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Analysis of Glycated and Ascorbylated Proteins by Gas Chromatography–Mass Spectrometry

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Proteins or poly-L-lysine which were incubated in the presence of ascorbic acid, dehydroascorbic acid (ascorbylation), or various sugars (glycation) were analyzed by gas chromatography–mass spectrometry (GC–MS). To also detect more labile reaction products, the Maillard modified proteins or poly-L-lysine were enzymatically hydrolyzed and reacted with *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide to form the *N*(*O*)-*tert*-butyldimethylsilyl (tBDMS) derivatives prior to GC analysis. Under these conditions, the known Maillard products N^ϵ-(carboxymethyl)lysine (**1**), oxalic acid mono-N^ϵ-lysinylamide (**2**), and N^ϵ-(carboxyethyl)lysine (**3**) could be simultaneously detected and quantified in glycated and ascorbylated proteins. Additionally, N^ϵ-(1-carboxy-3-hydroxypropyl)-L-lysine (**4**) was identified for the first time as a Maillard product of proteins. Under the conditions applied here, **4** was found only in ascorbylated proteins or poly-L-lysine, but not in glycated proteins. Maillard-modified poly-L-lysine was further subjected to high-performance liquid chromatography (HPLC) analysis after enzymatic hydrolysis and formation of the phenyl isothiocyanate derivatized amino acids. Using this method, N^ϵ-formyl-L-lysine (**5**), which cannot be distinguished from **2** by GC–MS analysis, was identified for the first time as a glycation product. Compound **5** is mainly formed from ribose, lactose, and fructose. The indicated Maillard products were quantified in β-lactoglobulin (GC–MS) or poly-L-lysine (HPLC) which were glycated or ascorbylated using different precursors.

KEYWORDS: Maillard reaction; glycation; ascorbylation; ascorbic acid; GC–MS

INTRODUCTION

Sugars and proteins undergo a nonenzymatic browning reaction commonly referred to as Maillard reaction or glycation. This reaction occurs during food processing, such as baking or roasting, and thermal treatment of milk, or during storage. Besides sugars, L-ascorbic acid can also form covalent protein adducts (ascorbylation). It can be deduced from model studies that proteins are ascorbylated in a Maillard-type reaction (*1*). During food processing or storage, protein ascorbylation can cause decrease of the nutritional value (*2*), discoloration (*3*, *4*), and off-flavor formation (*5*).

Protein glycation has been investigated using different methods (*6*) such as HPLC, amino acid analysis (*7*), or gas chromatography after acidic, alkaline, or enzymatic (*8*) protein hydrolysis. Furthermore, immunochemical methods have been successfully applied for analysis (*9*). In the course of these studies, various protein glycation products have been identified in model mixtures or in heated foodstuffs, and include the Amadori product, N^ϵ-(carboxymethyl)lysine (**1**) (*10*), oxalic acid mono-N^ϵ-lysinylamide (**2**) (*11*), pentosidine (*12*), or pyrraline (*13*). N^ϵ-(carboxymethyl)lysine and oxalic acid mono-N^ϵ-lysinylamide (*9–11*) have been used as markers for heat treatment of different food products. As protein ascorbylation seems to be of significance in food technology and in vivo, several investigations have been carried out to identify ascorbylation products. In model studies, L-ascorbic acid and alkylamines or free amino acids have been incubated under various conditions, and several reaction products have been identified (*14–17*). However, knowledge about ascorbylation of proteins is still limited. By immunochemical methods, oxalic acid mono-N^ϵ-lysinylamide has been detected on ascorbylated proteins (*18*). N^ϵ-(carboxymethyl)lysine, which is known to be an important glycation product, formed from different carbohydrate precursors, has been identified as a major product on ascorbylated proteins (*14*, *19*, *20*).

