ELSEVIER

Carbohydrate Polymers **23** (1994) 253–260

0144-8617/94/$07.00

Degradation of fully water-soluble, partially *N*-acetylated chitosans with lysozyme*

Ragnhild J. Nordtveit, Kjell M. Vårum & Olav Smidsrød

Norwegian Biopolymer Laboratory (NOBIPOL), Division of Biotechnology, The Norwegian Institute of Technology (NTH), University of Trondheim, N-7034 Trondheim, Norway

(Received 24 June 1993; accepted 5 August 1993)

Chitosans, prepared by homogeneous *N*-deacetylation of chitin, with degrees of *N*-acetylation ranging from 4 to 60% (F_A = 0·04 to 0·60) exhibiting full water solubility and known random distribution of acetyl groups, were degraded with lysozyme. Initial degradation rates (r) were determined from plots of the viscosity decrease ($\Delta 1/[\eta]$) against time of degradation. The time course of degradation of chitosans with lysozyme were non-linear, while the time course of degradation of chitosans with an oxidative–reductive depolymerization reaction (using H_2O_2) showed the expected linear relationship for a first-order, random depolymerization reaction, independent of the chemical composition of the chitosan.

The effect of lysozyme concentration and substrate concentration on the initial degradation rates were determined, showing that this lysozyme–chitosan system obeys Michaelis–Menten kinetics.

The initial degradation rates of chitosan with lysozyme increased strongly with increasing fraction of acetylated units (F_A). From a Michaelis–Menten analysis of the degradation data that assumes different catalytic activities of lysozyme for the different hexameric substrates in the polysaccharide chain, it is concluded that the hexameric substrates that contain three–four or more acetylated units contribute mostly to the initial degradation rate when lysozyme degrades partially *N*-acetylated chitosans.

A chitosan with a very low fraction of acetylated units (F_A = 0·010) was studied as an enzyme inhibitor. Initial degradation rates of chitosan (with different F_A values) decreased as the inhibitor concentration increased, while the relative rates stayed constant, indicating that the *ratio* between initial reaction rates for productive sites (hexamers containing three–four or more *N*-acetylated units) are unaffected by non-productive sites, as deduced from the theory of competing substrates.

INTRODUCTION

Chitosan, which is (partially) *N*-deacetylated chitin, contains 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc; **A** unit) and 2-amino-2-deoxy-β-D-glucopyranose (GlcN; **D** unit) residues linked through (1→4) glycosidic linkages, and may be considered as a binary heteropolysaccaride. We have shown (Vårum *et al.*, 1991*a*,*b*) that the **A** and **D** units are randomly distributed along the chains in water-soluble, partially *N*-acetylated chitosans.

*Preliminary results of this work were presented at the 'International Symposium on Chitin Derivatives in Life Sciences', Hokkaido University, Sapporo, Japan in October 1990, and published in the proceedings book *Chitin Derivatives in Life Science*, 1992.

Lysozyme (EC 3.2.1.17) hydrolyses β-(1→4) glycosidic linkages, and its natural substrate is certain bacterial cell wall peptidoglycans. In addition, chitin is also a substrate for lysozyme. It has, however, been shown that lysozyme also may hydrolyse partially *N*-acetylated chito-oligomers. Fully acetylated chito-oligomers are hydrolysed much more effectively than the less acetylated oligomers, while completely *N*-deacetylated chito-oligomers are not degraded by lysozyme (Amano & Ito, 1978). Lysozyme degradability is one of the important properties of chitosan for its potential use in pharmacy and medicine.

Hirano *et al.* (1989) have determined large differences in lysozymic degradation rates for partially *N*-acetylated chitosans, and found an optimum degradation rate with ≈80% *N*-acetylated chitosan. The enzymatic reactions

were, however, determined in a two-phase system at pH 6·8, with the partially *N*-acetylated chitosans as suspended particles. As the solubility of chitosans depends on the degree of *N*-acetylation (Sannan *et al.*, 1976; Vårum *et al.*, 1992), differences in degradation rates may also be caused by differences in substrate solubility. In this regard it is interesting that it has recently been shown that chitosans prepared by heterogeneous *N*-deacetylation of chitin may be separated into an acid-soluble fraction and an acid-insoluble fraction of different chemical composition (Vårum *et al.*, 1992). Sashiwa *et al.* (1990) found that the highest lysozyme susceptibility was obtained with a 30% *N*-acetylated soluble chitin; however, no comparable degradation rates were determined.

The active binding site of lysozyme binds six sugar rings, and the purpose of the present work was to obtain further understanding of the mechanism of lysozyme degradation of partially *N*-acetylated chitosans. Initial degradation rates were compared using chitosans of different chemical composition: that is, fraction of acetylated units (F_A), with known random (Bernoullian) distribution of acetyl groups, and full water solubility (one-phase system) at the chosen conditions for degradation. The initial degradation rates were determined by a viscosimetric assay, which monitors degrees of scission less than one percent (Smidsrød *et al.*, 1965). This low degree of scission makes it possible both to correlate the reaction rates to the chemistry of the intact chitosans and to neglect any transglycosylation reactions.

