13. COMBINED DEGRADATION OF CHITOSANS

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1. Introduction

Chitosan, high molecular weight natural compound of promising applications [1], is heteropolysaccharide containing 2-acetamido-2-deoxy-D-glucopiranose or 2-amino-2-deoxy-D-glucopiranose residues, randomly (in most cases) distributed along the polysaccharide chain. Chitosan obtained by standard de-N-acetylation of α-chitin has relatively high degree of polymerization (d.p.), and as this property has the main impact on its biochemical activity, the reduction of chitosan d.p. to desired level by controlled degradation methods using physical [2,3], chemical [4] and enzymatic agents [5] has been reported. Specially low molecular weight chitosan and chitooligomers with varying N-acetylation degree, expressed by mole fraction of N-acetylated units (F_A), have attracted the attention of scientists working in the field of applied chemistry [6], medicine [7], agriculture [8], biotechnology [9] and others [10, 11].

There are many papers [12–14] and some monographs [15] devoted to biochemical methods of production of chitooligomers, as the enzymatic methods of chitosan degradation are relatively simple to be carried out, fast, efficient, and environmental friendly. On the other hand, the construction of high efficient bioreactors, required to scale up the production, still seems to be technologically difficult and expensive [16]. Therefore, chemical and physical methods of chitosan degradation are still being studied and improved as well. Two of these are hydrogen peroxide induced and photochemical degradation methods, reported and described in detail in literature [17–20].

Hydrogen peroxide decomposes to hydroxyl radicals, which attack chitosan leading to the chain scission, what is the reason of the fast decrease in the value of d.p. There are also a few reports [21–22], which state that this is a very convenient method for chitool-
igomers production with good yield. Photochemical degradation by means of UV light is known as a convenient physical method for polymer degradation, which undergoes as a result of free radical reactions along the macromolecule [23].

As the free radical degradation method is relatively efficient and fast it seems to be authorized to study simultaneous action of the two degrading agents. The second reason for investigation of this type of degradation is the possibility of generation of oxidized products of degradation, what in the case of chitosan is not clearly established up to date. The aim of this study is to establish the yield and chemical structure of chitooligomers obtained by combined (hydrogen peroxide and UV) degradation of chitosan in homogeneous conditions, and confirm the mechanism of degradation (free radical or ionic, side reactions etc.)

2. Experimental

Materials. Chitosans with intrinsic viscosity (in 0.5 M CH$_3$COOH/0.3 M CH$_3$COONa at 30 °C) 2.7 dL·g$^{-1}$ and $F_A$ 0.01, and 4.9 dL·g$^{-1}$ and $F_A$ 0.20 from Primex (Island) were used for degradation. Other chemicals of p.a. grade (H$_2$O$_2$, CH$_3$COOH, CH$_3$COONa, CH$_3$COONH$_4$) or hplc grade (CH$_3$OH) from Aldrich, and bidistilled water were used in the solution preparation, GPC experiments and MALDI TOF MS analysis.

Degradation. 2% (w/w) H$_2$O$_2$ solution in 0.1 M CH$_3$COOH was used as a medium for chitosan degradation in ambient temperature (25 °C). UV lamp (254 nm, Philips) with low light intensity (~1 mW·cm$^{-2}$) was used for UV irradiation during H$_2$O$_2$-induced degradation. During degradation experiments the decrease of intrinsic viscosity was monitored by means of one-point method (of Matthes [24]) in Ubbelohde-type viscometer at 30 °C. The degradation was finished each time the relative viscosity approached the value of 1.05. Immediately after degradation the solution was freeze-dried at −55 °C and the material was collected for further analysis.

Methods. Viscometry was used for determination of intrinsic viscosities of starting material as described above (Terbojevich [25]) and for monitoring the run of degradation using the one-point method of Matthes [24] in Ubbelohde-type viscometer with the flow time of 99.0 s for the 0.5 M CH$_3$COOH/0.3 M CH$_3$COONa solvent system at 30 °C. GPC analysis was done in two 1 m columns with 25 mm in diameter with stationary phase of Biogel P4 and CH$_3$COOH/CH$_3$COONH$_4$ buffer system with pH 4.3. The flow rate was adjust to ~0.2 mL·min$^{-1}$ and fractions were collected every 20 min, and recognized using the refractive index detector. For the GPC experiments ~100 mg of the sample in the buffer was injected to the column each time.