The purpose of this study was to compare protein glycation by various carbohydrates with protein ascorbylation. Therefore, we developed a method to simultaneously detect major reaction products on Maillard-modified proteins. Protein modifications can be investigated by analyzing hydrolyzed proteins by gas chromatography–mass spectrometry (GC–MS) after appropriate derivatization. For sample workup, acidic or alkaline hydrolysis of proteins is commonly performed. Because many

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ascorbylation products, such as oxalic acid mono-N^ϵ-lysinyla-mide, are not stable under these conditions, we used an enzyme cocktail (*8*) consisting of pepsin, pronase E, and aminopeptidase M to effect the degradation of the proteins to the corresponding amino acids.

Derivatization of amino acids for GC analysis is frequently carried out by acylation of the α-amino group and esterification of the carboxyl group (*6, 14*). However, there are some negative aspects of this derivatization procedure, such as two incompat-ible reaction media which require an intermediate evaporation step, HCl catalysis, and high reaction temperatures (*21*), leading to degradation of labile Maillard products, such as oxalic acid mono-N^ϵ-lysinylamide. Therefore we silylated the samples, allowing quantitative derivatization of all functional groups of protein amino acids by a one-step reaction under mild condi-tions.

For our application, the introduction of the trimethylsilyl (TMS) function is not advisable as the resulting TMS derivatives are very unstable, easily hydrolyzed, and do not give reproduc-ible GC results (*22*). In addition, two different procedures are necessary to quantitatively derivatize all protein amino acids (*23*). Thus, we used the silylating agent *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) to form the *N*(*O*)-*tert*-butyldimethylsilyl (tBDMS) derivatives (**Figure 1**). The tBDMS function is considerably more stable than the TMS group (*24*). Furthermore, a single-step derivatization under mild conditions for all 22 protein amino acids can be achieved using MTBSTFA (*23*). The tBDMS derivatives of the amino acids were analyzed by GC–MS. During these studies, two novel ascorbylation products were identified.

MATERIALS AND METHODS

GC–MS. GC–MS was performed with a Hewlett-Packard gas chromatograph (model 5890, series 2) equipped with a Hewlett-Packard MS computerized system (model 5971A). A 25 m × 0.25 mm i.d., 0.25 μm, Optima 1701 (14% cyanopropyl-phenyl/86% dimethylpol-ysiloxan) fused silica capillary column (Macherey-Nagel, Düren, Germany) was used. The carrier gas (helium) flow rate was 1 mL/min. Injector and detector temperatures were 320 °C. The oven temperature was programmed as follows: 2 min at 100 °C, ramp to 300 °C at 10 °C/min, hold for 20 min at 300 °C. The mass spectrometer was operated in scan mode for compound identification, and mass spectra were recorded in the electron impact mode.

HPLC–DAD. Analytical HPLC with diode array detection (DAD) was performed with a Jasco LG-1580 gradient pump, equipped with a MD-1510 multiwavelength detector, an AS-1555 autosampler, and Borwin chromatography software. The mixtures were separated on a 125 mm × 3 mm, 5 μm Nucleosil 100 RP-18 column (Macherey Nagel) protected by a guard cartridge packed with the same material as that packed in the column. The gradient was programmed as follows: 0–7 min from 100 to 95% solvent A, 7.1–18 min from 95 to 70% A, 18.1–19 min from 70 to 10% A, hold for 5 min. (solvent A, 12.5 mmol sodium dihydrogen phosphate, pH 6.4; solvent B, 50% solvent A, 50% acetonitrile). The flow rate was 1 mL/min, and 40-μL aliquots of the samples were injected.

Reagents. All chemicals were purchased from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany) except D-fructose and maltose which were from Merck, (Darmstadt, Germany) and l-ascorbic acid which was from Roth (Karlsruhe, Germany). D-Glucosone was prepared according to the modified method of Salomon et al. (*25*) using D-glucose instead of D-xylose.

Enzymes. Pepsin (10 FIP U/mg) and Pronase E (4 000 000 PU/g) were obtained from Merck. Aminopeptidase M (20 U/mL) was purchased from Boehringer (Mannheim, Germany).

Preparation of Ascorbylated and Glycated Proteins. Incubations were carried out in a shaking water bath, in sterile 50-mL centrifuge tubes (Corning, Corning, NY) using 0.08 M phosphate buffered saline (PBS) (pH 7.4) as solvent.

