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ARTICLE

Model Studies on Chemical and Textural Modifications in Gelatin Films by Reaction with Glyoxal and Glycolaldehyde

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The present study investigated chemically modified gelatin biopolymer films. Gelatin solutions were treated with glyoxal and glycolaldehyde, respectively, at concentrations ranging from 0.25 to 7.5 wt % based on gelatin. From these solutions, films were produced under defined conditions and characterized with different chemical and physical methods. N^{ε}-carboxymethyllysine (CML), glyoxal-derived lysine dimer (GOLD), and 5-(2-imino-5-oxo-1-imidazolidinyl)norvaline (imidazolinone) were analyzed as chemical parameters for protein modification by reversed-phase high-performance liquid chromatography (RP-HPLC) and fluorescence detection after post-column *o*-phthaldialdehyde derivatization. An increase in the content of these substances with increasing concentrations of carbonyl modifiers correlated with the loss of available free lysine and arginine residues. Swelling, solubility, and mechanical properties (Young's modulus, stress and strain at break) showed the relationship with the degree of monovalent modification and cross-linking as well. The determination of unreacted glyoxal and glycolaldehyde suggested a different mechanism of cross-linking induced by glyoxal versus glycolaldehyde as reactive intermediates in Maillard chemistry.

KEYWORDS: Biopolymer film; gelatin; chemical modification; glyoxal; glycolaldehyde; N^{ε}-carboxymethyllysin; glyoxal-derived lysine dimer; Young's modulus

INTRODUCTION

The reaction of reducing sugars and their degradation products with amino groups of amino acids, peptides, and proteins is called the Maillard reaction or non-enzymatic browning. During this complex series of reaction pathways, proteins will be multi-modified to result in material of novel characteristics and functionalities (*1*, *2*). Glyoxal (GL) and glycolaldehyde (GLC) are known to be reactive intermediates toward nucleophilic amino acid residues of proteins (*3*). The carbonyl group reacts with the ε-amino group of lysine and forms the most important monovalent modification N^{ε}-carboxymethyllysine (CML) (*4*). The cross-link glyoxal-derived lysine dimer (GOLD) between two lysine residues can be used as a direct parameter of the covalent connection of proteins (*5*). After the formation of a Schiff base and an Amadori rearrangement (*6*), the reaction of GLC with amine residues involves an additional radicalic pathway (*7*) to result in the development of colored compounds and cross-links. A major arginine modification is 5-(2-imino-5-oxo-1-imidazolidinyl)norvaline (imidazolinone), arising from conversion of N^{7}-carboxymethylarginine and dihydroxyimidazolidine under strong acidic conditions (*8*). Similar to CML, imidazolinone presents a general quantitative important parameter for monovalent amino acid modification.

Beside unintentional modifications of proteins during the processing of food or under physiological conditions, the Maillard reaction was widely used to affect or create novel properties of different proteins. Sugars, such as ribose, glucose, or lactose, and polysaccharides, such as galactomannan, chitosan, and maltodextrin, were used to influence emulsions, foaming, gelling, and solubility of whey proteins, ovalbumin, and β-lactoglobulin (*9*−*13*).

In the food industry, gelatin is used as a technical additive or ingredient, and in the pharmaceutical industry, gelatin is used as soft and hard capsules or gelatin sponges (*14*). Gelatin forms biodegradable polymer films, which have been widely studied (*15*−*18*). To affect and control the mechanical properties of gelatin films, different cross-linking techniques and agents were applied. Beside thermal treatment, chemical and enzymatic cross-linking was performed in both solutions and films; e.g., modification with glutaraldehyde (*19*) led to an increase of stiffness with an increasing dicarbonyl concentration. Other amino-acid-reactive substances, such as genipin (*20*) or diisocyanates (*21*), reduced swelling behavior or increased the resistance against collagenases.