Table 1. Characterization of chitosan fractions used in this study. The fraction of acetylated units (F_A) and the diad frequencies were determined from high-field proton NMR spectroscopy (Vårum *et al.*, 1991*a*) and the intrinsic viscosities ([η]) of the chitosan (free-amine form) were determined as previously described (Draget *et al.* (1992). 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)
1	0·04	0·01	0·03	0·92	340
random	0·04	0·00	0·04	0·92	
2	0·12	0·03	0·09	0·80	370
random	0·12	0·01	0·11	0·77	
3	0·17	0·05	0·12	0·71	670
random	0·17	0·03	0·14	0·69	
4	0·27	0·08	0·20	0·52	450
random	0·27	0·07	0·20	0·53	
5	0·42	0·20	0·22	0·36	840
random	0·42	0·18	0·24	0·34	
6	0·47	0·24	0·23	0·30	610
random	0·47	0·22	0·25	0·28	
7	0·51	0·27	0·24	0·25	440
random	0·51	0·26	0·25	0·24	
8	0·53	0·30	0·23	0·24	460
random	0·53	0·28	0·25	0·22	
9	0·59	0·39	0·20	0·21	910
random	0·59	0·35	0·24	0·17	
10	0·60	0·40	0·21	0·18	820
random	0·60	0·36	0·24	0·16	

MATERIALS AND METHODS

Chitosans

Chitin was isolated from fresh shrimp shells. Chitosans prepared by homogeneous *N*-deacetylation of chitin (Sannan *et al.*, 1978) with degrees of *N*-acetylation from 4% to 60% (F_A = 0·04 to 0·60) were used. The chitosans were characterized by high-field NMR spectroscopy (Vårum *et al.*, 1991*a*,*b*) and by their intrinsic viscosities (Draget *et al.*, 1992). Table 1 shows the chemical compositions, diad frequencies and intrinsic viscosities for the different chitosans.

Inhibitor

A chitosan with a very low fraction of acetylated units was used as inhibitor. This chitosan was prepared by further heterogeneous *N*-deacetylation of a commercial chitosan (F_A = 0·15) obtained from Pronova Biopolymer (Drammen, Norway). The degree of *N*-acetylation was determined by proton NMR spectroscopy (Vårum *et al.*, 1991*a*) to be 1·00% (F_A = 0·010). The chitosan was then degraded using $NaNO_2$ (Allan & Peyron, 1989), and reduced conventionally with $NaBH_4$, to a number-average molecular weight of about 10 000 ([η] = 20 ml/g).

Lysozyme

Lysozyme from chicken egg white was used (Sigma L-6876, Grade I) without further purification.

Viscosity assays

Assay for enzymatic hydrolysis of chitosan

The desired concentration of chitosan (0·046–0·69 g/dl) was made by dissolving the chitosan in 1% acetic acid/sodium acetate buffer solution (6 ml) at pH 4·5 (in some experiments inhibitor was added) and diluting the chitosan solution with an equal volume of 0·2 M KCl. Lysozyme (0·040–0·77 mg/ml) was dissolved in buffer solution containing 0·1 M KCl. Lysozyme solution (0·35 ml) and chitosan solution (4·2 ml) were mixed, filtered through a 0·8 μm membrane filter (Millipore Corp., Bedford, USA), and the relative viscosities were determined in a Schott Geräte Ubbelohde capillary viscosimeter at 20°C as a function of time. The accuracy in the determination of the initial slope (k; see Theory section for details)

from the time course of degradation curves (see Fig. 2) for a chitosan with $F_A = 0{\cdot}60$ was determined to ±15%. However, when comparing degradation rates (r-values) for chitosans of different chemical composition, certain assumptions are also involved in converting the slope of the time course of degradation to actual degradation rates; i.e. the monomer molecular weight and the a-value are independent of the degree of acetylation (see the Theory section).

Assay for oxidative–reductive degradation (ORD) of chitosan with H_2O_2 in the presence of Fe^{3+}

Chitosan solutions (0·25 g/dl) were prepared as described in the enzymatic assay, and 8·82 M H_2O_2 (90 µl) and 10 mM $FeCl_3$ (0·7 ml) were added to the chitosan solution (6 ml). The relative viscosities were determined as a function of time.

RESULTS AND DISCUSSION

Characterization of chitosan fractions

The chemical composition (F_A), the diad frequencies, (F_{AA}, F_{AD} (=F_{DA}) and F_{DD}) together with the intrinsic viscosities for the different chitosans, which were prepared by homogeneous N-deacetylation of chitin (Sannan *et al.*, 1978), are given in Table 1. The diad frequencies determined from the high-field proton NMR spectra of the chitosan samples were close to the calculated random (Bernoullian) diad distribution. Diad and triad frequencies determined from high-field ^{13}C-NMR spectra (data not shown) confirmed the random distribution of **A** and **D** units, as previously described (Vårum *et al.*, 1991*b*).

