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Degradation of partially *N*-acetylated chitosans with hen egg white and human lysozyme

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Fully water-soluble, partially *N*-acetylated chitosans with fractions of *N*-acetylated units (F_A) from 0.12 to 0.60 (degrees of acetylation from 12 to 60%) and known random distribution of acetylated and deacetylated units were degraded with human milk lysozyme at pH 4.5 and ionic strength 0.16 (M). The initial degradation rates (r) of human milk lysozyme were determined as a function of F_A, and were found almost identical to the double logarithmic plot of r versus F_A determined previously for hen egg white (HEW) lysozyme (Nordtveit *et al., Carbohydr. Polym.*, **23**, 253–260, 1994), suggesting that the substrate specificities of human and HEW lysozyme with respect to partially *N*-acetylated chitosans are indistinguishable.

Human lysozyme degradation rates of two neutral-soluble chitosans (F_A of 0.42 and 0.60) were compared at pH 4.5 and 7.0. The initial degradation rate at pH 4.5 was about five times higher than the rate at 7.0. However, the ratio between the r-values for the two chitosans at pH 4.5 and 7.0 were almost identical, indicating very similar substrate specifcities at the two pH-values.

In a more detailed study with HEW lysozyme, the effect of pH on the rate of degradation was determined for three chemically different chitosans, showing similar pH-dependence with a rather broad optimum around pH 4 for all samples. The parallel behaviour of the three chitosans as substrates with relative degradation rates independent on pH indicate, as with human lysozyme, substrate specificities largely independent on pH. The substrate specificities of HEW and human lysozyme were shown to be quite independent of ionic strength, whereas the absolute rates of degradation for the chemically different chitosans increased with ionic strength up to 0.2 M, as expected for a positively charged enzyme attacking a positively charged substrate. Since the relative degradation rates of chitosans with widely different F_A-values, and thereby charge densities, were independent both on pH and ionic strength, in a pH-range affecting the charge density of both enzyme and substrate, it follows that short-range interactions (i.e. hydrogen bonding, van der Waals interactions and electrostatic forces in the binding site) rather than long-range electrostatic interactions between the positively charged lysozyme and the positively charged chitosan substrate are of importance for determining substrate specificities.

Our data suggests that tailor-made chitosans with a predetermined degradation rate in the human body can be made by simply controlling their F_A-values.

INTRODUCTION

Chitosan is a linear binary heteropolysaccharide composed of (1→4) linked 2-acetamido-2-deoxy-ß-D-glucopyranose (GlcNAc; **A**-unit) and 2-amino-2-deoxy-ß-D-glucopyranose (GicN; **D**-unit) residues. Chitin, a homopolymer of (1→4)-linked **A**-residues, is a common constituent of shells of crustaceans, insect exoskeletons and fungal cell walls. In nature, chitosan is much less plentiful than chitin, but certain fungi contain chitosan in their cell walls (Araki & Ito, 1975; Davis & Bartnicki-Garcia, 1984).

Generally, all chitosans are soluble in aqueous media at acidic pH-values, which distinguishes chitosan from chitin. Commercial high-molecular weight chitosans with fractions of acetylated units (F_A) between 0 and 0.2 are only soluble at pH-values below the apparent pK_a-value of 6.5 of the **D**-units (Domard, 1987; Anthonsen

& Smidsrød, 1995) where the polysaccharide will be highly positively-charged. However, high-molecular weight chitosans with F_A between 0.4 and 0.6 have been shown to be fully soluble at neutral pH-values (Sannan *et al.*, 1976), and we have recently demonstrated that the neutral-solubility of chitosans can be controlled by careful selection of their chemical composition (F_A) and molecular weight (Vårum *et al.*, 1994). Of particular importance for application of chitosan in the human body is that high-molecular weight chitosans with F_A from 0 to 0.4 precipitate between pH 6 and 7.5, but the chitosans show increasing solubility at higher pH-values with increasing F_A. Such solubility data are obviously of importance when studying enzymatic degradation rates of chitosans as a function of pH.