In the literature, chemically modified gelatin films are characterized almost exclusively on the basis of their resulting physical properties, with little or no knowledge of the underlying mechanisms or modifications. Because the possibility to tailor fit the solubility and mechanical, thermal, and barrier characteristics of gelatin is highly desirable, the present study for the first time correlates chemical parameters and mechanical properties of

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gelatin films modified by GL and GLC. Quantification of CML, GOLD, and imidazolinone allowed for deeper insights into the mechanism of protein Maillard modification.

MATERIALS AND METHODS

Materials. Chemicals of the highest quality available were obtained from Aldrich (Taufkirchen, Germany), Fluka (Taufkirchen, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), and Sigma (Taufkirchen, Germany), unless otherwise indicated.

CML and GOLD were synthesized according to Glomb and Pfahler (*22*), and imidazolinone was synthesized according to Glomb and Lang (*8*).

Film Preparation. Biopolymer films were obtained from gelatin gels by drying under defined conditions. Gelatin (type A, GELITA AG, Ebersbach, Germany) was dissolved in distilled water for 20 min at 60 °C. The gelatin content of the solution was 4% (w/v). GL and GLC were added, respectively, at concentrations ranging from 0.25 to 7.5 wt % related to gelatin and were stirred for further 15 min at 60 °C. Aliquots of these solutions were applied to rectangular forms. After jellification for 2 h at room temperature, the gels were dried for 20 h at 30 °C. The mass per unit area of the resulting films was 0.01 g/cm^2. Chemical, physical, and mechanical properties were determined from gelatin films produced by four independent replications for each carbonyl concentration.

Swelling Properties and Water Solubility. A piece of gelatin film with defined dimensions (5 × 5 cm) was stored in water at 40 °C for 30 min. After centrifugation and subsequent removal of the liquid, the gelatin film was weighed. The swelling properties were calculated from the increase in weight. After drying the hydrated gelatin films at 30 °C to a constant mass, the weight loss was calculated from the difference between the initial and resulting weight (see eqs **1** and **2**).

$$\mathrm{WA}\ (\%) = 100\%\frac{m_W}{m_F} - 100\% \qquad (1)$$

$$\mathrm{WL}\ (\%) = 100\% - \frac{m_D}{m_F}100\% \qquad (2)$$

where WA is the water absorption (%), WL is the weight loss (%), m_F is the mass of gelatin film before water storage (g), m_W is the mass of gelatin film after water storage (g), and m_D is the mass of gelatin film after drying (g).

Determination of Lysine, Arginine, CML, GOLD, and Imidazolinone. The content of the amino acids and the amino acid modifications was obtained after acid hydrolysis of the gelatin films. A total of 5 mg of gelatin films was dissolved in 2 mL of 6 N HCl and heated for 20 h at 110 °C after the removal of oxygen by degassing with argon. Volatiles were removed in a vacuum concentrator, and the residues were diluted with 0.1 N HCl to concentrations appropriate for high-performance liquid chromatography (HPLC) analysis.

Quantitative results were obtained from external calibration (coefficient of determination > 0.99) with commercially available amino acids or synthesized reference material. Contents of amino acids showed standard deviations < 0.03 mmol/g of gelatin film, resulting in coefficients of variation < 5%. Contents of amino acid modifications showed standard deviations < 40 mmol/mol of lysine, resulting in coefficients of variation < 30%.

Determination of GL. Free and reversibly bound GL in modified gelatin films was determined as quinoxaline after derivatization with *o*-phenylenediamine (*o*-PD). A total of 50 mg of powdered gelatin film was dissolved in 2 mL of 0.1 M *o*-PD solution, and 0.6 M perchloric acid (pH 2.3) was added to give a total volume of 5 mL. The solution was kept at room temperature (RT) for 5 h (conversion of dicarbonyls to quinoxalines under this reaction conditions was 100% in line with ref *23*). After centrifugation and filtration, an aliquot of the filtrate was diluted with H_2O for HPLC analysis. Quantitative results were obtained from external calibration (coefficient of determination > 0.99) with commercially available quinoxaline. Contents of GL showed standard deviations < 18 μmol/g of gelatin film, resulting in coefficients of variation < 13%.