Time course of a random degradation of chitosans with different chemical composition

For a first-order, random depolymerization of a relatively stiff polysaccharide (Mark–Houwink–Sakurada constant a close to 1), one would expect a linear curve when $\Delta(1/[\eta])$ is plotted against time of degradation (Smidsrød *et al.*, 1967; see the Theory section). This was confirmed by determining the time course of degradation of chitosans with different chemical compositions by H_2O_2 in the presence of $FeCl_3$, as shown in Fig. 1. Linear plots were obtained independent of the fraction of acetylated units (F_A). It has recently been shown that the stiffness of the chitosan chain is dependent on its chemical composition (Wang *et al.*, 1991; Anthonsen *et al.*, 1993). An increase in the a-value from 0·58 ($F_A = 0$) to 1·06 ($F_A = 0{\cdot}6$) has been reported (Anthonsen *et al.*, 1993). However, these differences in chain stiffness between chitosans do not seem to influence the linearity of their time course of degradation for a random depolymerization of the chitosans.

Time course of lysozyme degradation of chitosans of different chemical composition

Figure 2 shows $\Delta(1/(\eta_{sp}/c))$ plotted against the time of degradation for a series of chitosans with increasing F_A, using a constant concentration of lysozyme. It is clearly seen that the degradation rates increase strongly when F_A increases. The curvature in the plots indicates a non-random degradation of chitosan by lysozyme, parallel to what has been reported for a non-random hydrolysis of cellulose (Marchessault & Rånby, 1959). The curvature in the plots is present for all chitosans (not easily seen from

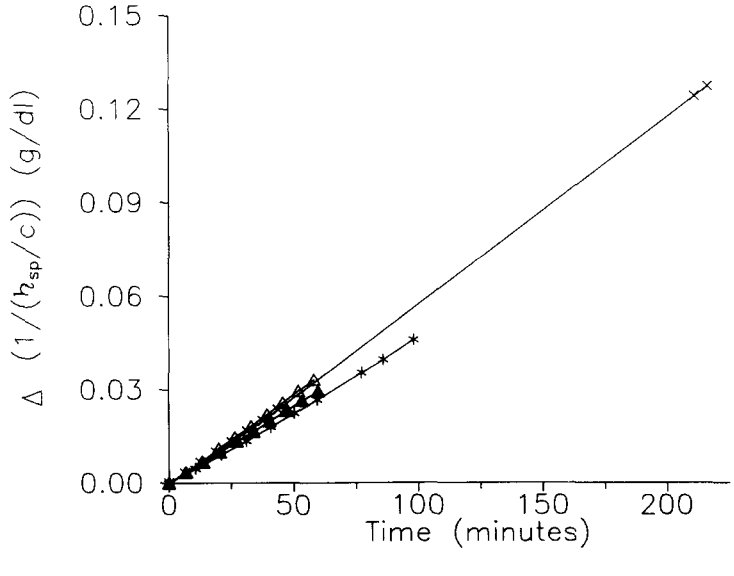

Fig. 1. Time course of degradation of chitosans with different chemical composition by H_2O_2 in the presence of $FeCl_3$. ×, $F_A = 0{\cdot}12$; ▲, $F_A = 0{\cdot}27$; *, $F_A = 0{\cdot}47$; △, $F_A = 0{\cdot}53$.

Fig. 2). Thus, the chitosan chains contain faster cleaving points for lysozyme hydrolysis which are regularly distributed along the polymer.

Kinetics of lysozyme degradation of chitosans

The effect of substrate concentration on the degradation rate (r) was investigated for three chitosans of different chemical composition (Fig. 3). Substrate saturation is observed at chitosan concentrations above $c = 0{\cdot}2\%$ for all three chitosans. The slight reduction in r at the higher substrate concentrations is probably due to the error made by assuming that the reduced viscosity (η_{sp}/c) is equal to the intrinsic viscosity ([η]) (see the Theory section). This was confirmed by comparing degradation rates obtained from plots of $\Delta(1/(\eta_{sp}/c))$ versus time with $\Delta(1/[\eta])$ versus time for a chitosan with $F_A = 0{\cdot}53$. The value of [η] was obtained from the value of (η_{sp}/c) using the Flory–Huggins equation (Tanford, 1961), with a Huggin's constant of 0·5 (mean value from intrinsic viscosity determinations in our laboratory). The r-value increased by 15% at the highest substrate concentration (0·69%), while the increase was negligible at the lowest substrate concentration of 0·09%, when converting reduced viscosities to intrinsic viscosities.

Figure 4 shows the effect of the concentration of lysozyme on the degradation rates for two chitosans of different chemical composition. The rate increased in direct proportion to the concentration of lysozyme. Thus, it is concluded that the degradation of chitosans with different chemical compositions by lysozyme appears to follow Michaelis–Menten-type kinetics.