Both chitin and chitosan are substrates for hen egg white (HEW) lysozyme (Berger & Weiser, 1957; Amano & Ito, 1978), an enzyme which is positively charged at physiological pH-values (pI = 11), and which natural substrates are ß-(1→4) glycosidic linkages in the negatively charged polysaccharide backbone of certain bacterial cell walls.

Human lysozyme is found in various body fluids and tissues, in concentrations from 4 to 13 mg/l in serume (Henry, 1991) and from 450 to 1230 mg/l in tears (Temel *et al.*, 1991). It was found by X-ray analysis that human lysozyme has a main chain conformation very similar to that of HEW lysozyme (Banjard *et al.*, 1974), and NMR studies suggest the presence of similar binding subsites in human and HEW lysozyme (Cohen, 1969). However, human lysozyme shows replacement of 53 amino acid residues of the HEWL molecule. The most important substitution is that Trp62 in HEW lysozyme, which participates in the binding of the substrate, is replaced by Tyr in human lysozyme. The apparent binding constants of fully acetylated chitooligosaccharides (monomer to hexamer) to human lysozyme were reported to be of the order of one-tenth of those in the case of HEW lysozyme (Teichberg *et al.*, 1972). Fukamizo *et al.* (1982) have reported that human lysozyme has a 1.2 times higher rate constant for the cleavage of glycosidic linkages in a pentamer of ß-(1→4)-linked GlcNAc, compared to HEW lysozyme. Thus, literature data suggests that there may be differences between human and HEW lysozyme in binding affinities and degradation rates of certain substrates.

The influence of pH and ionic strength on degradation rates for several lysozymes have been studied for a number of substrates (Davies *et al.*, 1969; Saint-Blancard *et al.*, 1970; Kuramitsu *et al.*, 1975). However, these substrates are either uncharged (i.e. chitin and chitin oligomers) or negatively charged (i.e. cell suspensions of *M. lysodeikticus*) in contrast to the positively charged chitosans.

We have recently demonstrated (Nordtveit *et al.*, 1994) that the initial rate of degradation of fully water-soluble chitosans with HEW lysozyme increases dramatically with increasing F_A as determined *in vitro* at pH 4.5 and ionic strength 0.16 (M) using a viscosimetric assay. The object of the present paper is to compare previous chitosan degradation data on HEW lysozyme with comparable data on human lysozyme. Furthermore, we wanted to study the effect of pH and ionic strength when chitosans are degraded by lysozyme, in order to judge our previous conclusions on site specificity of HEW lysozyme to biodegradation in human tissue.

MATERIALS AND METHODS

Chitosans

The chitosans with varying F_A were prepared as previously described (Nordtveit *et al.*, 1994).

Lysozyme

Lysozyme from hen egg white (Sigma L 6876) and human milk (Sigma L 6394) was used without further purification.

Viscosity assay for enzymatic hydrolysis of chitosan

The chitosan solutions (0.25 g/dl) were prepared by dissolving the chitosan in 1% acetic acid/sodium acetate buffer solution (pH 4.5), and then diluting the solution with an equal volume of 0.2 M KCl. In the experiments where pH was varied, small volumes of concentrated HCl or NaOH were used to achieve the desired pH. When ionic strength was varied, 0–2 M KCl was added. The degradation of chitosan with hen egg white lysozyme at varying pH were performed at an ionic strength of 0.4 (M). Hen egg white lysozyme (7.5 mg/ml; ~95% protein) and human milk lysozyme (19.3 mg/ml; ~10% protein) were dissolved in deionized water. The viscosity assay and the determination of initial degradation rates were performed as described in Nordtveit *et al.* (1994).