Determination of GLC. Free GLC in the gelatin films was extracted as *O*-benzylhydroxyloxime after derivatization using *O*-benzylhydroxylamine (BH). A total of 20−50 mg of powdered gelatin films with 2 mL of 50 mM BH solution and 0.5 mL of 1 mM phenylacetaldehyde (PAA) solution as the internal standard was incubated in 10 mL of 0.2 M phosphate buffer (pH 7.4) for 3 h at 37 °C. A total of 250 μL of 6 M HCl was added to the sample solution, and BH oxime derivates were extracted with diethyl ether. The extracts were concentrated to dryness, and the residues were reacted with 100 μL of *N*,*O*-bis(trimethylsilyl)acetamide (BSA) in 100 μL of pyridine for silylation of the OH groups. A total of 1 μL of the pyridine−BSA solution was injected into gas chromatography−mass spectrometry (GC−MS). Quantitative results were obtained from external calibration (coefficient of determination > 0.99) using different volumes of a GLC solution with a known amount of analyte instead of the powdered gelatin film. Contents of GLC showed standard deviations < 10 μmol/g of gelatin film, resulting in coefficients of variation < 35%.

HPLC. For determination of the amino acids and their modifications, a Jasco (Gross-Umstadt, Germany) ternary gradient unit 980-PU-ND with a degasser DG-980-50, an autosampler 851-AS, a column oven set at 15 °C, and a fluorescence detector FP-920 was used. Chromatographic separations were performed on stainless-steel columns (VYDAC 218TP54, 250 × 4.6 mm, RP18, 5 μm, Hesperia, CA) using a flow rate of 1.0 mL/min. The mobile phase used was water (solvent A) and MeOH/water [7:3 (v/v); solvent B]. To both solvents (A and B), 1.2 mL/L of heptafluorobutyric acid (HFBA) was added. For determination of lysine, arginine, imidazolinone, and CML, samples were injected at 2% B and run isocratic for 25 min and the gradient then changed to 100% B in 5 min, was held at 100% for 10 min, then changed again to 2% B in 5 min, and was held for 20 min. For determination of GOLD, samples were injected at 10% B and run isocratic for 10 min and the gradient then changed to 18% B in 50 min, then changed to 100% B in 5 min, was held at 100% for 20 min, then changed again to 10% B in 5 min, and was held for 15 min. The fluorescence detector was attuned to 340 nm for excitation and 455 nm for emission. Prior, a post-column derivatization reagent was added at 0.5 mL/min. This reagent consisted of 0.8 g of *o*-phthaldialdehyde, 24.73 g of boric acid, 2 mL of 2-mercaptoethanol, and 1 g of Brij35 in 1 L of H_2O adjusted to pH 9.75 with KOH.

For determination of GL quinoxaline, a Jasco PU-2089 Plus quaternary gradient pump with a degasser was used combined with a Jasco AS-2055 Plus autosampler. The column oven was set at 20 °C, and a UV detector Jasco UV-2075 Plus was used (all Jasco, Gross-Umstadt, Germany). Chromatographic separation was carried out on a stainless-steel column (Knauer Eurospher 100-5 C18, 250 × 4.6 mm, Berlin, Germany) using a flow rate of 1 mL/min. The mobile phase used was water (solvent A) and water/MeOH [3:7 (v/v), solvent B]. To both solvents, 0.6 mL/L of HFBA was added. Samples were injected at 20% B gradient, changed linear to 30% B in 35 min, changed to 40% B in 5 min, changed to 70% B in 15 min (held for 5 min), changed to 100% B in 30 min (held for 10 min), and then changed to 20% B in 5 min (held for 15 min). The UV detector was attuned to 320 nm.