Table 2 shows the initial lysozyme degradation rates of chitosans with different chemical compositions. The data show clearly that the initial degradation rates of chitosan are extremely dependent on their chemical composition (F_A), which must be caused by some site-specificity of the enzyme.

Michaelis–Menten analysis of lysozyme degradation of chitosans

We have analysed our experimental degradation data in more detail in order to gain some understanding of the *mechanism* of lysozymic degradation of chitosans with a known chemical composition (F_A) and random distribution of **A** and **D** units.

We assume that the reverse reaction between E and P is negligible:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_{cat}} E + P.$$

Thus, the *initial* degradation rate is:

$$r' = k_{cat} \cdot [ES]. \tag{1}$$

The *dissociation* constant for the ES-complex is, assuming that k_{cat} is low compared to k_1 and k_{-1}, as assumed in the original analysis of enzyme kinetics by Michaelis and Menten:

$$K_S = \frac{[E]\,[S]}{[ES]} \tag{2}$$

or

$$[ES] = \frac{[E]\,[S]}{K_S}. \tag{3}$$

It is well known that the active site of lysozyme is able to bind six sugar rings, i.e. lysozyme contains a hexameric binding site. We now consider chitosan,

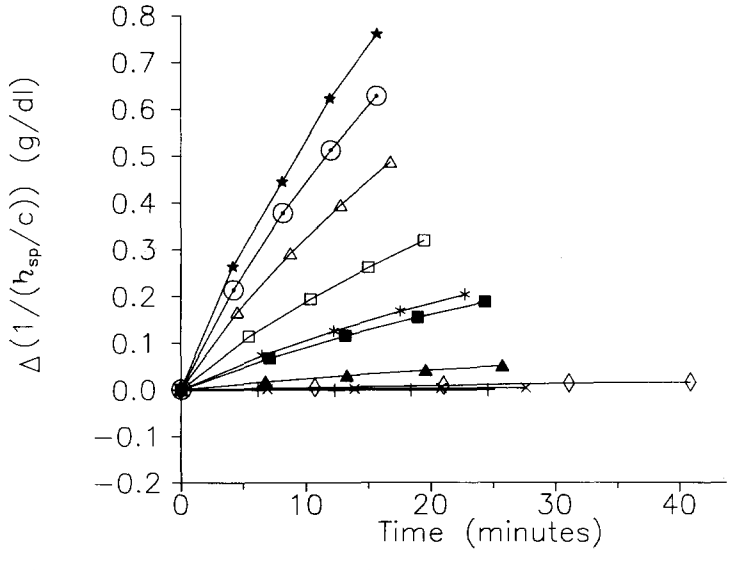

Fig. 2. Time course of degradation of chitosans with different chemical composition by lysozyme. The concentration of chitosan was 0·23 g/dl, and lysozyme concentration was 0·58 mg/ml. +, $F_A = 0{\cdot}04$; ×, $F_A = 0{\cdot}12$; ◇, $F_A = 0{\cdot}17$; ▲, $F_A = 0{\cdot}27$; ■, $F_A = 0{\cdot}42$; *, $F_A = 0{\cdot}47$; □, $F_A = 0{\cdot}51$; △, $F_A = 0{\cdot}53$; ★, $F_A = 0{\cdot}59$; ⊙, $F_A = 0{\cdot}60$.

Table 2. Lysozyme degradation rates (r) and relative rates of degradation (with degradation rate of chitosan with $F_A = 0.12$ equal to 1) of chitosans with increasing degree of acetylation (F_A), compared at the same lysozyme concentration (0·58 mg/ml) and chitosan concentrations (0·23 g/dl)

F_A	r ($g^2/(dl^2\,min)$)	Relative rate of degradation
0·05	(0·002)[a]	(0·033)
0·12	0·052	1
0·17	0·12	2
0·27	0·61	12
0·42	2·31	44
0·47	2·73	53
0·51	6·50	125
0·53	8·77	169
0·59	14·56	280
0·60	18·68	359

[a] This rate was lower than detectable in the viscosimetric assay, and the values given are extrapolated using the linear relationship in Fig. 5.

composed of randomly distributed acetylated (**A**) and deacetylated (**D**) units, as a polymer substrate for lysozyme. There are $2^6 = 64$ possible hexamers of different composition in the chitosan chain, from the fully deacetylated hexamer D–D–D–D–D–D to the fully acetylated hexamer A–A–A–A–A–A. Accordingly, there are 64 different K_i and $k_{cat(i)}$ for binding and degradation of the 64 substrates. Equation (3) then becomes:

$$[ES] = \sum_{i=1}^{64} [ES]_i = \sum_{i=1}^{64} \frac{[E] \cdot [S]_i}{K_i} \tag{4}$$

