Enzymatically hydrolysed chitosan oligomer solutions – Effects on intrinsic viscosity and molecular weight

Reidar Barfod Schüller[1] and Berit Bjugan Aam[2]

[1]Norwegian University of Life Sciences, Department of Chemistry, Biotechnology and Food Science, 1432 Ås, Norway

[2]BioCHOS AS, Høyskoleveien 12, 1432 Ås, Norway

ABSTRACT

Chitosan polymers were enzymatically hydrolyzed to yield chitosan oligomer mixtures of varying average molecular weights (MW_n)/ average degrees of polymerizations (DP_n). As the enzymes cleaved the chitosan polymers, the DP_n/MW_n was reduced and thereby also the intrinsic viscosity. The chitosan oligomer samples were analyzed by nuclear magnetic resonance spectroscopy (NMR) to find the DP_n. The intrinsic viscosity was determined in the normal way by extrapolating data series of specific viscosity divided by concentration to zero concentration. Five chitosan oligomer samples of different DP_n/MW_n were analyzed. The DP_n of the samples were 84, 57, 38, 26, and 14, corresponding to MW_n of 14 kDa, 9.5 kDa, 6.3 kDa, 4.3 kDa and 2.3 kDa, respectively.

The molecular weight (MW) obtained from the intrinsic viscosity by means of the Mark-Houwink-Sakurada (MHS) equation correlate well with the MW_n determined from the ^{1}H-NMR measurements.

Amplitude sweeps showed that the chitosan oligomer solutions exhibited a structure being viscoelastic solids at low strains. The elastic modulus of the solutions in the linear viscoelastic region clearly decreased when the MW_n decreased. The structures were weak, but did not break before the strain exceeded approximately 10%.

INTRODUCTION

Chitin is a naturally occurring biopolymer, which is most commonly extracted from shrimp or crab shells. Chitosan is produced from chitin, by chemically removing partly or completely the acetyl groups on the N-acetyl glucosamine (GlcNAc) residues in chitin. The result is chitosan heteropolymers consisting of a mixture of GlcNAc and glucosamine (GlcN) residues, where the amine groups on the glucosamine residues have the potential to be positively charged, depending of pH in the surroundings. The chitosan polymers are soluble in weak acids, unlike the crystalline chitin material.

Chitosan are linear polymers with a non-reducing end, internal sugar-residues, and a reducing end. Chitosan polymers are characterized by its number of GlcNAc (A) versus GlcN (D) residues. This can be denoted fraction of acetylation (F_A). Both the F_A and the MW / degree of polymerization (DP) of the chitosan is important for its properties.

Chitinases and chitosanases are enzymes capable of cleaving the glycosidic bonds between two residues in chitosan polymers, to generate chitosan oligomers. The main difference between the two different enzyme types is that chitinases requires a GlcNAc residue in the -1 position of its active site, whereas chitosanases requires a GlcN residu in the -1 subsite. Chitosanases are therefore

most active in hydrolyzing chitosans, where there are a large fraction of GlcN residues.

The product of an enzymatic hydrolysis of chitosan is a mixture of chitosan oligomers of varying DP. Such a mixture of chitosan oligomers is characterized by its DP_n. We can find the DP_n of a chitosan oligomer mixture with ^{1}H-NMR analysis[1].

It is well known that the intrinsic viscosity can be linked to the molecular weight of the dispersed molecules[2], and Kasaai and co-workers showed excellent agreement for chitosan polymer samples with the Mark-Houwink-Sakurada (MKS) equation:

$$[\eta] = KM_v^a \qquad (1)$$

where $[\eta]$ is the intrinsic viscosity, M_v the viscosity average molecular weight and K and a are constants for given solute-solvent system and temperature.

$$\begin{aligned}[\eta] &= KM_v^a = 1.57 \cdot 10^{-4} M_v^{0.79} \\ &= 1.57 \cdot 10^{-4} q_{MHS} M_w^{0.79} \\ &= 1.49 \cdot 10^{-4} M_w^{0.79}\end{aligned} \qquad (2)$$

Knowledge of the polydispersity factor (for Chitosan $q_{MHS} = 0.95$ according to Kasaai et al[2]) has been used to express the intrinsic viscosity as a function of the weight average molecular weight.

METHODS

Preparation of samples

Chitosan oligomer samples were prepared from KitoFlokk™ chitosan (Teta Vannrensing, previous Norwegian Chitosan, Kløfta Norway). This chitosan has a F_A of 0.15, meaning that the chitosan consists of 85% GlcN residues and 15% GlcNAc. A chitosanase (Chimax-35, purchased from Amicogen, South Korea) was used in the enzymatic hydrolysis of the chitosan, to yield mixtures of chitosan oligomers with a decreasing DP_n.

