Synthesis and Characterization of Gelatin Fragments Obtained by Controlled Degradation

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Summary: Telechelic oligomers are attractive starting materials for the preparation of materials with tailorable properties. Here, peptide chains with defined molecular weight were obtained by controlled degradation of gelatin with hydroxylamine, which resulted in the cleavage of asparaginyl-glycine bonds and formation of new aspartyl hydroxamates and amino endgroups. The reaction of gelatin with hydroxylamine resulted in fragments with molecular weights of 15, 25, 37, and 50 kDa (determined by SDS-PAGE) independently of the reaction time and conditions. The fragment mixture showed typical single and triple helical organization in WAXS spectra, but rheological studies showed lower G' and G'' values for gels from the fragment mixture than from gelatin, and lower gel-sol transitions temperatures. A more narrow distribution was found for the fragment mixture ($M_n = 25$ kDa, PDI = 1.4) than for commercial gelatin.

Keywords: gelatin; hydroxylamine; oligomer synthesis; polymer degradation; telechelics

Introduction

A synthesis strategy to obtain materials with tailorable mechanical properties and controlled degradation rate is to use telechelic oligomers with defined molecular weight. Two general strategies for the synthesis of telechelics are known, synthesis from monomers[1] and controlled degradation of high molecular weight polymers. Synthetic techniques such as ionic polymerization, polycondensation[2] or radical polymerization[3] can be employed for the formation of telechelics, when suitable monomers are available.[4,5] Degradative methods have extensively been applied to polyhydroxyalkanoates such as poly(3-R-hydroxybutyrate) (P3HB) or copolyesters, whereby especially trans-esterification[6] or the controlled degradation of copolymers with weak links have been studied.[7–10] Low molecular weight P3HB can also be obtained by degradation of high molecular weight P3HB, via acid-catalyzed methanolysis,[11] acid/ base hydrolysis[12] or thermal degradation.[13] The degradation mechanism (e.g. random chain scission or endgroup scission) has a strong influence on the structure of the obtained telechelic.[14] By monitoring the reaction by gel permeation chromatography and stopping the reaction at the right time point, fragments with the desired molecular weight are obtained.

In protein chemistry, some selective chemical methods for peptide bond cleavage are known, e.g. treatment with CNBr.[15] Hydroxylamine specifically cleaves the bond between asparagin and glycine residues leading to the formation of aspartyl-hydroxamates and amino endgroups (Scheme 1). Such a method is fast, likely easy to scale up, and inexpensive. Gelatin is produced by partial hydrolysis of collagen and consists of a mixture of polypeptide fragments, including α-chains, β-chains (two α-chains covalently crosslinked) and y-chains (three covalently crosslinked α-chains).[16] Bornstein *et al.* reported the presence of hydro-

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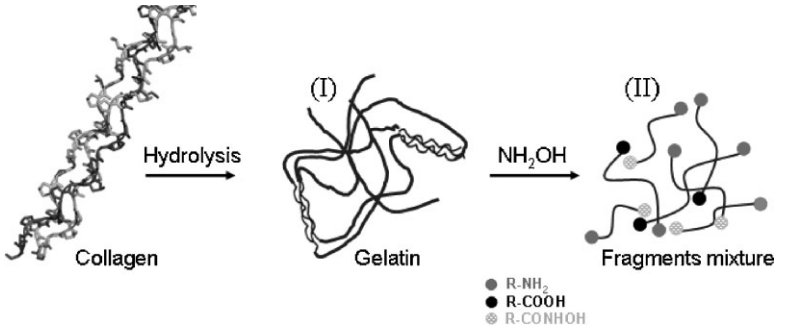

Scheme 1.
Gelatin is produced via the partial hydrolysis of collagen[18] (I); by the use of NH_2OH fragments with defined molecular weight and endgroups can be obtained (II).

xylamine sensitive bonds in collagen.[15] Therefore, here it was investigated whether hydroxylamine mediated degradation of gelatin leads to telechelic oligomers with defined molecular weight and endgroups, which are potentially useful in the synthesis of segmented polymers with tailorable properties.[17] The degradation of gelatin with hydroxylamine was performed under different conditions and the molecular weight of the fragments mixture was determined by SDS-PAGE, gel permeation chromatography (GPC) and MALDI-TOF MS. The physical properties of the fragments mixture and the chain molecular organization were investigated by WAXS and rheological measurements and compared with the high molecular weight gelatin.