For preparation of ascorbylated proteins (dehydroascorbic acid–proteins), 100 mg of β-lactoglobulin (from bovine milk), human serum albumin (fraction V), or bovine pancreatic ribonuclease A (Serva, Heidelberg, Germany), respectively, and 87 mg of L-dehydroascorbic acid (0.5 mmol) were dissolved in 10 mL of PBS. The reaction mixtures were sterile-filtered through 0.2-μm filters into the reaction tubes. After incubation for 14 d at 37 °C, the reaction mixtures were dialyzed three times against pure water and lyophilized.

β-Lactoglobulin was also ascorbylated by incubating 88 mg of L-ascorbic acid (0.5 mmol) with 100 mg of β-lactoglobulin as described above with the exception that the solution was adjusted to pH 7.4 with diluted NaOH prior to incubation.

To obtain glycated β-lactoglobulin, 0.5 mmol D-glucose, D-fructose, D-ribose, maltose, or lactose, respectively, and 100 mg of β-lactoglo-bulin were dissolved in 10 mL of PBS. The reaction mixtures were filtered through 0.2-μm filters into 50-mL tubes. After incubation for 14 d at 37 °C, the reaction mixtures were dialyzed three times against pure water and lyophilized.

Advanced glycation end product (AGE)–β-lactoglobulin was pre-pared according to the literature (*26*). Briefly, 50 mg/mL β-lactoglobulin was incubated in PBS containing 0.5 M glucose for 60 d at 37 °C, dialyzed three times against pure water, and lyophilized.

The corresponding unmodified control proteins were obtained by incubating 100 mg of protein in 10 mL of PBS and treating the reaction mixture as described above.

Preparation of Ascorbylated and Glycated Polylysine. A 100-mg portion of poly-L-lysine hydrobromide (MW 15.000–30.000) was dissolved in 10 mL of PBS (pH 7.4). Then 0.5 mmol of l-ascorbic acid, L-dehydroascorbic acid, D-glucose, D-glucosone, maltose, lactose, D-ribose, or d-fructose was added, and the reaction mixtures were sterile-filtered through 0.2-μm filters into 50-mL centrifuge tubes. Incubation and further treatment were carried out as described above for the proteins. For HPLC experiments, the incubation time for glucose, lactose, and maltose was 5 weeks, and for ascorbic acid, fructose, and ribose was 3 weeks. Unmodified control polylysine was prepared by incubation of poly-L-lysine for 5 weeks under the same conditions.

Enzymatic Hydrolysis. The proteins and poly-L-lysine were hy-drolyzed enzymatically as described in the literature (*8*) with some modifications: thymol and prolidase were not added; and instead of Tris buffer pH 8.20, ammonium acetate buffer was used. After adding 250 μL of 2 M ammonium acetate buffer, the pH of the sample solution was adjusted to 7.50 with NH₃. After hydrolysis, enzymes were separated from the hydrolyzed proteins by centrifugal concentrators (NANOSEP 10K, Pall Filtron, Northborough, MA). Quantitative or close-to-quantitative protein degradation by application of these enzymes was reported in the literature (*27, 28*). The use of prolidase did not improve the hydrolyzation rate in our procedures, which seems to be confirmed by other studies (*28*).

GC–MS Derivatization. Half of the enzymatically hydrolyzed sample was used for derivatization. After the sample was freeze-dried, it was suspended in 1 mL of anhydrous ethanol. Afterward the ethanol was evaporated in a vacuum at room temperature. This step was repeated twice to dry the sample completely. Acetonitrile (100 μL) and 100 μL of *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) were added to the dry sample. The reaction mixture was heated at 70 °C for 30 min. After cooling to room temperature, the suspension was filtered through cotton. This solution was injected directly onto the GC column. The most common MS fragments in EI mass spectra of the *N*(*O*)-*tert*-butyldimethylsilyl amino acids are the following: $[M - 15]^+$ −CH₃; $[M - 57]^+$ -C(CH₃)₃; $[M - 85]^+$ −C(CH₃)₃−CO; $[M - 131]^+$ −OtBDMS; $[M - 159]^+$ −COOtBDMS; $[M - 131 - 159]^+$ −OtBDMS−COOtBDMS.