GC. GLC samples were analyzed on a Thermo Finnigan Trace GC Ultra coupled to a Thermo Finnigan Trace DSQ (both Thermo Fisher Scientific GmbH, Bremen, Germany). The GC column was a HP-5 (30 m × 0.32 mm; film thickness, 0.25 μm; Agilent Technologies, Palo Alto, CA) with the following conditions: injector, 220 °C; split ratio, 1:10; and transfer line, 250 °C. The oven temperature program was as follows: 100 °C (0 min), 5 °C/min to 200 °C (0 min), and 10 °C/min to 270 °C (10 min). Helium 5.0 was used as the carrier gas in a constant flow mode (linear velocity, 40 cm/s; flow, 1 mL/min). Mass spectra were obtained with EI at 70 eV (source, 210 °C) in full-scan mode (mass range, m/z 50−650). Quantifications were performed in SIM mode using m/z 117 for silylated GLC BH oxime and m/z 225 for PAA BH oxime. The dwell time was 100 ms in each case.

Tensile Test. Stress−strain curves of strip-shaped (10 mm wide, with thickness around 75 μm) films were recorded using a testing machine ZWICK 1425 (Zwick GmbH and Co. KG, Ulm, Germany) with a load cell 2 kN after DIN EN ISO 527-1-3 at a cross-head speed of 1 mm/min. The initial distance between the clamps was 70 mm. The Young's modulus, E, the stress at break, σ_b, and strain at break, ε_b, were evaluated on the basis of six measurements for each concentration of cross-linking agent.

RESULTS AND DISCUSSION

Physical and Mechanical Properties. Swelling properties of the gelatin films were accessed by storing pieces of films in water

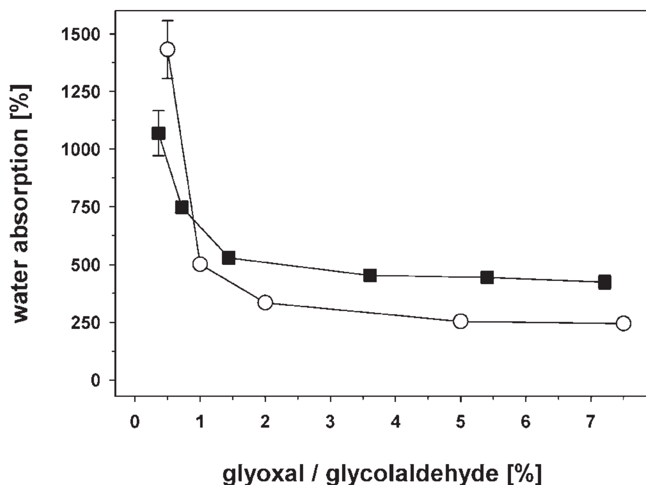

Figure 1. Swelling properties of chemically modified gelatin films after storing in water at 40 °C for 30 min (■, GL; ○, GLC).

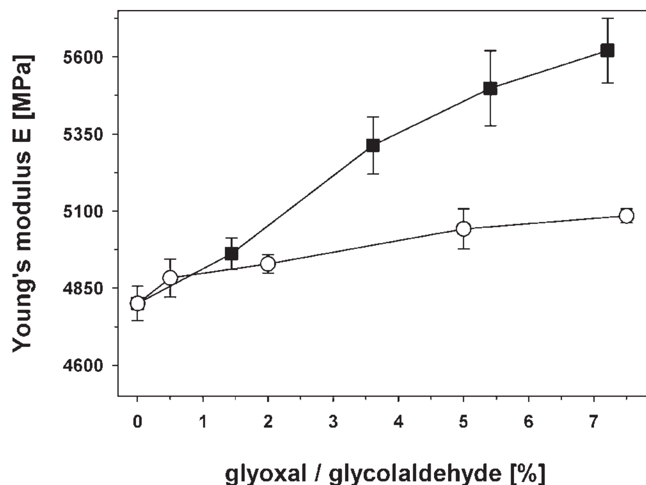

Figure 3. Young's modulus of chemically modified gelatin films (■, GL; ○, GLC).