Experimental Part

Hydroxylamine Cleavage

An aqueous solution of gelatin Type A (pig skin, 300 bloom, Sigma Aldrich) (5 mg/mL) was incubated at 45 °C or room temperature with 1 M, 2 M or 4 M aq. hydroxylamine solution (Merck). The pH of the mixture was adjusted to 9.5 by adding a 1 M aq. Na_2CO_3 solution. The reaction was performed for 2 or 24 hours. Subsequently, the hydroxylamine was removed by dialysis against water and the product was lyophilized. The amine content was determined by the use of a spectrophotometric assay.[19]

Sodium dodecyl sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE)

The molecular weight distribution was determined by SDS-PAGE on 4–20% Ready Gels (Bio-Rad Laboratories) in a Mini-Protean system (Bio-Rad). A prestained SDS-PAGE standard was used for the determination of molecular weight. Samples of gelatin were dissolved in distilled water at 40 °C for 30 min at a concentration of 2 mg/mL. All samples were diluted 1:1 with a 2x sample buffer, then heated to 90 °C for 5 min and cooled down. 15 μL of each sample were loaded onto their respective lane in the gel and a current of 120 V was applied for approximately 45 min. Protein bands were visualized after a 60 min staining (0.1 wt.-% Comassie Blue, 10 Vol-% acetic acid and 40 Vol.-% methanol in water) and 60 min treatment in a destaining solution (10 Vol.-% glacial acetic acid, 20 Vol.-% methanol in water). The molecular weight of the gelatin fragments was approximated by measuring the relative mobility of the standard protein molecular weight markers.

Rheological Measurements

The rheological measurements were performed with a stress controlled rheometer MARS (Thermo Haake, Germany) equipped with a plate-plate geometry of 20 mm and a Peltier temperature controlled system. 6 wt.-% solution of gelatin or gelatin fragments in distilled water prepared at 40 °C were introduced between the rheometer

plates and cooled down to 4 °C. After the gel formation, the rheological measurements were performed. A solvent trap was used to prevent solvent evaporation. Preliminary stress-sweep experiments were carried out to determine the linear viscoelastic range. The elastic (G′) and viscous (G″) moduli were determined as a function of the frequency from 0.01 to 10 Hz at a stress of 3 Pa at 4, 15, and 25 °C. Temperature sweep measurements were performed from 4 to 45 °C at a heating rate of 1 °C/min.

Wide Angle X-Ray Scattering (WAXS)

WAXS measurements were performed on a Bruker D8 Discover (Bruker AXS, Germany). The X–ray generator was operated at a voltage of 40 kV and a current of 20 mA. A copper anode and a graphite monochromator produced Cu K_α radiation with a wavelength (λ) of 0.154 nm. WAXS images were collected from gelatin samples in transmission geometry with a collimator-opening of 0.8 mm at a sample-to-detector distance of 15 cm. The exposure time employed was 900 s. Integration of the two-dimensional scattering data gave the intensity as a function of the scattering angle 2θ. The measurements were performed on gelatin and gelatin fragment films. The films were prepared by dissolving 0.5 g of sample in 5 mL water at 40 °C, the solutions were casted in a Petri dish and dried at room temperature over night.

Gel Permeation Chromatography

The molecular weight analysis of gelatin fragments was carried out by multi-detector gel permeation chromatography equipped with a light scattering detector (RALS), differential viscosimetry detector and a refractive index detector. Gelatin solutions were loaded into the column at a concentration of 1.5 mg/mL. The analyses were carried out using two columns (A6000 and A7000, 300 mm × 7.8 ID) connected in series with a A7 guard column, and eluting with aqueous sodium nitrate solution (0.1 M) at a flow rate of 0.7 mL/min. The light scattering detector was calibrated using a dextran standard. The molecular weight of the sample was directly determined with the light scattering detector.

Results and Discussion

The treatment of gelatin with hydroxylamine led to the formation of a mixture of fragments with molecular weight of 15, 25, 37, and 50 kDa. The reaction was performed using different molar concentration of hydroxylamine, specifically 1, 2, and 4 M, at 45 °C or room temperature, for 2 hours and 24 hours respectively. The molecular weight distribution was determined by SDS-PAGE (Figure 1). Gelatin contains α1 and α2 chains (100 kDa) and β chain (200 kDa) as major constituents. These high-molecular weight bands are not visible in any sample treated with hydroxylamine. According to the gel electrophoresis, hydroxylamine cleavage resulted in smaller fragments compared to the non-treated gelatin. Bands at 50 kDa, 37 and 20 and 15 kDa were found in all samples, independent of the reaction time, temperature or hydroxylamine molar concentration, which shows the specificity of the reaction. The fragment mixture was analyzed also by MALDI-TOF mass spectrometry, in which however it was not possible to detect the larger fragments seen in the SDS-PAGE, possibly due to complex spectra because of multiple charges and/or smaller intensities of the larger fragments. However, it was possible to detect fragments of ca. 13.5 and 14.5 kDa, which correspond to the band at 15 kDa in the SDS-PAGE, as well as several peaks below 10 kDa not being resolved in the SDS-PAGE.