MALDI TOF MS analysis was carried out by using REFLEX II MALDI-TOF spectrometer (Bruker) at Interdisciplinary Centre for Mass Spectroscopy of Biopolymers at Potsdam University in Germany.
3. Results and discussion

3.1. Degradation rate

Typical kinetic runs of combined degradation are presented in Figure 1, as a function of intrinsic viscosity versus time. For comparison kinetic curves are also shown for hydrogen peroxide and UV degradation. As it is shown, obviously after 120 min the degradation was interrupted when the relative viscosity approached the value of ~1.05. The higher the concentration and/or the $F_A$ value, the longer time of degradation, what indicates that amine groups present along the chain facilitate the degradation. In Table 1. numerical values of the rate constants, calculated from the slope of the dependence of $(\eta_t^{-1/a} - \eta_0^{-1/a})$ vs. time of degradation are collected, where $\eta_t$ and $\eta_0$ are the intrinsic viscosities after time $t$, and before degradation, respectively, and $a$ is the Mark-Houwink-Kuhn-Sakurada parameter [25]. The difference $(\eta_t^{-1/a} - \eta_0^{-1/a})$ is then proportional to the number of scissions per chain. The synergistic effect of UV irradiation and hydrogen peroxide is clearly shown. As the experiments were carried out in dark place, it is clearly shown that the hydrogen peroxide decomposition requires the UV-light action, indicating the free-radical mechanism of chitosan degradation. UV-light or hydrogen peroxide itself are not efficient degradation agents in applied experimental conditions. This indicates that low dose of UV light cannot cause the chain scission by producing the macromolecular radicals, however the presence of $\text{H}_2\text{O}_2$, which rapidly decomposes in the presence under UV-irradiation, results in hydroxyl radical action, attacking the glycosidic bond. $\text{NH}_3^+$- or $\text{NH}_2$-groups present at C(2) position in glucopyranose rings have the strong electronic sucking effect, what facilitates the degradation by weakening of anomeric C–H and C–O– bonds. Therefore, the hydrogen

![Figure 1. Intrinsic viscosities vs. time of combined or $\text{H}_2\text{O}_2$ or UV degradation for chitosans with different $F_A$ values (indicated in square brackets) and solution concentrations.](image)
abstraction and macroradical reorganization leading to the chain scission are faster in the case of chitosan[0.01]. This also was observed for wider spectrum of $F_A$ [26].

Table 1. Values of $[\eta]^{-1/a} - [\eta]_0^{-1/a}$ ($S$) for chitosans with varying $F_A$ value and in varying solution concentrations. $a = 0.76$ in 0.5 M CH$_3$COOH/0.2 M CH$_3$COONa [25].

<table>
<thead>
<tr>
<th>$F_A$</th>
<th>concentration, % (m/v)</th>
<th>$S$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>1.0</td>
<td>0.0645</td>
</tr>
<tr>
<td>0.01</td>
<td>2.0</td>
<td>0.0254</td>
</tr>
<tr>
<td>0.2</td>
<td>1.0</td>
<td>0.0340</td>
</tr>
</tbody>
</table>

3.2. GPC profiles

In order to analyze the yield and relative degree of polymerization of chitooligomers obtained in the investigated process of chitosan degradation, the GPC analysis was adopted. The results for chitosan [0.01] are presented as chromatogram in Figure 2, together with MALDI TOF MS identification of the degree of polymerization. The yield of the production of chitosan oligomers was calculated as the weight ratio between oligomer fraction and the whole mass of degradation products. For about 200 mg sample mass the yield of chitooligomer production was ~70 mg, e.i. the degradation products contained 35 % (w/w) of the oligomer fraction. However, as it is seen in the chromatogram, the resolution of separate oligomers is relatively poor in comparison to other analysis made in the same chromatographic system [27].

![Figure 2. MALDI TOF MS spectrum of chitooligomer fraction of chitosan [0.01] degraded in H$_2$O$_2$/UV-irradiation system. Inserted Figure: Fragment of GPC profile of degradation products together with d.p. recognized by MALDI TOF MS.](image)
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san[0.2] (data not shown) the yield of chitooligomers was relatively low and the chromatogram did not indicate individual signals for separate chitooligomers. The presence of oligomers was confirmed only by MS analysis. This could indicate the presence of interactions between the material being separated and the stationary phase (silica gel) on the column. The nature of these interactions is not clearly explained, however the MS analysis could give us the basic information about the molecular structure of obtained products, which should contains some additional polar (ionic) group along the chain to be able to adsorb at the surface of stationary phase in the chromatographic column.