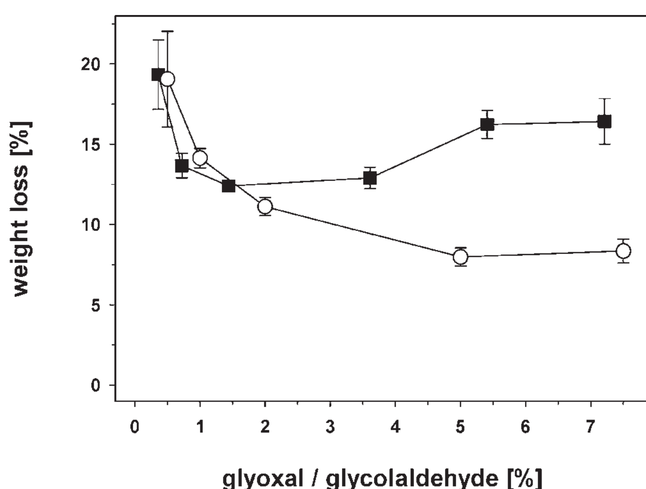

Figure 2. Solubility of chemically modified gelatin films after storing in water at 40 °C for 30 min (■, GL; ○, GLC).

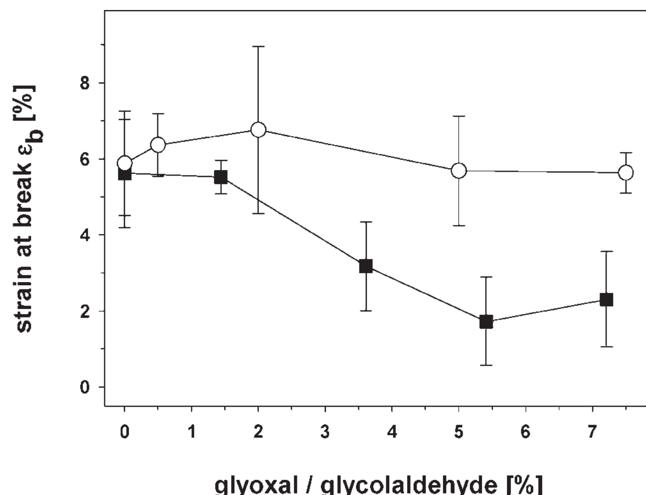

Figure 4. Strain at break, σ_b, of chemically modified gelatin films (■, GL; ○, GLC).

under defined conditions and subsequent differential weighing. The water solubility was determined by drying the same specimen. Gelatin films produced without any GL/GLC were completely dissolved in water under the given experimental settings. **Figure 1** shows the decrease in weight, designated as water absorption, in relation to carbonyl modifier. With increasing GL and GLC concentrations, the ability to absorb water decreased significantly to reach a minimum at about 2% carbonyl concentration. However, significant differences between GL- and GLC-modified gelatin films could be observed. While GL reduced the water absorption to 424%, GLC reached a value of 244%. Further differences between GL- and GLC-modified films are shown in **Figure 2**. Whereas the loss of weight of GLC films decreased with an increasing GLC concentration to 8%, films with GL reached a minimum of 12% at a GL concentration of 1.44% relative to gelatin. With an increasing GL concentration, the loss of weight increased again to 16%. The effect of the reduced gelatin swelling ability with increasing carbonyl concentrations was already observed, e.g., glutaraldehyde, and showed a similar relationship (*19*). However, a comparison of absolute values is not possible because of different experimental settings. In the present paper, the increase in the carbonyl reagent led to an increase in covalent bonds between gelatin chains. The covalent cross-linking of the protein network increased stiffness and reduced the ability to incorporate water and resulted in a decrease of swelling properties. This can also be seen in the change of solubility, which, in comparison to unmodified gelatin films, was significantly repealed. The presence of a minimum with GL films and a later increase at a higher modifier concentration was exactly covered by the release of excess unreacted or reversibly bound GL (e.g., 4% of free GL in 7.21% film). In contrast, the modification of gelatin with GLC led to irreversible binding of the carbonyl reactant to the biopolymer matrix.