and

$$r' = \sum_{i=1}^{64} \left(k_{cat(i)} \frac{[E]\,[S]_i}{K_i} \right) \tag{5}$$

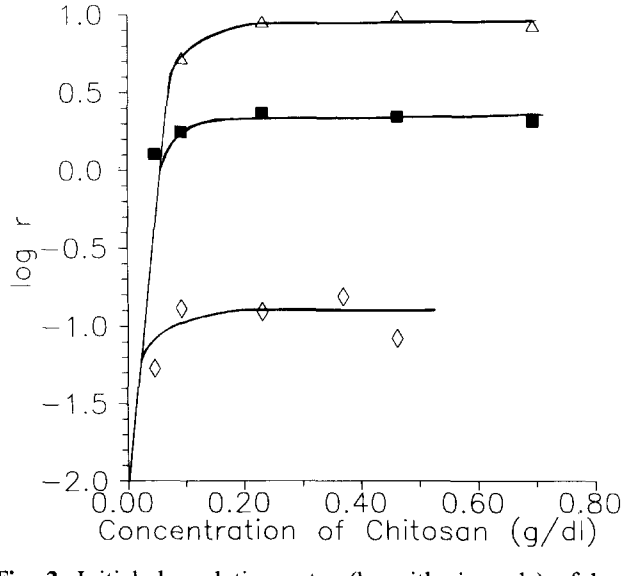

Fig. 3. Initial degradation rates (logarithmic scale) of lysozyme on three chemically different chitosans as a function of chitosan concentration. Lysozyme concentration was 0·58 mg/ml. ◇, $F_A = 0.17$; ■, $F_A = 0.42$; △, $F_A = 0.53$.

In order to simplify eqn (5), we assume in our model that the different K_S-values are of equal magnitude while the $k_{cat(i)}$-values are very different, resulting in the experimentally observed differences in the degradation rates (see Table 2). No experimental data on the binding of chitin/chitosan *hexamers* to lysozyme have been reported. However, Fukamizo *et al.* (1992) recently reported on the binding of partially acetylated trisaccharides to lysozyme. Although they found no evidence of binding of the fully deacetylated trimer to lysozyme, their data indicated that the deacetylated units are also able to interact with lysozyme. Excluding the fully deacetylated hexamer (D–D–D–D–D–D), the K_i-values of the partially acetylated hexamers may be of equal magnitude. Furthermore, from Fig. 3 it is also seen that substrate saturation occurs at about the same chitosan concentration, independent of F_A. If the K_i-values of the partially acetylated hexamers were very different, the less acetylated chitosan would be expected to show substrate saturation at a higher concentration compared with the less acetylated chitosan (Kristiansen, Vårum & Grasdalen, unpublished results). Thus, with $(K_1) = K_2 = K_3 = \ldots = K_{64} = K_S$ (where K_1 is the dissociation constant for the fully deacetylated hexamer), eqn (5) becomes

$$r' = \frac{[E]}{K_S} \cdot \sum_{i=1}^{64} ([S]_i \cdot k_{cat(i)}). \tag{6}$$

Using the total enzyme concentration, $[E]_T$, which equals $[E] + [ES]$, we obtain

$$r' = \frac{[E]_T \sum_{i=1}^{64} ([S]_i \cdot k_{cat(i)})}{\sum_{i=1}^{64} [S]_i + K_S}. \tag{7}$$

The concentration of each hexamer ($[S]_i$) is just the fraction of the hexamer (F_i) times the total substrate concentration ($[S]$):

$$[S]_i = F_i \cdot [S] \tag{8}$$

Combining, we obtain

$$r' = \frac{[E]_T \cdot [S] \cdot \sum_{i=1}^{64} (F_i \cdot k_{cat(i)})}{[S] + K_S}. \tag{9}$$

Interpretation of the degradation rates of chitosans with different chemical composition

From eqn (9) it is seen that the degradation rate is proportional to the sum

$$\sum_{i=1}^{64} (F_i \cdot k_{cat(i)}) = (F_{DDDDDD} \cdot k_{cat(1)}) + F_{DDDDDA} \cdot k_{cat(2)} + \ldots + F_{AAAAAA} \cdot k_{cat(64)}. \tag{10}$$

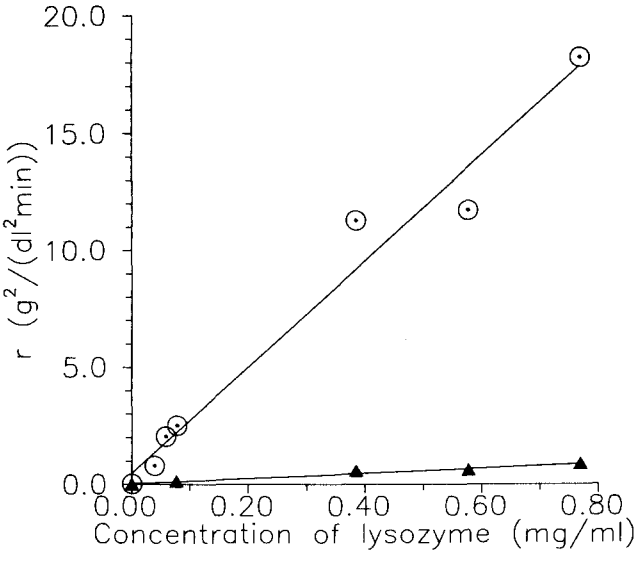

Fig. 4. Initial degradation rates of lysozyme on two chemically different chitosans as a function of lysozyme concentration. Chitosan concentration was 0·23 g/dl. ▲, $F_A = 0{\cdot}27$; ⊙, $F_A = 0{\cdot}60$.