Quantification by GC–MS. The reaction products **1**, **2**, **3**, and **4** were quantified with the help of an internal standard substance (*S*-carboxymethyl-L-cysteine) that was added before enzymatic hydrolysis. A standard mixture containing known quantities of β-lactoglobulin, *S*-carboxymethyl-L-cysteine, **1**, **2**, **3**, and **4** was also submitted to hydrolysis and derivatization. As there are two different derivatization products for compound **1** (three or four silyl groups), both peaks were
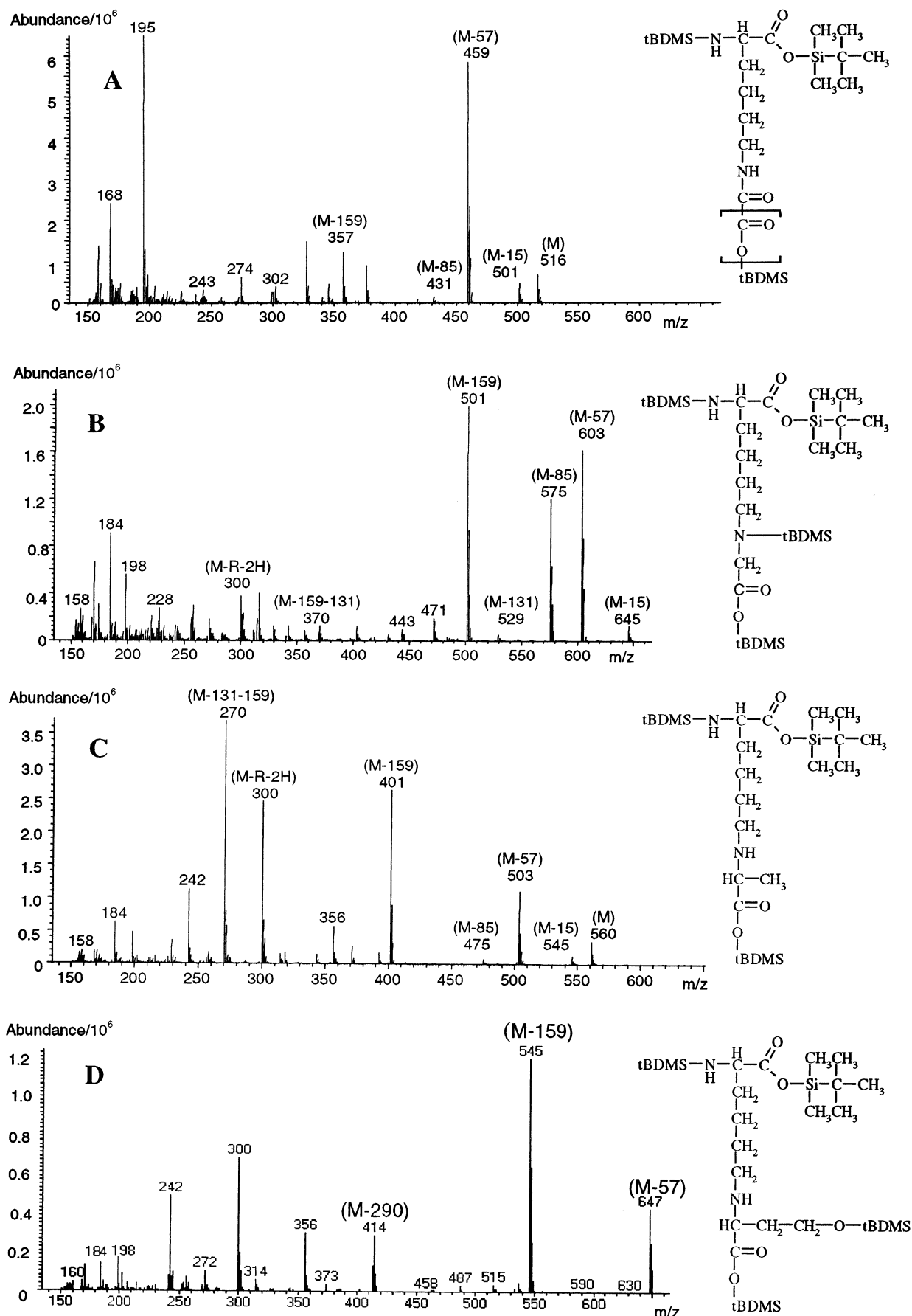
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Figure 1. Mass spectra of *N(O)-tert*-butyldimethylsilyl (tBDMS) derivatives of (A) oxalic acid mono-N^ϵ-lysinylamide, (B) N^ϵ-(carboxymethyl)lysine, (C) N^ϵ-(carboxyethyl)lysine, and (D) N^ϵ-(1-carboxy-3-hydroxypropyl)-L-lysine, obtained by electron impact ionization GC–MS.