The chitosan (final concentration of 2%) was dissolved in 0.5% HCl, and the pH was adjusted to 5.5 by adding 1M NaOH. Chimax-35 (0.1 mg/g chitosan) was added to the solution and incubated at 37°C and 200 rpm. Samples were taken out at different time points (30 min, 1h, 2h, 3h, and 6h) and the reactions were stopped by adding HCl to decrease the pH in the solutions to 2.5. The reactions were heated to 60°C to permanently inactivate the enzyme, sterile filtrated and lyophilized.

Intrinsic viscosity measurements

The lyophilized chitosan oligomer samples were dissolved in 0.1M sodium acetate buffer (pH 4.5) prior to analysis on the rheometer. The viscosity of the chitosan oligomer samples is decreasing with decreasing DP_n, and the samples with the lowest DP_n could therefore be dissolved at a higher concentration than the samples with the higher DP_n.

The viscosity of different concentrations of chitosan oligomer samples in water was determined, and the intrinsic viscosity was determined according to common methods, extrapolating to zero concentration.

The intrinsic viscosity is defined by:

$$[\eta] = \lim_{c \to 0} \left(\frac{\eta_{sp}}{c} \right) \qquad (3)$$

$$\eta_{sp} = \frac{\eta - \eta_0}{\eta_0} \qquad (4)$$

$$\eta_r = \frac{\eta}{\eta_0} \qquad (5)$$

Where η_{sp} is the specific viscosity, η is mixture viscosity, η_0 is viscosity of the

continuous phase and c is the concentration of dispersed material. η_r is relative viscosity.

The MW_n was calculated from Eqn. 2, having determined the intrinsic viscosity.

NMR measurements

The chitosan oligomer samples were analyzed by ^{1}H-NMR, with an Ascend^{TM} 400 instrument from Bruker, to find the DP_n[1].

The NMR spectra gives individual signals for the reducing end GlcNAc ´s, internal and nonreducing end GlcNAc´s, reducing end GlcN ´s, and internal and non-reducing ends GlcN´s. The α- and β-anomers of the reducing end sugars also gives separate signals. This information can be used for finding the DP_n of a chitosan oligomer mixture by using the formula DP_n = (Aα+Aβ+A+Dα+Dβ+D)/(Aα+Aβ+Dα+Dβ) where Aα is the α-anomer of the GlcNAc reducing end residues, Aβ is the β-anomer of the GlcNAc reducing end residues, A is the signal from the internal and non-reducing end GlcNAc´s, Dα is the α-anomer of the GlcN reducing end residues, Dβ is the β-anomer of the GlcN reducing end residues, and D is the signal from the internal and non-reducing end GlcN´s.

The degree of polymerization, DP_n, is the average number of sugar units in the chitosan oligomer mixture. Each of these sugar residues has an MW_n of 167 Da. The MW_n of the oligomers can hence be calculated.

Amplitude sweeps

Amplitude sweeps were made to observe if a linear viscoelastic region existed for the oligomer solutions. The following set up was used: Angular frequency = 10 rad/s, 0-100 % strain

Dynamic data from frequency sweeps

Frequency sweeps were recorded with the following settings: strain = 0.1 % which is well within the LVR region determined from the amplitude sweeps. Angular frequency varied from 500 to 0.05 rad/s.

RESULTS AND DISCUSSION

Intrinsic viscosity and molecular weight reduction

An example plot showing determination of intrinsic viscosity is shown in Figure *1* where the intrinsic viscosity is determined from the average of the crossover points of the y-axis of η_{sp}/c and $ln(\eta_r)/c$.

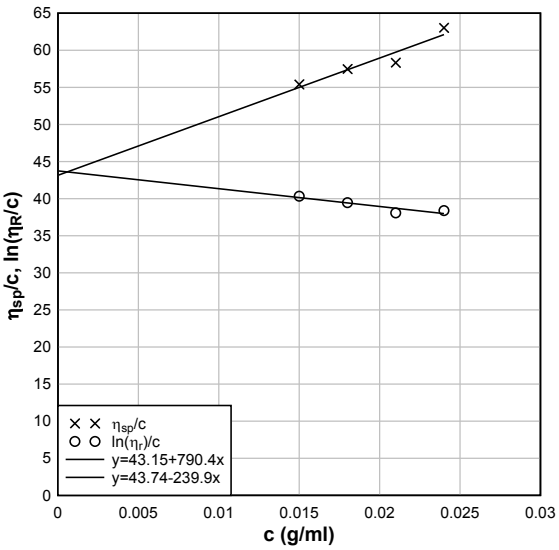

Figure 1: Example plot showing determination of intrinsic viscosity as the average of the intercepts: (43.15+43.74)/2=43.45.