The carbonyl-induced protein modification was further visualized by recording the stress−strain curves of modified gelatin films. Important characteristics are the Young's modulus and stress and strain at break. The Young's modulus, which characterizes the stiffness of material, increased as expected with an increasing carbonyl content of the films (**Figure 3**). The Young's modulus of GL-modified films rose by 17%. In contrast, the modulus of GLC-modified films increased by only 6%. Despite high variations in the values of stress and strain at break, which are typical for brittle foils, the results point to the tendency of decreasing strain at break with an increasing GL content of the films (**Figure 4**). The decrease of the strain at break with an increasing cross-linking agent was more pronounced in GL-modified films than in GLC-modified films. This result correlated with the stronger increase of the Young's modulus of GL films

Table 1. GL and GLC Lead to Gelatin Modification

	added carbonyl		lysine	arginine	CML	GOLD	imidazolinone	free/reversibly bound carbonyl	carbonyl conversion (%)	
	(%)[a]	(μmol/g)	(μmol/g)	(μmol/g)	(μmol/g)	(μmol/g)	(μmol/g)	(μmol/g)	amino acids[b]	carbonyls[c]
native	0	0	258.0	438.4	nd[d]	nd[d]	nd[d]	0	0	0
	0.72	125.1	247.1	370.2	10.3	0.3	28.1	33.6	63	73
GL	3.61	625.5	212.4	156.6	50.9	2.1	120.6	320.7	52	49
	7.21	1251.0	192.7	72.9	64.1	2.8	153.1	702.5	34	44
	0.50	83.3	233.5	376.1	2.5	nd[d]	2.7	1.0	104	99
GLC	2.00	333.1	199.5	376.8	4.7	nd[d]	6.7	4.0	36	99
	7.50	1249.0	134.6	363.4	8.6	nd[d]	7.2	23.5	16	98

[a] Relative to gelatin. [b] Calculated from the sum of modified lysine and arginine related to added carbonyl. [c] Calculated from free carbonyl related to added carbonyl. [d] nd = not detectable.

than GLC films. In comparison to the literature, the determined Young's modulus of unmodified gelatin films showed higher values. This difference must be attributed to the usage of glycerin as a plasticizer (*24*) or a prior immersion and swelling of the specimen in a mixture of water/ethanol (*16*). The general effects of cross-linking reagents on the mechanical properties (increase of E and decrease of ε_b with an increasing modifier content) have already been published for glutaraldehyde (*19*), but no distinct explanation on a molecular basis was given. The different increases of the Young's modulus in GL versus GLC films and swelling and solubility characteristics implied a different mechanism of modification.

Chemical Properties. This conclusion was first supported on a molecular basis by the determination of unreacted carbonyls in the films. Because of their different chemical nature, two analytical strategies were applied. GL was quantified as a quinoxaline after derivatization with *o*-PD by HPLC. GLC was measured as the silylated BH oxime by GC. The results of the analysis are given in **Table 1**. With an increasing GL concentration, the rate of carbonyl conversion decreased. In films produced with a GL concentration of 0.72% (125.1 μmol/g), an unreacted carbonyl concentration of 33.6 μmol/g of gelatin film was determined, which relates to reacted carbonyl of about 73%. At 7.21% (1251.0 μmol/g), GL resulted in a carbonyl conversion of 44%. Consequently, 702.5 μmol/g was detected by the *o*-PD method, which correlates to a weight of 4.1%. This was exactly matched by the difference between the weight loss (16.4%) of films using 7.21% GL and the minimum (12.4%) at 1.44% GL. Thus, the increase of the weight loss in GL films has to be explained by the release of free and reversibly bound GL during the swelling process.