used for quantification. Quantification was performed in the SIM mode. The following target ions were used: m/z 302 (*S*-carboxymethyl-L-cysteine), 256 (compound **1**, three silyl groups), 575 (compound **1**, four silyl groups), 459 (compound **2**), 270 (compound **3**), and 414 (compound **4**). Retention time of lysine was 17.3 min under these conditions. To improve sensitivity, m/z was recorded between 18.5 and 24 min for quantification. The calculation was carried out by the MS Chemstation G1034 software (Hewlett-Packard). Each protein was analyzed three times.

HPLC Derivatization. For phenyl isothiocyanate derivatization, 1 mg of enzymatically hydrolyzed polylysine was dissolved in 100 μL of ethanol (50%). To 5 μL of this solution 10 μL of reaction solution (71% ethanol, 8% water, 20% triethylamine, and 1% phenyl isothiocyanate) was added, and the mixture was incubated at room temperature for 20 min. After lyophilization, the samples were diluted in 0.5 mL of HPLC buffer A (12.5 mmol sodium dihydrogen phosphate, pH 6.4) and submitted to HPLC analysis. Each sample was derivatized two times.

Quantification by HPLC. Synthesized **2** and compound **5** (Sigma-Aldrich, Deisenhofen, Germany) were derivatized, and different quantities were subjected to HPLC–DAD analysis. A standard curve was established and used for quantification of the samples.

Syntheses of Reference Compounds. Compound **2** (*18*) and compound **1** (*19*) were prepared according to the literature. Compound **3** was prepared according to the modified method of Büttner et al. (*29*) using N^α-*tert*-butyloxycarbonyl-L-lysine instead of N^α-acetyl-L-lysine. Compound **4** (*30*) was synthesized as follows: 5 mmol of D,L-homoserine, 5 mmol of pyridoxal hydrochloride, and 2.5 mmol of cupric chloride dihydrate were diluted in 25 mL of 0.1 N sodium acetate buffer. The pH was adjusted to 5 with sodium hydroxide, and the solution was incubated at 100 °C for 20 min. After the solution cooled and the pH was adjusted to 3 with acetic acid, the copper ions were removed with the help of a cation exchange column (Dowex 50, H$^+$-form, 50–100 mesh), and the reaction product 2-keto-4-hydroxybutyrate was isolated. A 3-mmol portion of this was diluted in 30 mL of methanol, 2.6 mmol of N^α-*tert*-butyloxycarbonyl-L-lysine were added, and the solution was stirred at room temperature for 30 min. After adding 2.5 mmol of sodium cyanoborohydride, the solution was stirred for 24 h at room temperature. To destroy remaining sodium cyanoborohydride and to remove the protection group, concentrated HCl was added. The isolated reaction product was separated by chromatography on silica gel.

RESULTS AND DISCUSSION

The purpose of this study was to develop a mild gas chromatographic method to analyze glycated proteins and to use the method to compare protein glycation by different carbohydrates with ascorbylation. After the reactions, proteins were hydrolyzed by the use of an enzyme cocktail. The hydrolysate was silylated by *N*-methyl-*N*-(*tert*-butyldimethyl-silyl)-trifluoroacetamide to obtain the *N(O)*-*tert*-butyldimethylsilyl derivatives prior to GC–MS analyses.