RESULTS AND DISCUSSION

Determination of human lysozyme degradation rates of chitosan with increasing F_A

Human lysozyme demonstrated the same non-linear time course of degradation of chitosan as HEW lysozyme (Nordtveit *et al.*, 1994), as can be observed from the plot of $\Delta (1(\eta_{sp}/c))$ versus time shown in Fig. 1. The initial rates of degradation (r) were determined from the initial slopes by fitting the curves to a second-order polynom as previously described (Nordtveit *et al.*, 1994). A strong increase in r-values with increasing F_A of the chitosan is observed also for human lysozyme parallel to our previous observations on HEW lyso-

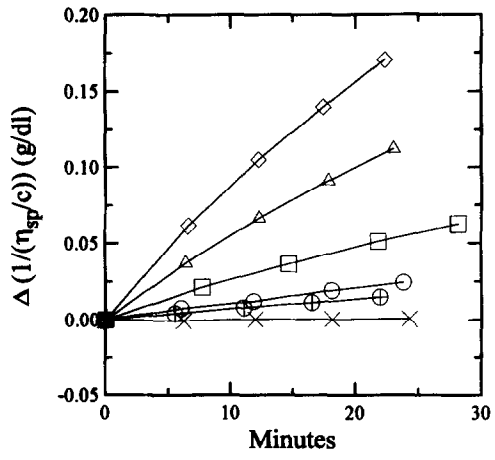

Fig. 1. Time course of degradation of chitosans with different chemical composition by human milk lysozyme. Concentration of chitosan was 0.23 g/dl and concentration of lysozyme was 0.15 mg/ml. X, F_A = 0.12; ⊕, F_A = 0.27; ○, F_A = 0.34; □, F_A = 0.42; △, F_A = 0.51; ◇, F_A = 0.60.

Table 1. Human lysozyme initial degradation rates of two chitosans, F_A = 0.60 (r_1) and F_A = 0.42 (r_2), and relative rates of degradation (r_1/r_2) at pH 4.5 and 7.0

pH	r_1 (g^2/dl^2 min)	r_2 (g^2/dl^2 min)	r_{rel} (r_1/r_2)
4.5	2.351±0.235	0.575±0.058	4.1±1.2
7.0	0.529±0.053	0.105±0.011	5.0±0.7

zyme. In order to compare the present degradation data on human lysozyme with our previous degradation data on HEW lysozyme, a double logarithmic plot of initial degradation rates of human lysozyme versus F_A of the chitosans is given in Fig. 2. The plot is linear with a slope of 3.5, almost identical to the slope of 3.6 in the same plot for HEW lysozyme. This means, as suggested for our analysis of HEW lysozyme degrading partially *N*-acetylated chitosans, that hexameric binding sites

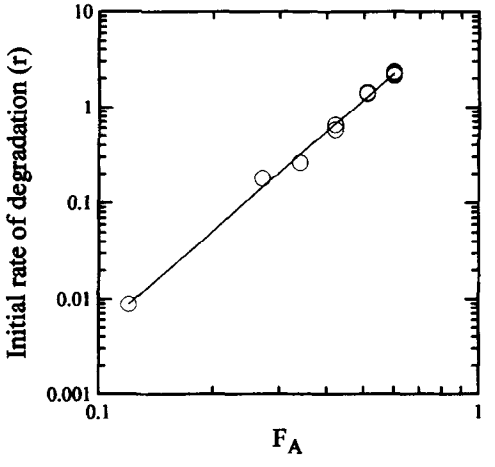

Fig. 2. Double logarithmic plot of initial degradation rates of human milk lysozyme on chitosans vs the fraction of acetylated units (F_A) of the chitosans. Concentrations of chitosan and lysozyme as in Fig. 1.

containing three to four acetylated units contributes mostly to the initial rate of degradation. However, it should be noted that the tendency for a higher slope at the highest F_A as determined for HEW lysozyme previously is not observed for human lysozyme.

Literature data have suggested different binding constants and degradation rates of chitin oligomers for HEW and human lysozyme (Teichberg *et al.*, 1972; Fukamizo *et al.*, 1982); however, our results do not support a difference in substrate specificities of human and HEW lysozyme towards partially *N*-acetylated chitosans.

Comparison of human lysozyme degradation rates of chitosans at pH 4.5 and pH 7.0.