In unmodified films, a lysine content of 258 μmol/g and an arginine content of 438 μmol/g were determined. The good correlation of carbonyl conversion based on the sum of free lysine and arginine related to that of unmodified films strongly suggested lysine and arginine to be the most preferred amino groups targeted. In contrast, in GLC-modified films, there was only about 1−2% unreacted GLC detectable, while the carbonyl conversion based on modified amino acids reduced from almost 100 to only 16%. However, these disparting results from GL and GLC quantification could be generated from the derivatization reaction itself. While *o*-PD forms stable quinoxalines even from reversibly bound α-dicarbonyls (*23*), the formation of BH oxime might be limited to available free carbonyl. In the case of modified gelatin films, GL imines could have reacted to GL quinoxaline and lysine during derivatization, while BH was not able to form oximes from GLC imines. These considerations therefore underline the impact of analytical methods on carbonyl determination and might relativate a critical discussion on quantitative carbonyl data. The method of amino acid quantification focused on lysine, arginine, and respective modifications also allowed for alternative

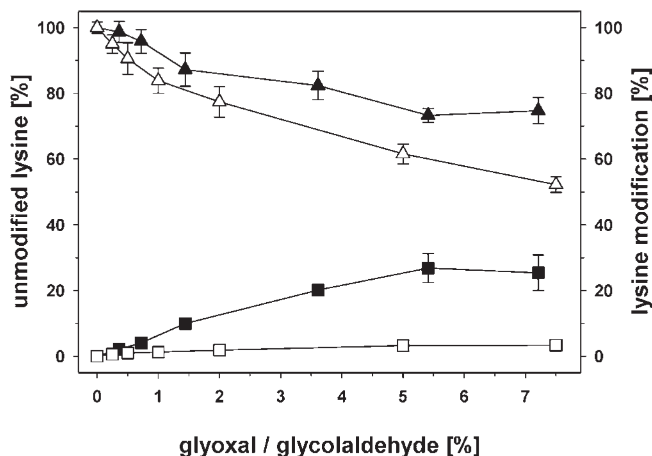

Figure 5. Ratio of unbound lysine and their modifications CML and GOLD (▲ and △, unbound lysine related to the lysine content of native gelatin; ■ and □, lysine modifications as the total of CML and GOLD related to the lysine content of native gelatin; ▲ and ■, GL films; △ and □, GLC films).

explanations. Higher concentrations of GLC might lead to increasing reactivity toward other amino acids or labile lysine/arginine compounds, resulting in unmodified amino acids under the present conditions of acid protein hydrolysis.

Table 1 depicts that with GL about 50% of the total modified lysine/arginine residues were identified as CML, GOLD, and imidazolinone. With GLC, the specific parameters described only 10% of the modification. These results were analyzed in more detail. In **Figure 5**, the content of unmodified lysine and related modifications is plotted against the carbonyl concentration. The degree of lysine modifications was calculated as the sum of CML and GOLD and related to the lysine content of unmodified gelatin film. The amount of unmodified lysine was calculated likewise. It is obvious that in GL films lysine modifications can be quantitatively described as CML and GOLD after acid hydrolysis. The Maillard reaction of GL also leads to other cross-links, such as GODIC (*5*) and GOLA (*22*). However, beside their minor relevance, these substances cannot be analyzed in proteins after acid hydrolysis because of their chemical composition. Model reactions showed GOLD to be the most important GL−lysine−lysine cross-link. The formation of GOLD in comparison to other cross-links was dependent upon the GL/lysine ratio (*22*). It is evident that an increase in GL promotes the formation of GOLD. Our investigations confirmed these results. In gelatin films produced at low GL concentrations (0.36%), the CML/GOLD ratio was 149:1, while at a concentration of 7.21% GL, the ratio decreased to 46:1. In contrast, in GLC gelatin films, GOLD could not be detected. The CML concentration in these films was about a 10th of the concentration compared to GL-modified films. These results confirmed the mechanical properties described