The ‘macromolecular part’ of the chromatogram contains two overlapping signals, which could be attributed to undegraded high molecular weight polymer fraction and fraction of chitooligomers physically or chemically adsorbed at macromolecules. This was also checked by MALDI TOF MS analysis.

3.3. MALDI TOF MS analysis

The results of analysis of chitooligomer products obtained in degradation of chitosan [0.01] and chitosan [0.2] by means of hydrogen peroxide in the presence of UV-light are presented in Fig. 3 and 4. As it is shown, the main products in the chitooligomer fraction of the GPC chromatogram of chitosan[0.01] degradation products (Figure 3.) are completely de-N-acetylated chitooligomers with regular structure, what is indicated by values of the ratio mass to charge (m/z). Indicated molar masses can be attributed to oligomers with the degree of polymerization between 4 and 15. There are also m/z ratios present in the spectrogram which cannot be straightly recognized as regular chitooligomers. In these cases the m/z values are always lower by 2 than ratios for chitooligomers with regular structure. This could be due to the presence of oxidized –OH groups on the reducing end of chitooligomer chains, which forms the lactone ring. Opened-ring acid form of the reducing end is also possible, however differentiating between additional potasiated signals (present in each spectrogram made by this technique) and open-ring form signal is not possible because of the same value of the m/z ratio (both give signals shifted by +16 in comparison to the regular sodiated signals of not oxidized chitooligomers).

In the case of chitosan[0.2] degradation products (Figure 4.), additionally to the signals of completely de-N-acetylated chitooligomers, there are signals of partly N-acetylated chitooligomers present in the spectrum. As indicated in Figure 4., up to 3 2-acetamido-2-deoxy-D-glucopiranose units per chitooligomer chain of the value of d.p. up to 8 are confirmed to be present. However, the analysis of the sequence was not carried out because of the small quantity of the chitooligomers and relatively poor separation of them (data not shown). Figure 5, with MS spectrum of “macromolecular” part of the degradation fraction, shows that there are also present chitooligomers eluting together with high molecular weight chitosan, what confirms the assumption made on the basis of GPC elution curve. In this fraction the chitooligomers with the value of d.p. of 3-5 were identified to be adsorbed physically (m/z ratio not changed in comparison to “original” chitooligomers with the regular chemical structure) at the macromolecules.
**Figure 3.** MALDI TOF MS analysis of chitosan[0.01] combined degradation products. D-2-amaA2-deoxy-D-glucopiranose units.

**Figure 4.** MALDI TOF MS analysis of chitosan[0.2] combined degradation products. D-2-amaA2-deoxy-D-glucopiranose units, A-2-acetamido-2-deoxy-D-glucopiranose units.
This indicates strong attraction forces (probably of electrostatic nature) present in the system between oxidized chitooligomers (carboxylic groups –COO\(^-\) could be present) and positively charged chitosan chains (with –NH\(_3^+\) groups).

4. Conclusion

Chitosan is a high molecular weight compound sensitive for combined action of hydrogen peroxide and UV-irradiation. The strong synergistic effect between these two degrading agents was observed: degradations by H\(_2\)O\(_2\) or UV-light conducted separately (in applied experimental conditions) were completely insufficient to decrease the d.p. of chitosan in reasonable time of degradation in comparison to combined degradation. The degradation is induced by hydroxyl radical formed as a result of photo-destruction of hydrogen peroxide:

\[
\text{H}_2\text{O}_2 \rightarrow 2 \text{HO}^* \tag{1}
\]

These radicals abstract the hydrogen atom from anomeric C(1) atom, what begins the macroradical re-organization leading to the chain scission, what is typical for cellulose-type macromolecules [23]. The yield of chitooligomers in the case of fully de-N-acetylated chitosan is relatively high (35%), however the increase of F\(_A\) causes decrease of chitooligomer yield. Oxidation products present in the chitooligomers mixture are responsible for strong interaction with high molecular weight chains and chitooligomers.
5. References

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