of initial degradation rates of lysozyme (0.058 mg/ml) on CM-chitins (0.23 g/dl) vs the fraction of acetylated units (F_A) of the substrates.The number above the symbols is the ratio 6/3-substitution, while the number below is the total fraction of substitution, *F*.

The effect of *O*-carboxymethylation may be seen in Fig. 9 where the initial rate of degradation is plotted against F_A in a log–log plot with an expanded F_A-axis. All experimental points except one are grouped into two areas in Fig. 9 marked area (a) and area (b). In area (a) the fraction of carboxymethylation (marked below the labels in the figure) is relatively low and increases rather systematically with increasing F_A. The ratio between 6 and 3 substitution (marked above the labels in the figure) is high and relatively constant in this area. In area (b) the fraction of carboxymethylation is generally higher and increases with F_A. The ratio between 6 and 3 substitution decreases with F_A to values lower than in area (a). The sample which is outside both areas (sample 10) is the only sample with both a high ratio between 6 and 3-substitution and a high fraction of carboxymethylation. This sample corresponds to the lowest initial degradation rate. Although the number of experimental points in Fig. 9 is limited, increasing r with increasing F_A is clearly seen in area (a), whereas an effect of decreasing r with increasing carboxymethylation is seen in area (b). The results inside area (b) also suggest that carboxymethylation in position 3 has a larger effect in reducing the degradation rate than carboxymethylation in position 6. Sample 10 also obeys the same rules, as the rate is rather high compared with sample 1, owing to a higher ratio between 6 and 3 substitution and taking into account the lower F_A-value for sample 10 in comparison with sample 1.

Furthermore, we want to give a physical interpretation of our data. An A-unit carboxymethylated in position 3 is chemically related to the *N*-acetyl-D-muramic acid (**M**) residue (with $-CH(CH_3)-COO^-$ substituted with a CH_2-COO^- group at position 3) in the natural substrate of lysozyme. The chemical structure of CM-chitin and the repeating unit of lysozyme's natural substrate is shown in Fig. 10a and Fig. 10b, respectively. It is known that the **M**-unit is responsible for the absolute specificity of binding of a **M**-unit (and not an **A**-unit) in subsites B, D, and F on lysozyme. Hence, from structural similarities it is reasonable to assume that the 3-carboxymethylated **A**-unit can only bind to subsite B, D and F. It follows that an increased fraction of 3-carboxymethylation would reduce the concentration of hexameric substrates which can be bound and cleaved with the highest rate, in accordance with our experimental results. Nishimura *et al.* (1986) found that lysozyme degradation of CM-chitins decreased when the fraction of carboxymethylation increased from 0.8 to 1.2, which was attributed to the increased carboxymethylation at position 3. Additionally, Miyazaki and Matsushima (1968) claimed that the new reducing ends of the saccharides produced by hydrolysis with lysozyme were an A-unit or an unit carboxymethylated in 3-position, but never a 6-carboxymethylated unit; i.e. a 6-carboxymethylated residue was never found at subsite D. It was suggested that the 6-carboxymethylated unit would only fit into subsite A, C and E. However, these strong restrictions could never fit with our data. We assume that a 6-carboxymethylated residue is excluded from subsite D, in agreement with Phillips (1966), that carbon atom 6 and its adjacent oxygen atom in the sugar residue bound to subsite D make uncomfortably close contacts with atoms in the enzyme molecule, unless this sugar residue is distorted from its normal conformation, which has the appearance of a chair. With these assumptions the probability, P, that a certain hexamer will fit into the catalytic site of lysozyme assuming a random carboxymethylation (Ho & Klosiewicz, 1980) may be expressed as:

$$P = (1 - f_3)^3(1 - f_6)f$$

where f is a scaling factor ($f = 1.5$). The data have been rated against $F_A = 0.95$, by multiplying the observed rates with the factor (0.95/the actual F_A)^4 where a requirement of four acetyl groups in the binding site of lysozyme is used. This is shown in Fig. 11, where the logarithm of the corrected initial rate of degradation is plotted against the total fraction of carboxymethylation. The plot shows a maximum at a total fraction of substitution (F) of 0.9, with decreasing rates at both lower and higher F-values. Although this result fits the above presented model in which steric hindrance is taken into consideration, the decrease in rates for the two samples with the lowest F-values does not agree with the model. Both the viscometric assay and the enzyme action could be affected, to reduced observed degradation rates by some limited solubility or microaggregation in solution. The low fraction of carboxymethylation could be the cause of this. In order to support this hypothesis by experimental results, four selected chitosans with increasing F-values (0.77, 1.06, 1.44, 1.48) were subjected to analysis by size exclusion chromatography (SEC) combined with low-angle laser light scattering (LALLS). It was found that the CM-chitin with the lowest F (0.77) contained a small fraction of microaggregates (about 5% by weight). However, it is difficult to relate the low r-values of the CM-chitins with the lowest F-values to this small fraction of microaggregation. Hydrophobic association of highly

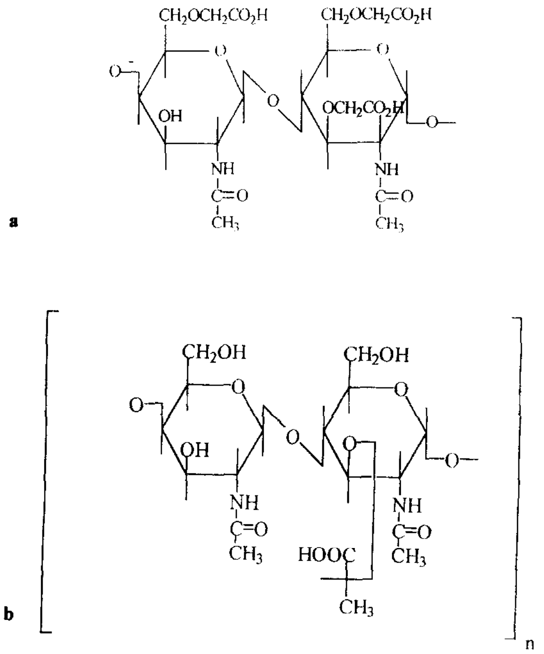

Fig. 10. (a) Chemical structure of CM-chitin. (b) Chemical structure of the repeating glycan unit of lysozyme's natural substrate, the cell wall peptidoglycans of certain bacteria.

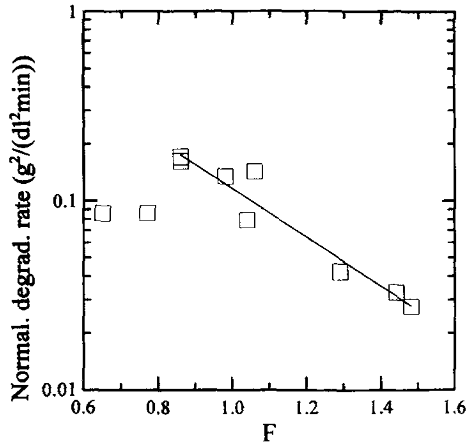

Fig. 11. The logarithm of the normalised initial degradation rates of lysozyme on CM-chitins vs the total fraction of carboxymethylation, *F*. The observed rates have been normalised to $F_A = 0.95$, by multiplying the observed rate with the factor (0.95/the actual $F_A)^4$ where a requirement of four acetyl groups in the binding site of lysozyme is used. The solid line shows the probability, *P*, that a certain hexamer will fit into the catalytic site of lysozyme; $P = (1-f_3)^3(1-f_6)\,f$, where f is a scaling factor ($f = 1.5$). The concentrations are as in Fig. 9.

acetylated chitosans has also been found (Ottøy *et al.*, 1996), and such interactions may slow down or hinder the action of lysozyme, even if the interaction is not strong enough to cause microaggregates which are visible in a SEC–LALLS experiment.

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