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Note

In vitro degradation rates of partially *N*-acetylated chitosans in human serum

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Abstract

The initial degradation rates (r) in human serum of three chitosans with $F_A = 0.42$, 0.51, and 0.60 were determined by measuring the decrease in viscosity as a function of time. A strong increase in r with increasing F_A of the chitosans was observed, with r increasing proportionally to $F_A^{4.5}$. With increasing concentrations of lysozyme added to the reaction mixtures of chitosan and serum, the relative increase in degradation rate of chitosans with increasing F_A was almost the same as that without lysozyme added. Addition of the chitinase inhibitor allosamidin (50 μM) did not inhibit the degradation rate of chitosan ($F_A = 0.60$) by human serum. The results suggest that chitosans are actually mainly depolymerized by lysozyme in human serum, and not by other enzymes or other depolymerization mechanisms.

Keywords: Chitosan; Degradation; Serum; Lysozyme; Chitinase

Chitosan is a linear binary heteropolysaccharide consisting of (1 → 4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc; **A**-unit) and 2-amino-2-deoxy-β-D-glucopyranose (GlcN; **D**-unit). The **A**- and **D**-units have been shown to be randomly distributed in water-soluble, partially *N*-acetylated chitosans prepared from chitin by alkaline deacetylation [1,2]. Water-soluble chitosans can be prepared with fractions of *N*-acetylated units (F_A) from 0 to 0.6 [3,4].

Chitosan can be enzymatically degraded by e.g. chitinases, chitosanases, and lysozymes. In addition, as with all polysaccharides, depolymerization may occur by acid hydrolysis and by an oxidative-reductive depolymerization (ORD) reaction. To our knowledge, no experimental evidence on which degradation mechanisms are responsible for the degradation of chitosans in the human body has been published. In this respect, the recent reports of the purification and characterization of chitinases from human serum are interesting [5–7].

By use of a viscometric assay, we have recently shown that both hen egg white and human lysozyme degradation rates (r) of partially *N*-acetylated chitosans increase strongly with increasing F_A, i.e. proportional to $F_A^{3.6}$ [8], in accordance with recently reported sequence specificities of lysozyme towards partially *N*-acetylated chitosans [9,10]. We now report in vitro degradation rates of partially *N*-acetylated

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Table 1
Chemical composition (F_A) and intrinsic viscosity [η] of chitosans

F_A	[η] (mL/g)
0.42	840
0.51	450
0.60	500

chitosans in human serum, pH 5, suggesting that hydrolysis by lysozyme is the most important depolymerization mechanism of chitosans in human serum.

Three chitosans with $F_A = 0.42$, 0.51, and 0.60 were selected for this study (Table 1). Chitosans with lower F_A values were also tested, but caused precipitation reactions in serum, and could not be used in the viscometric assay. Fig. 1 shows a plot of the increase in the inverse reduced viscosity, $\Delta(1/(\eta_{sp}\,c))$, versus time. The initial rate of degradation (r) was determined from the initial slopes of the curves according to Nordtveit et al. [4]. A strong increase in r with increasing F_A of the chitosans is seen for the degradation of chitosans in serum, with r increasing proportionally to $F_A^{4.5}$ (Fig. 2, lower curve). These results show an even stronger increase in r with increasing F_A than our previously reported results on hen egg white and human lysozyme, where r increased proportionally to $F_A^{3.6}$ [4,8]. However, it should be noted that those results were obtained as an average exponent for chitosans with a broader range

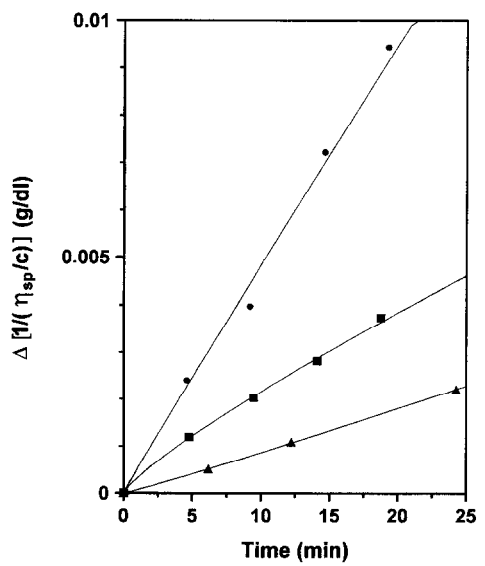

Fig. 1. Time course of degradation of chitosans with human serum. (▲) $F_A = 0.42$, (■) $F_A = 0.51$, (●) $F_A = 0.60$.