The intrinsic viscosity and the corresponding MW_n after different enzyme exposure times are shown in Figure *2*. The effect of enzyme activity is clearly observed. The MW_n is more than halved after six hours enzyme activity.

NMR measurements

The NMR spectra after different times of enzyme exposure are shown in Figure *3*. The DP_n of the samples were 84, 57, 38, 26, and 14, corresponding to MW_n of 14 kDa, 9.5 kDa, 6.3 kDa, 4.3 kDa and 2.3 kDa, respectively. The value of the DP_n clearly reduced as time increased as shown in Figure *5*, where also

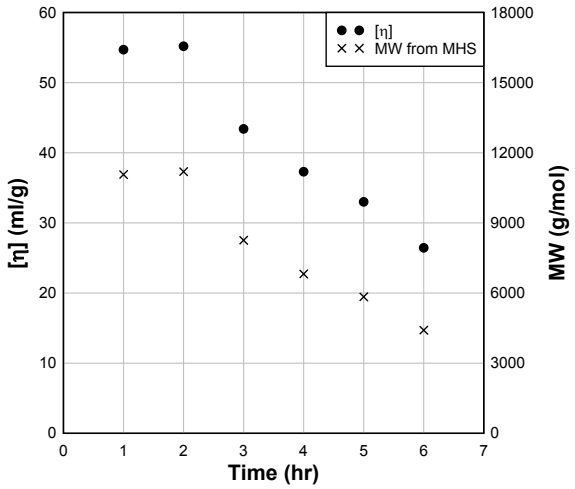

Figure 2: Comparison between intrinsic viscosity and MW_n from MHS equation after different times of enzyme activity.

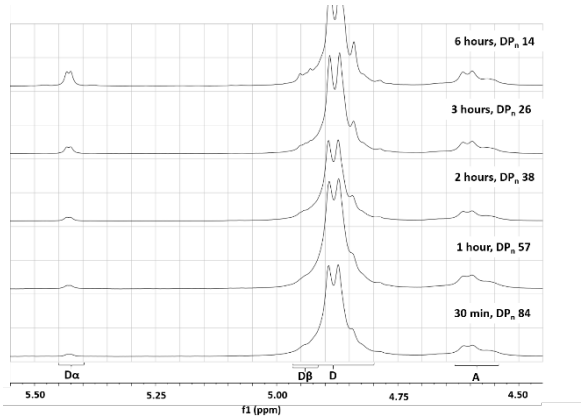

Figure 3: ^{1}H- NMR spectra after different times of enzyme exposure. The integral of the individual peaks was inserted in the equation (DP_n = (A+ Dα+Dβ+D)/ (Dα+Dβ) to find the DP_n of the different chitosan oligomer samples.

the product of G' and concentration is shown. A comparison between MW from intrinsic viscosity measurements and DP_n is shown in Figure *4* and Figure *5*. The MW_n obtained from DP_n correlates well with the MW obtained from the intrinsic viscosity. The linear regression, shown in Figure *4*, has a correlation coefficient equal to 0.9779. We observe that there is a systematic difference, where the MW determined from DP_n is approximately 78% of the value determined from MHS.

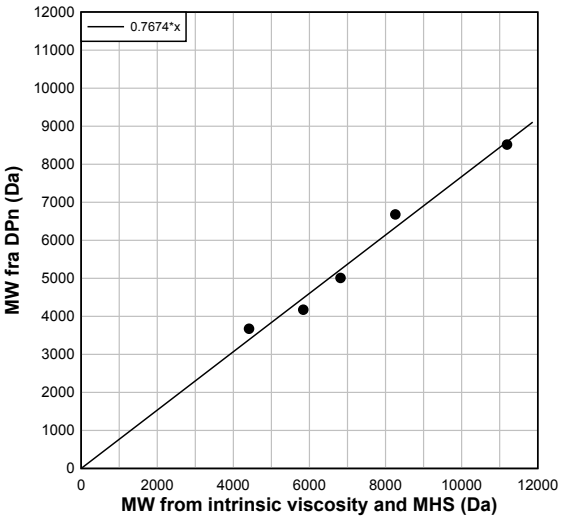

Figure 4: Comparison between molecular weight from intrinsic viscosity measurements and DP_n.