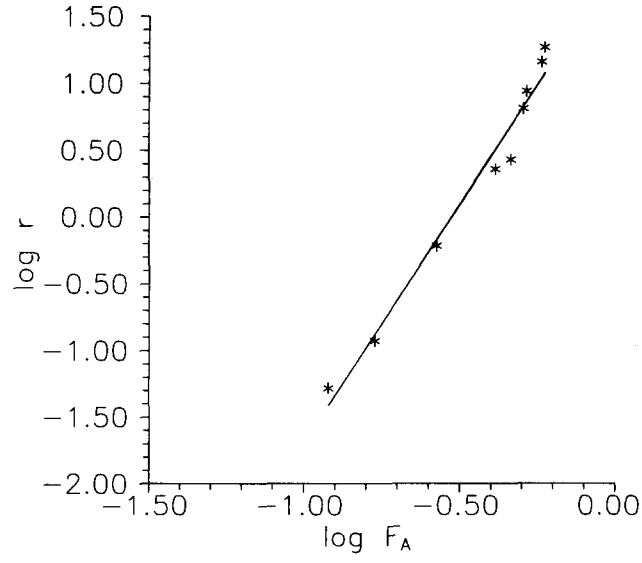

Fig. 5. Double logarithmic plot of initial degradation rates of lysozyme on chitosans (r) versus the fraction of acetylated units (F_A) of the chitosans. Concentrations of chitosan and lysozyme as in Fig. 2.

Amano & Ito (1978) reported on the degradation rates of partially acetylated chito-oligosaccharides with lysozyme. The highest rates were reported for the fully acetylated chito-oligomers. We tested the correlation between *the number of acetylated units* on lysozyme's hexameric binding site and the degradation rates. As the distribution of **A** and **D** units in the chitosans is random, the fraction of any hexamer can easily be calculated. As an example, we consider the hexamers containing four **A** units and two **D** units, where the fraction representing the sum of all hexamers with such a composition is simply $F_A^4 \cdot F_D^2$. A log–log plot of the lysozyme degradation rates on chitosans versus F_A is given in Fig. 5. The linearity in the plot, with a slope of 3·6, suggests that $k_{cat(i)}$ is highest for hexameric binding sites that contain three–four acetylated units. A tendency for a positive curvature in Fig. 5 suggests that $k_{cat(i)}$ for hexamers containing five or six acetylated units may be even higher. It should be stressed that the similarity in the form of eqns (5) and (6) does not allow us to separate strictly between variations in K_i and $k_{cat(i)}$ from the type of experimental data which is plotted in Fig. 5. Further binding studies are necessary in order to study the effect of variations in K_i.

Inhibition of lysozyme activity by a chitosan with a very low fraction of acetylated units ($F_A = 0{\cdot}010$)

Negligible lysozyme degradation rates were observed for highly deacetylated chitosan ($F_A = 0{\cdot}04$, see Table 2). However, Fukamizo *et al.* (1992) reported that **D** units on a partially acetylated trimer also may interact with lysozyme. A chitosan with a very low fraction of acetylated units, i.e. with a very low probability of more than single acetylated units occurring in the hexamer binding site of lysozyme, would therefore be expected to inhibit lysozyme degradation of chitosans. Figure 6 shows a plot of the logarithm of the initial degradation rates versus the concentration of inhibitor, and it is seen that the degradation rates decrease as the inhibitor concentration increases for all chitosans. The relative decrease in the degradation rates for chitosan substrates of increasing F_A (i.e. increasing amount of productive binding sites) with increasing inhibitor concentration (increasing amount of non-productive binding sites) is almost the same, which is seen as nearly parallel lines in Fig. 6. Thus, the *ratio* between the initial reaction rates for productive sites (hexamers containing three or more **A** units) are unaffected by the presence of non-productive sites (hexamers containing only one **A** unit), as has been deduced by Fersht (1985) in the theory for enzyme competition between different substrates.

The monotonous increase in the rates of lysozyme degradation with increasing fraction of acetylated units reported in this work has been found earlier (Hirano *et al.*, 1989), but with a much less pronounced increase in the degradation rates with F_A. However, these enzymatic reactions were carried out under heterogeneous conditions, and the *accessibility* of the substrate (chitosan) to the enzyme would be as important as its chemical composition.