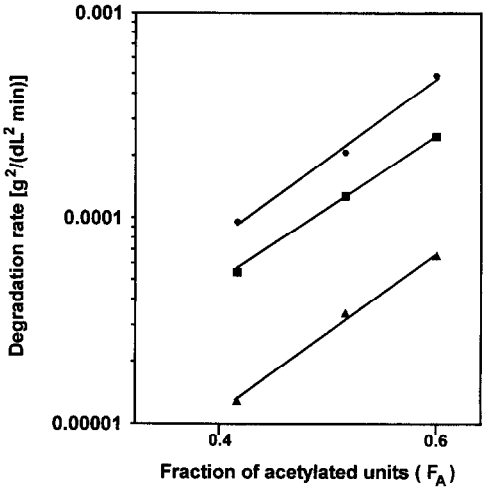

Fig. 2. Degradation rates as function of F_A (double logarithmic scale) for degradation of chitosans in serum (▲), in serum + 5 μg/mL serum of lysozyme (■), and in serum + 10 μg/mL serum of lysozyme (●).

of F_A (0.12–0.60), and that a tendency for a higher exponent was observed at the highest F_A, in accordance with the present results. In order to test this result further, a known amount of lysozyme was added to each of the reaction mixtures of chitosan and serum, and the degradation rates were determined. The results are shown in Fig. 2 in a double logarithmic plot of degradation rates versus F_A (three chitosans) in serum, and in serum with increasing amounts of lysozyme added. The relative increase in degradation rate of chitosans with increasing F_A is almost the same, as is reflected in the slopes of 4.5 (serum), 4.2 (serum + 5 μg/mL serum of lysozyme), and 4.5 (serum + 10 μg/mL serum of lysozyme).

Furthermore, addition of the chitinase inhibitor allosamidin (50 μM) did not inhibit the degradation rate of chitosan by human serum. This experiment was also performed with freshly prepared serum, as the stability of human chitinases in serum preparations has, to our knowledge, not been reported. The degradation rates of the chitosan with $F_A = 0.60$ in human serum were determined to be $(4.25 \pm 0.54) \times 10^{-4}$ g^2/dL^{2}min without added allosamidin, and $(4.49 \pm 0.30) \times 10^{-4}$ g^2/dL^{2}min with 50 μM allosamidin. This result suggests that human chitinases are not responsible for the in vitro degradation of chitosans in serum, as human lysozyme is unaffected by allosamidin [11] while human serum chitinase is strongly inhibited by allosamidin [5,6].

It has previously been found that the rate of depolymerization of chitosans by the ORD-reaction did not show any systematic variation with F_A [4]. We

also tested the rate of acid hydrolysis of the three partially *N*-acetylated chitosans (pH 1, 60 °C). The results (data not shown) showed only a moderate increase in r with increasing F_A (r increased proportionally to F_A). Moreover, when the absolute rates in acid and serum were compared, they were quite similar in spite of the higher temperature in acid (60 °C compared to 37 °C) and the four orders of magnitude higher proton concentration in acid (pH 1 compared to pH 5). From these results we can exclude the acid degradation mechanism as responsible for the in vitro degradation of chitosans at pH 5.

We conclude that the in vitro degradation rates of human serum at pH 5 of water-soluble chitosans increase strongly with increasing F_A, suggesting that the polysaccharides are actually mainly depolymerized by lysozyme in human serum, and not by other enzymes or other depolymerization mechanisms. Since we found earlier that the substrate specificities of lysozyme in the degradation of soluble chitosans are rather independent for pH between 4.5 and 7, it may be inferred that these substrate specificities also operate in vivo at physiological conditions.

1. Experimental

Chitin was isolated from fresh shrimp shells. Three chitosans prepared by homogeneous *N*-deacetylation of chitin [12], with fractions of *N*-acetylated units of 0.42, 0.51, and 0.60, were used. The F_A-values of the chitosans were determined by high-field ^1H NMR spectroscopy [1], and their intrinsic viscosities as previously described [13]. Table 1 shows the chemical composition and the intrinsic viscosities for the different chitosans.

The human serum viscosity assay was performed as follows: Human serum was obtained by allowing whole human blood to clot prior to centrifugation. The supernatant (human serum) was stored at 4 °C, and was stable for at least 5 days. Human serum (2.5 mL) was mixed with 0.1 mL HOAc (10%), and the pH was adjusted to 5.0 by HOAc or NaOAc. Chitosan solutions (3 mg/mL) were prepared in 0.04 M NaOAc/HOAc buffer (pH 5.0) containing 0.3 M NaCl. An equal volume (2.3 mL) of human serum (pH 5) and chitosan solution was mixed, and 0.1 mL Glanapon (diluted 1:100 with buffer) was added to prevent foaming in the Ubbelohde viscosimeter. The reaction mixture was filtered through a 0.8 μm Millipore membrane filter, and the decrease in viscosity was measured as a function of time in a capillary viscosimeter at 37 °C. The initial rates of degradation were measured as previously described [4].

Chicken egg white lysozyme (L 6876) was obtained from Sigma, and was used without further purification.

Note Added in Proof

In two earlier papers (refs. [4] and [8]), absolute values of degradation rates based on a similar viscometric as used here are given which are one thousand times too high. This does not affect any of the conclusions in the two papers or in the theoretical treatment of the degration rates in a subsequent paper (ref. [9]), which are all based on relative degradation rates.

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