The presence of hydroxylamine sensitive bonds in collagen was observed during the investigation of its primary structure.[20,21] Butler[22] and Bornstein[15] established that collagen chains were cleaved with a high degree of specificity at the asparyginyl-glycyl bond. The cleavage at this particular bonds result from the tendency of the asparaginyl side chain to cyclize into a substituted succinimide, which is susceptible to nucleophilic attack by hydroxyla-

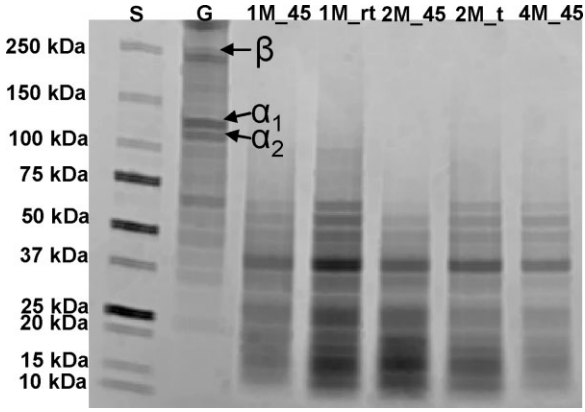

Figure 1.
SDS-PAGE of gelatin before and after treatment with NH_2OH. S: Standard; G: gelatin; 1M_45, 1M_rt, 2M_45, 2M_rt and 4M_45: Gelatin after NH_2OH mediated cleavage varying the reaction conditions.

mine. The sensitivity of Asn-Gly bonds to hydroxylamine compared to other asparaginyl bonds can be likely explained by the ease of cyclization of the asparaginyl side chain in the absence of the steric hindrance imposed by the side chain of the next amino acid.[23] The proposed mechanism of the hydroxylamine cleavage is depicted in Figure 2.

The cleavage of gelatin with hydroxylamine yielded a mixture of fragments with aspartyl-hydroxamates and new amino glycine endgroups. The amino content of gelatin before and after treatment with hydroxylamine was determined by the use of a spectrophotometric assay (TNBS). The amino content of gelatin was $27 \cdot 10^{-5}$ mol, and increased to $32 \cdot 10^{-5}$ mol for the gelatin fragments. The increase of free amino groups corresponds to the proposed mechanism.[23]

The rheological behavior of gelatin and fragment mixture solutions (6 wt.%) was investigated. In order to assess the strength of the physical gels, frequency sweep measurements were carried out at 4, 15 and 25 °C in a frequency range from 0.01 to 1 Hz (Figure 3). At the same weight concentration of gelatin and the fragments mixture, the elastic modulus (G′) of gelatin was higher than the G′ of the fragment mixture and did not depend on the frequency.

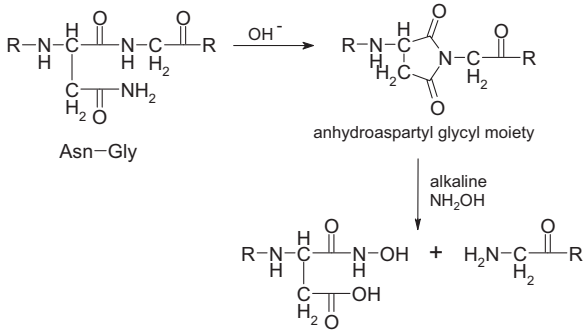

Figure 2.
Proposed mechanism of NH_2OH-mediated cleavage of the Asn-Gly bond. The anhydroaspartyl moiety can be cleaved by hydroxylamine yielding fragments resulting in aspartyl hydroxamate C-terminus and an amino-terminal glycine.[23]

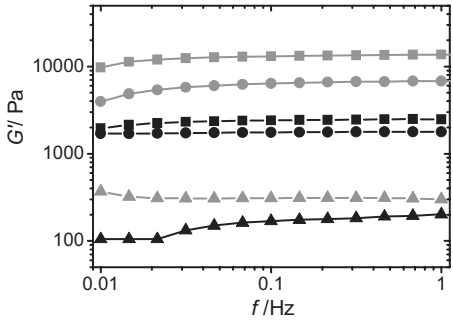

Figure 3.
Frequency sweep measurements of gelatin (■-●-▲) and fragments mixture (■-●-▲) solutions (6 wt. %) at 4 °C (■) 15 °C (●) and 25 °C (▲).