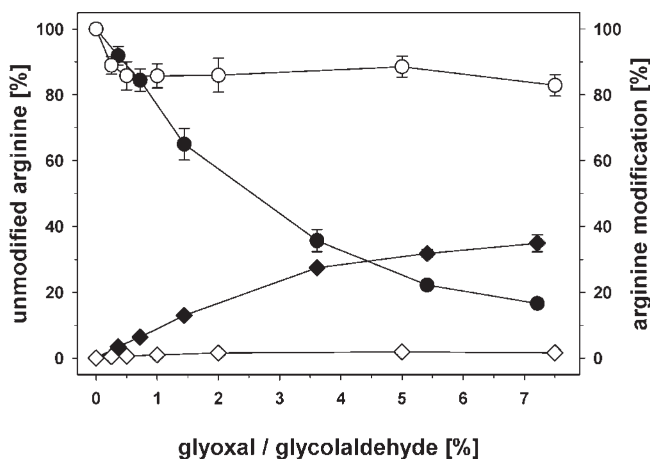

Figure 6. Ratio of unbound arginine and their modification imidazolinone (● and ○, unbound arginine related to the arginine content of native gelatin; ◆ and ◇, arginine modification imidazolinone related to the arginine content of native gelatin; ● and ◆, GL films; ○ and ◇, GLC films).

above. The formation of GOLD correlated directly with the stiffness of the protein network. The absence of this cross-link and the lack of a decrease of strain at break in GLC films must therefore be attributed also to a low level of other covalent cross-links. In contrast, cross-linking of RNase by GLC was reported (*6*), and resulting covalent lysine structures elucidated as pyrazinium radical cations (*25*). On the other hand, incubation of bovine serum albumin with GL and GLC, respectively, led to higher concentrations of CML for GL (*3*). The reason must be the additional prerequisite step to oxidize GLC or GLC imines to GL or derivatives. Beside the low concentration of measured lysine modifications in GLC films, the amounts were far from accounting for the loss of unmodified lysine. This discrepancy clearly indicates the presence of other GLC-specific lysine modifications, which are currently not described in the literature and are obviously of monovalent nature. The lower content in cross-link structures compared to GL films should lead to higher triple-helix formation during renaturation and, thus, resulted in lower water absorption, as shown in **Figure 1**. The correlation of higher triple-helix content to decreased water absorption has been shown before (*16*).

Differences in the mechanism of protein modification between GL and GLC also became obvious in the conversion of arginine (**Figure 6**). While GL strongly modified arginine residues up to 80%, in GLC gelatin films there were independent of the GLC content about 85% unmodified arginine detectable. The detection of acid-induced imidazolinone confirmed these differences (**Table 1**). While in GL films up to 35% of modified arginine was analyzed as imidazolinone, in GLC films, only 2% imidazolinone was determined. The constant content of unmodified arginine and the low level of modification in films modified with GLC again strongly emphasized the oxidation of GLC to GL as a necessary step toward arginine conversion. Beside the occurrence of other arginine modifications, explanations for the discrepancy between the decrease of unmodified arginine and the formation of imidazolinone in GL films are given in the literature (*8*, *26*). Imidazolinone is a parameter for the overall extent of GL−arginine modifications formed under acidic conditions from dihydroxyimidazolidine and N^7-carboxymethylarginine. However, under the conditions of acid protein hydrolysis, imidazolinone was not stable and partly degraded to arginine.

On the basis of these results and the literature, it can be stated that arginine is the major target to be modified by GL (*27*), while GLC almost exclusively reacts with lysine and other amino groups. Small losses of free arginine and the detection of imidazolinone in GLC-modified gelatin films must be due to the oxidation of GLC to GL. This was confirmed by the determination of 1−2% GL based on added GLC (**Table 1**). We were able to show that, in the case of GL, all modified lysine residues could be detected as CML and GOLD. While the GLC reaction with lysine was even more intensive, it must be explained by other structures. Arginine modification in GL-modified films could at least in part be related to the formation of imidazolinone.

In conclusion, our investigation clearly proves the possibility to affect physical and mechanical properties of gelatin films by chemical modification with GL and GLC. Because these are important intermediates of the Maillard reaction, the results can be extended to the degradation of higher sugars in a broader sense. We could unequivocally display the dependency of water solubility and stiffness on the carbonyl concentration. Chemical analysis of the films provided insights to the mechanism of cross-linking reactions and allowed for a plausible correlation to mechanical properties. While the modification of gelatin films with GL could be elucidated in detail by the determination of different known lysine and arginine modifications, GLC-induced modification showed great discrepancy in these parameters. Further investigations are needed to understand the mechanism and structure of GLC protein modifications.

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