Both lysozyme and chitosan are positively charged at pH 4·5, and the formation of the enzyme–substrate complex involves a direct contact between two polycations. Lysozyme hydrolysing soluble chitin has a pH optimum near 5 (Marzotto & Galzigna, 1969), which is believed to be caused by the different pK-values of Asp-52

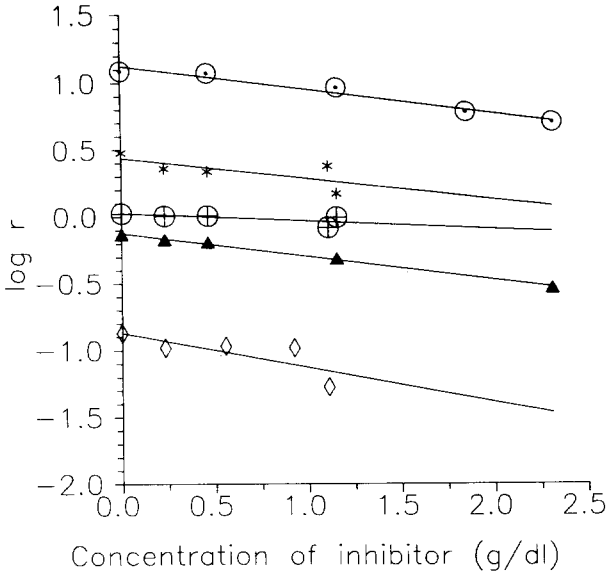

Fig. 6. The logarithm of the initial degradation rates of lysozyme on five chemically different chitosans as a function of increasing concentrations of a low-molecular-weight, almost fully *N*-deacetylated chitosan (inhibitor). Concentrations of chitosan and lysozyme as in Fig. 2. ◇, $F_A = 0{\cdot}17$; ▲, $F_A = 0{\cdot}27$; ⊕, $F_A = 0{\cdot}34$; ∗, $F_A = 0{\cdot}47$; ⊙, $F_A = 0{\cdot}60$.

and Glu-35 (acting as proton acceptor and proton donor, respectively) at the cleavage site of lysozyme. The site specificity may depend on pH and ionic strength, and further work along the lines presented here with well-characterized, soluble, model chitosans is necessary for clarifying the site specificity in a broader sense. In particular, for the potential in-vivo use of chitosan in medicine, degradation at physiological conditions is important. In such cases, partial solubility and two-phase systems have to be taken into account in addition to the question of site specificity alone. A validation of the site specificity of human lysozyme compared with hen egg white lysozyme used in this study towards partially *N*-acetylated chitosans is also of medical and pharmaceutical interest, and further studies are in progress.

CONCLUSION

Our data suggest that hexamers containing three–four or more acetylated units contribute mostly to the initial degradation rate when lysozyme degrades partially *N*-acetylated chitosans. This conclusion is firmly based upon a Michaelis–Menten type of analysis of the degradation data assuming different catalytic activity of lysozyme for the different hexameric substrates in the polymer chain. Our data do not allow us to separate fully between differences in formations of — or differences in rates of cleavage of — the different lysozyme–hexameric binding-site complexes, and further studies of the binding of lysozyme to hexamers of different composition and sequence are necessary.

THEORY

Determination of rates of degradation

Viscosity was used to determine initial rates of degradation by using the Mark–Houwink–Sakurada (MHS) equation to convert the loss in viscosity to the degree of scission of the glycosidic linkages. For a first-order or pseudo-first-order reaction, the degradation rate (r') can be expressed as

$$r' = -\frac{d[S]}{dt} = [S]_0 \cdot \frac{d\alpha}{dt} \tag{11}$$

where $[S]_0$ and $[S]$ are the molar concentrations of glycosidic linkages at the start of the reaction and at time t, respectively, and α is the degree of scission ($\alpha = 0$ for molecular weight $= \infty$ and $\alpha = 1$ for molecular weight equal to the weight of the monomeric unit).

If $\overline{DP_{n0}}$ and $\overline{DP_{nt}}$ are the number-average degrees of polymerization at the start of the reaction (time 0), and at the given (time t), respectively, it has been shown (Marchessault & Rånby, 1959) that for a first-order random-depolymerization reaction, the fraction of bonds broken ($\Delta\alpha$) is initially proportional to time:

$$\Delta\alpha = \frac{1}{\overline{DP_{nt}}} - \frac{1}{\overline{DP_{n0}}} = k' \cdot \Delta t \tag{12}$$

where k' is a constant.