The viscoelastic behaviour was investigated as a function of the temperature as well (Figure 4). The solutions were prepared at 40 °C, placed between the plates and equilibrated at the temperature of the rheometer, forming a physical gel. The gel formed by gelatin was stronger and stable in a wide range of temperature, whereas the elastic modulus of the fragment mixture started to decrease at approximately 10 °C. The sol-gel transition temperature taken as the crossover point between G′ and G″, occurred at higher temperature in gelatin gels than in the fragments mixture. The different behaviour could be likely due to the difference in molecular weight and to the relative content of α and β chain components.[24]

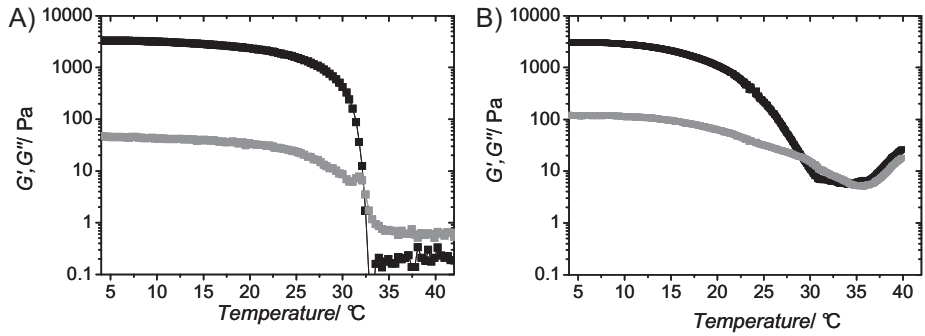

Figure 4.
Temperature sweep of gelatin (A) and gelatin fragments mixture (B) from 4 to 42 °C, (—) G′ and (—) G″.

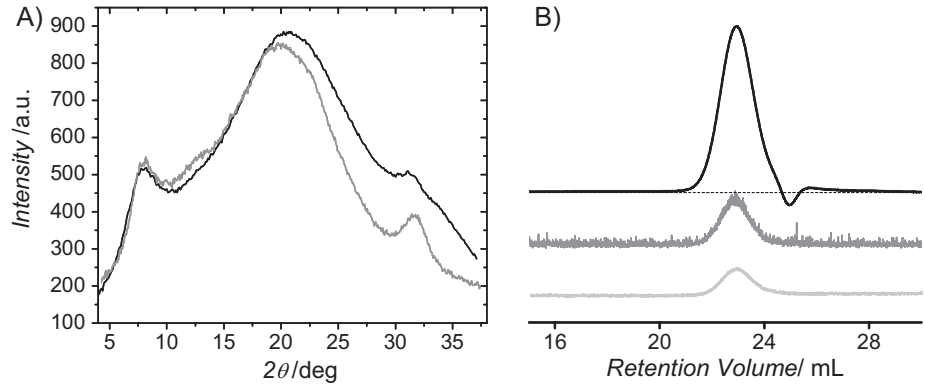

Figure 5.
(A) WAXS spectra of gelatin (—) and gelatin fragments (—). (B) Gel permeation chromatography of gelatin fragments solution, (—) Refractive Index, (···) Right Angle Light Scattering, (—) Viscometer.

The molecular organization of gelatin before and after treatment with hydroxylamine was determined by WAXS. The WAXS spectra of gelatin shows three peaks at $2\theta = 8^\circ$, 20° and 31°, which correspond to the diameter of the triple helix, the amorphous halo, and the amino acids contact along the axis of single helices, repectively.[25–27] The fragmentation of gelatin had no influence on the molecular organization (Figure 5). Attempts to separate individual fragments were made by using membrane filtration and GPC; however in both cases no separation was obtained.

The molecular weight distribution of gelatin and gelatin fragments was analyzed by GPC. The commercial gelatin had a M_n of 45700 and a polydispersity of 3.02, whereas for gelatin fragments a M_n of 25000 and a polydispersity of 1.4 were determined. Even though the separation of the fragments was not possible, the GPC profile showed a more narrow distribution of the gelatin fragments compared to the commercial gelatin.

Conclusion

Telechelic oligomers having defined endgroups were obtained by controlled degradation of gelatin using hydroxylamine. The cleavage of gelatin led to the formation of a mixture of fragments with either amino and carboxyl endgroups or amino and aspartylhydroxamate endgroups. Furthermore, due to the proteinic structure, various side chain functional groups including alcohols and carboxylic acids are present. The treatment of gelatin from different batches gave the same fragmentation. Separation of individual fragments remains a challenge, however the GPC profile of the fragments mixture showed a more homogeneous composition compared to the commercial gelatin. These telechelic oligomers are planned to be used for the building up of segmented polymers and polymer networks.

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