For applications of chitosan in medicine and pharmacy, the human lysozyme degradation rates at physiological pH-values are of great importance. The apparent pK_a-value of the amino group in partially *N*-acetylated chitosans is 6.5 (Domard, 1987; Anthonsen & Smidsrød, 1995), and in order to study the effect of (partial) neutralization of the positively-charged amino group, we compared the lysozyme degradation rates of two chitosans (F_A = 0.60 and F_A = 0.42) at pH 4.5 and pH 7.0. Both chitosans were fully water-soluble also at pH 7.0 (Vårum *et al.*, 1994). The results are given in Table 1, showing that the initial degradation rate at pH 4.5 is about five times higher than the rate at pH 7.0. However, the *relative* degradation rates between the two chitosans at the two pH values are the same, within the accuracy of the experimental r-values (±10%), suggesting that the substrate specificity of human lysozyme towards chitosans at pH 4.5 is also valid at physiological pH, when the solubility of the substrate is not changed.

Influence of pH on HEW lysozyme degradation rates of chitosans with increasing F_A

In our previous work, we degraded chitosan by HEW lysozyme at pH 4.5, to ensure full water-solubility of the substrates and that the amino-groups were fully charged (Nordtveit *et al.*, 1994). The influence of pH on the HEW lysozyme degradation rates when hydrolyzing three chitosans with increasing F_A (F_A = 0.17, 0.42 and 0.60) was examined at an ionic strength of 0.26 (M), and the results are shown in Fig. 3. It is observed that the shape of the activity vs pH curves are similar for all three chitosans, with a broad optimum near pH 4. The activity versus pH curves for HEW lysozyme have previously been determined for uncharged and fully soluble substrates (chitin oligomers), showing an optimum in activity around pH 5 (Banerjee *et al.*, 1973; Davies *et al.*, 1969). In view of our conclusion that hexameric sites containing three to four acetylated units contribute mostly to the initial degradation rate as determined here, and the recent observation that both

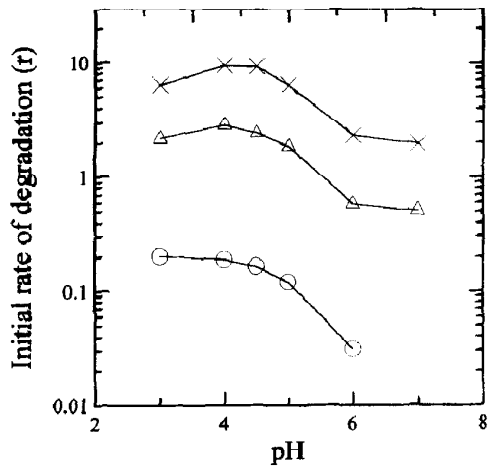

Fig. 3. Initial degradation rates (logarithmic scale) as a function of pH when HEW lysozyme degrades three chemically different chitosans. Chitosan concentration was 0.23 g/dl and lysozyme concentration was 0.55 mg/ml. ○, F_A = 0.17; △, F_A = 0.42; X, F_A = 0.60.

the reducing and non-reducing ends formed by lysozyme degradation of a partially *N*-acetylated chitosan are acetylated units (Stokke *et al.*, 1995), the similarity in the lysozyme activity vs pH curve of partially *N*-acetylated chitosans and soluble chitin oligomers is conceivable. It should, however, be noted that the net positive charges of both substrate and enzyme are decreased when the pH is increased from pH 3 to 7. Thus, the relative rate of degradation, and hence the substrate specificity of HEW lysozyme towards partially *N*-acetylated chitosans, seems independent of pH when the solubility of the substrate is not changed, as found for human lysozyme.

Effect of ionic strength on HEW degradation rates of chitosans with increasing F_A

As both chitosan and lysozyme are positively charged at pH 4.5, a strong dependence of the degradation rate with ionic strength was expected. We have determined and compared the effect of ionic strength on initial degradation rates for HEW and human lysozyme. Figure 4 shows the logarithm of the HEW initial degradation rate, log r, plotted against the ionic strength, I, for three chitosans with increasing F_A. The three curves are similar in shape, and the rates of degradation increase with increasing ionic strength up to about 0.2 (M), where it levels off. The constant activity above I = 0.2 (M), for all three chitosans with different F_A and thereby different charge densities, is attributed to the screening of the long range electrostatic interactions between the two positively charged reacting species (Laidler, 1982).