The oligomer solutions had variations in concentrations. The product of G' and concentration is shown in Figure *6*. The value seems to increase for the first two hours, and then the values reduces with time. A plausible explanation for this is that the observed structure requires some time to be established. After 2 hours we clearly observe that the stiffness of the structure reduces with time.

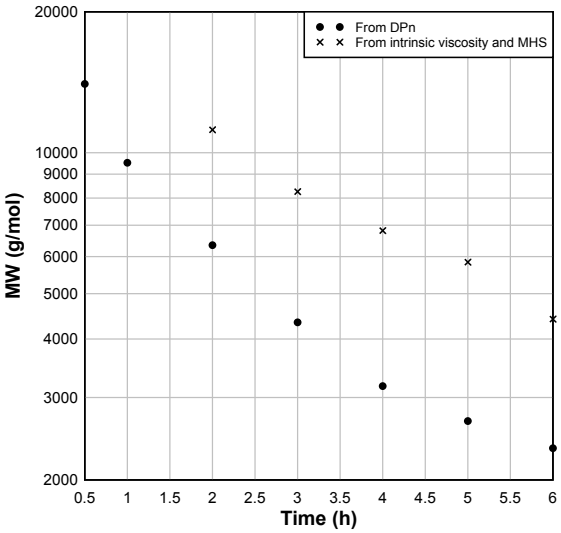

Figure 5: Molecular weights from intrinsic viscosity and DP_n.

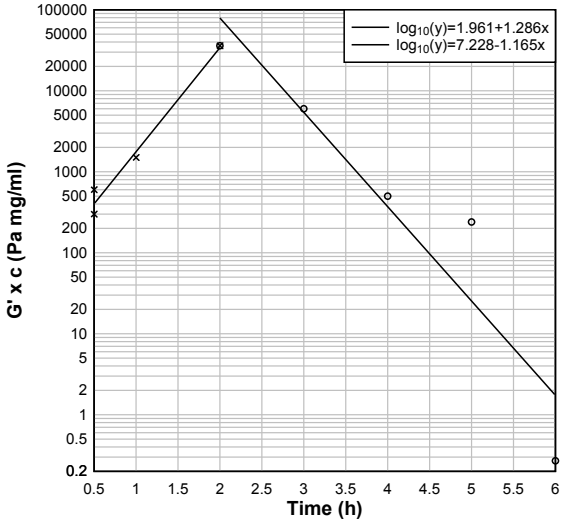

Figure 6: G' x c versus time.

Amplitude sweep results

The tests showed that the polymer solutions all exhibited viscoelastic behaviour with G' > G'' at low strains. The limiting strain of the LVR was larger than 10%. An example is shown in Figure 7.

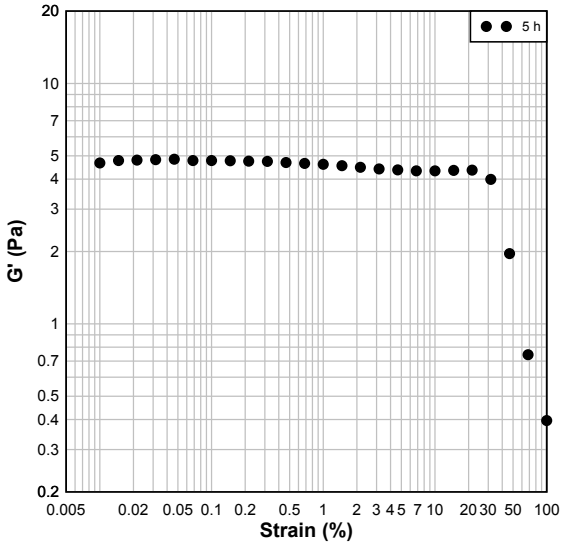

Figure 7: Amplitude sweep results showing G' versus strain.

Frequency sweep results (not shown) show that the solutions behave as viscoelastic solids at low frequencies, and G' and G'' cross as the frequency is increased, thus breaking the structure. The molecular weight distribution can therefore not be determined from dynamic data according to the established methods[3-5]. The reason for this is that the oligomers forms a viscoelastic solid when dissolved in water.

The known molecular weight of the chitosan oligomer samples correlates well with the results from the intrinsic viscosity measurements.

CONCLUSIONS

The results of this study are summarized as follows:

- The MW_n of the dispersed chitosan oligomer samples reduces with enzyme reaction time.
- The reduction in MW_n is seen from reduction in intrinsic viscosity calculated by the MHS-equation.
- The reduction in MW_n is also seen from ^{1}H-NMR spectra giving the value of DP_n.
- MW distribution from dynamic data were not obtained for these oligomer solutions.

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