Amino acid analyses of glycated or ascorbylated proteins revealed that the reaction leads mainly to loss of lysine but also includes loss of arginine and histidine (*31*). In the first experiments, we focused therefore on modification of lysine residues. To simplify the chromatograms, poly-L-lysine was incubated with L-dehydroascorbic acid, ascorbic acid, or D-glucose, respectively. The modified poly-L-lysine was hydrolyzed enzymatically and derivatized. GC–MS analysis resulted in chromatograms with a predominant lysine peak (not displayed in the figure) and several minor peaks. Analyzing the mass spectra, three peaks could be identified as oxalic acid mono-N^ϵ-lysinylamide (**2**), N^ϵ-(carboxymethyl)lysine (**1**), and N^ϵ-(carboxyethyl)lysine (**3**) (**Figure 2**). For compound **1**, two peaks appeared in the chromatogram, representing the triple and quadruple silyl derivatives, respectively. To confirm the identity of these three reaction products, the reference compounds were
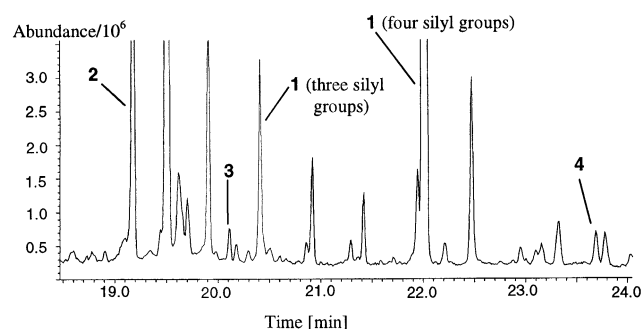

Figure 2. GC–MS chromatogram obtained from poly-L-lysine incubated with dehydroascorbic acid. Retention time of lysine was 17.3 min. To improve sensitivity, the spectra were recorded in the displayed time frame.

synthesized, silylated, and analyzed by GC–MS. Retention times and mass spectra of the products were identical to those of the synthesized reference compounds (**Figure 1, A–C**). In all samples of modified poly-L-lysine, compound **1** was detected in the highest concentrations. Additionally, minor amounts of compounds **2** and **3** were detected. These results show that **1**, **2**, and **3** are formed from various precursors including glucose and ascorbic acid. Compounds **1** and **3** have been identified before as glycation or ascorbylation products (*14*, *32*). As shown previously (*11*), compound **2** is formed not only during ascorbylation (*18*) but under oxidative conditions also from various sugars. Therefore, it can be concluded that the formation of **2** from glucose is highly dependent on the reaction conditions, particularly on the presence of oxidative stress, so that **2**, similar to **1**, can be referred to as a glycoxidation product of glucose. Furthermore, poly-L-lysine was incubated with glucoson, an oxidation product of glucose, and results obtained were similar to those obtained for incubation with glucose in the presence of oxygen.

In addition to **1**, **2**, and **3**, a previously unidentified peak was detected in ascorbylated poly-L-lysine, which was not found in the control mixtures or in glycated poly-L-lysine. The GC–MS spectrum of the peak suggested the structure of N^ϵ-(1-carboxy-3-hydroxypropyl)-L-lysine (**4**) (**Figure 1D**). Therefore, compound **4** was synthesized as a reference compound which showed retention time and GC–MS spectrum identical to those of the new peak. Thus, **4** could be identified for the first time as a new Maillard product. The findings indicate that **4** is preferentially formed as an ascorbylation product, whereas it was not detected in reaction mixtures of glucose or other sugars. However, further experiments are required to establish if **4** is exclusively formed from ascorbic acid and can be used as a marker for protein ascorbylation. Proteins which had been incubated with dehydroascorbic acid, ascorbic acid, or various sugars were then analyzed in the same way. Formation of **1**, **2**, **3**, and **4** were quantified. The results are shown for β-lactoglobulin in **Table 1**.