The dependence of degradation rates on ionic strength for human lysozyme is shown in Fig. 4b, showing similar curves as for HEW lysozyme. The

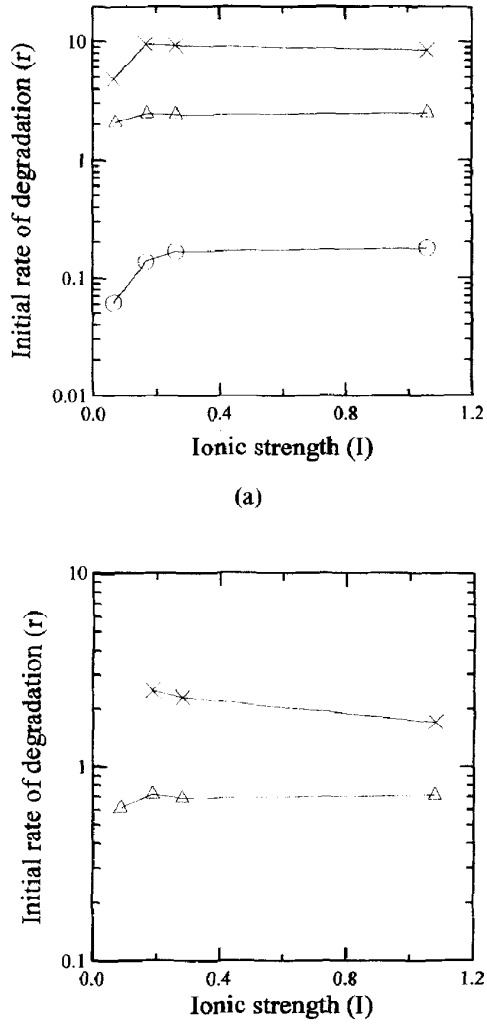

Fig. 4. Initial degradation rates (logarithmic scale) as a function of ionic strength when HEW lysozyme (a), and human milk lysozyme (b), degrade chemically different chitosans. Concentration of chitosan was 0.23 g/dl. HEW lysozyme concentration was 0.55 mg/ml and human milk lysozyme concentration 0.15 mg/ml. Symbols as in Fig. 3.

decrease in the degradation rate at low ionic strength for human lysozyme is less pronounced than for HEW lysozyme, which is contrary to our expectations, as human lysozyme has a higher content of charged amino acids compared to HEW lysozyme. We conclude that the relative degradation rates for both HEW and human lysozyme, and hence the site specificity, are independent of the ionic strength.

For the potential *in vivo* use of partially *N*-acetylated chitosans in medicine, it is of importance to observe that the lysozyme degradation at the ionic strength of physiological conditions (0.155 M) has reached its maximum.

The relative human and HEW lysozyme initial degradation rates of chitosans with increasing F_A (decreasing charge densities) have been found to be

independent of both ionic strength and pH. We conclude that long range electrostatic interactions between the two positively charged macromolecules (substrate and enzyme) are not important for lysozyme's substrate specificity, which must rather be determined by short range interactions inside the active cleft of lysozyme (i.e. hydrogen bonding, van der Waals interactions and electrostatic forces) as is to be expected by the current key-lock model of enzyme mechanisms.

When discussing *in vivo* degradation of chitosans with increasing F_A-values, the solubility of the chitosans at physiological pH-values must also be taken into consideration in addition to the substrate specificities discussed here. The neutral solubilities of chitosans generally increase with increasing F_A (Vårum *et al.*, 1994), which may further enhance the marked increase in the degradation rates with increasing F_A. For insoluble chitosans, other parameters like the size and shape of particles (determining the specific surface area), the crystallinity of the solid phase etc. will also affect the degradation rate of a chitosan with a given F_A. However, for chain scission to occur, a hexameric part of the chitosan chain most probably must be contained in the binding site of lysozyme at some stage resulting in the same chemical substrate specificity as found for soluble chitosans. Moreover, since the neutral solubilities of chitosans increase with decreasing molecular weight (Vårum *et al.*, 1994), the degraded chitosan fragments will eventually become soluble with the same substrate behavior as the soluble chitosans studied in this paper.

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