For a given type of molecular weight distribution, the intrinsic viscosity ($[\eta]$) is related to the molecular weight through the MHS equation:

$$[\eta] = K \cdot \overline{M_n}^{\,a} \tag{13}$$

where $\overline{M_n}$ is the number-average molecular weight and K is a constant. By combining eqns (11), (12) and (13) and assuming that the exponent (a) equals unity and that M_0, the monomer weight ($\overline{M_n} = M_0 \cdot \overline{DP_n}$) is independent of the degree of *N*-acetylation, we obtain

$$k' = \frac{\Delta\alpha}{\Delta t} = \left(\frac{1}{[\eta]_t} - \frac{1}{[\eta]_{t0}}\right) \cdot K{\cdot}M_0/\Delta t$$

$$= \Delta\frac{1}{[\eta]} \cdot K{\cdot}M_0/\Delta t. \tag{14}$$

For low concentrations of chitosan (c) it can be assumed that the reduced viscosity equals the intrinsic viscosity ($\eta_{sp}/c \approx [\eta]$). Since we measure *initial* degradation rates at very low degrees of scissions in the viscosity assay, $d\alpha/dt$ equals $\Delta\alpha/\Delta t$ and we obtain

$$r' = k' \cdot [S]_0. \tag{15}$$

It should also be noted that since we plot our degradation data as $\Delta 1/(\eta_{sp}/c)$ versus time, the experimentally determined slope (k) is related to k' (eqn (14)) through $k \approx k'/(K{\cdot}M_0)$.

For a first-order random degradation of relatively stiff polysaccharides (a close to 1)), it has been shown that a plot of $\Delta(1/(\eta_{sp}/c))$ against t is linear in a number of cases (Smidsrød *et al.*, 1967). The plots of $\Delta(1/(\eta_{sp}/c))$ versus time for lysozyme degradation of chitosan were non-linear, and the initial slope (k) was determined by fitting the curves to a second-order polynom.

In our degradation experiments we prefer to use weight concentrations, c (in g/dl) of chitosan, and we express the rate, r, as

$$r = k \cdot c. \tag{16}$$

The units for the reaction rates will then be $g^2/(dl^2\,min)$, when the reduced viscosities are given in dl/g. Reaction rates r and r' are related through the following equation:

$$r' \approx r \frac{[S]_0 \cdot K \cdot M_0}{c}. \tag{17}$$

ACKNOWLEDGMENTS

Professor H. Grasdalen is thanked for valuable discussions. Financial support has been provided by the Nordic Fund for Technology and Industrial Development (NI), the Norwegian Fisheries Research Council (NFFR), the Norwegian Council for Scientific and Industrial Research (NTNF), Pronova Biopolymer (Norway) and Astra Hässle (Sweden).

REFERENCES

Allan, G.G. & Peyron, M. (1989). In *Chitin and Chitosan: Sources, Chemistry, Biochemistry, Physical Properties and Applications*, eds G. Skjåk-Bræk, T. Anthonsen & P. Sandford. Elsevier Applied Science, London, UK, pp. 443–66.

Amano, K. & Ito, E. (1978). *Eur. J. Biochem.*, **85,** 97–104.

Anthonsen, M.W., Vårum, K.M. & Smidsrød, O. (1993). *Carbohydr. Polym.*, **22,** 193–201.

Draget, K.I., Vårum, K.M., Moen, E., Gynnild, H. & Smidsrød, O. (1992). *Biomaterials*, **13,** 635–8.

Fersht, A. (1985). *Enzyme Structure and Mechanism.* W. H. Freeman and Co., New York, p. 112.

Fukamizo, T., Ikeda, Y., Ohkawa, T. & Goto, S. (1992). *Eur. J. Biochem.*, **210,** 351–7.

Hirano, S., Tsushida, H. & Nagao, N. (1989). *Biomaterials*, **10,** 574–6.

Marchessault, H. & Rånby, B.G. (1959). *Svensk Papperstid.*, **62,** 230.

Marzotto, A. & Galzigna, L.Z. (1969). *Physiol. Chem.*, **350,** 427–30.

Sannan, T., Kurita, K. & Iwakura, Y. (1976). *Makromol. Chem.*, **177,** 3589–600.

Sannan, T., Kurita, K., Ogura, K. & Iwakura, Y. (1978). *Polymer*, **19,** 458–9.

Sashiwa, H., Saimoto, H., Shigemasa, Y., Ogawa, R. & Tokura, S. (1990). *Int. J. Biol. Macromol.*, **12,** 295–6.

Smidsrød, O., Haug, A. & Larsen, B. (1965). *Acta Chem. Scand.*, **19,** 143–57.

Smidsrød, O., Haug, A. & Larsen, B. (1967). *Carbohydr. Res.*, **5,** 482–5.

Tanford, C. (1961). *Physical Chemistry of Macromolecules.* John Wiley & Sons, New York, pp. 392–3.

Vårum, K.M., Anthonsen, M.W., Grasdalen, H. & Smidsrød, O. (1991*a*). *Carbohydr. Res.*, **211,** 17–23.

Vårum, K.M., Anthonsen, M.W., Grasdalen, H. & Smidsrød, O. (1991*b*). *Carbohydr. Res.*, **217,** 19–27.

Vårum, K.M., Anthonsen, M.W., Ottøy, M.H., Grasdalen, H. & Smidsrød, O. (1992). In *Advances in Chitin and Chitosan*, eds C.J. Brine, P.A. Sandford & J.P. Zikakis. Elsevier Applied Science, New York, pp. 127–36.

Wang, W., Bo, S., Li, S. & Qin, W. (1991). *Int. J. Biol. Macromol.*, **13,** 281–5.