GC–MS analysis of synthesized compound **2** revealed that during chromatography, decarboxylation of the oxalyl group takes place resulting in a single peak for N^α-formyl-L-lysine (**5**) (**Figure 1A**), most likely because of the high temperatures in the injector block. It has been reported that N^α-formyl modifications are formed by copper-catalyzed decomposition of the Amadori product of glucose (*33*). Therefore, it was investigated if this peak in the chromatogram solely represents compound **2** or if traces of free metal in the incubation mixtures also lead to the formation of formyl residues during the Maillard reaction. For this purpose, poly-L-lysine which had been incubated with various carbohydrates was degraded by enzymatic hydrolysis and subjected to HPLC analysis. Prior to chromatography, the

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Table 1. Concentrations (mmol/mol Lysine) of Compounds **1–4** in β-Lactoglobulin Incubated with Various Sugars, L-Ascorbic Acid, or L-Dehydroascorbic Acid as Determined by GC–MS (n.d., not detectable; +, detectable, but below quantification limit)[a]

	compound **1**[b]	compound **2**	compound **3**	compound **4**
dehydroascorbic acid–lactoglobulin	33.8	15.2	1.1	+
ascorbic acid–lactoglobulin	20.4	10.2	2.1	+
ribose–lactoglobulin	146.0	60.0	3.1	n.d.
fructose–lactoglobulin	30.3	26.7	2.1	n.d.
glucose–lactoglobulin	8.2	n.d.	n.d.	n.d.
lactose–lactoglobulin	3.0	n.d.	n.d.	n.d.
AGE–lactoglobulin	167.2	37.9	3.5	n.d.

[a] Detection limit of **2** was 2.5 mmol/mol lysine and of **1**, **3**, and **4** was 0.25 mmol/mol lysine. [b] Apparent concentrations.
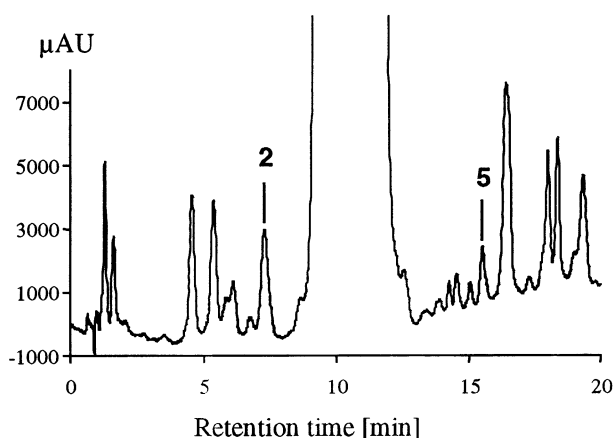
Figure 3. HPLC chromatogram obtained from poly-L-lysine incubated with ribose (AU = absorption units).

Table 2. Concentrations (mmol/mol Lysine) of Compound **2** and Compound **5** in Poly-L-lysine Incubated with Various Sugars, L-Ascorbic Acid or L-Dehydroascorbic Acid as Determined by HPLC (n.d., not detectable)

	compound **2**	compound **5**
dehydroascorbic acid–polylysine	2.32	0.27
ascorbic acid–polylysine	1.83	0.26
ribose–polylysine	3.93	2.65
fructose–polylysine	0.49	0.84
glucose–polylysine	0.78	0.32
lactose–polylysine	0.29	0.82
maltose–polylysine	0.33	n.d.

amino acid mixtures were derivatized by phenyl isothiocyanate to allow UV detection. Under these conditions, **2** and **5** are stable and can clearly be distinguished (**Figure 3**). The results are summarized in **Table 2**.

When poly-L-lysine was incubated with dehydroascorbic acid, ascorbic acid, or maltose, concentrations of **2** exceeded by far the concentration of **5** (ratio compound **2**/compound **5** $\geq$ 7:1). Reaction with glucose results also mainly in the formation of **2**, but some **5** can also be detected (compound **2**/compound **5**, 5:2). On the other hand, ribose, fructose, and lactose proved to be very good precursors for both products (compound **2**/compound **5**, 3:2/2:3/1:3). Thus, it was shown for the first time that compound **5** is an important Maillard product. Taking these results into consideration, it has to be assumed that the concentrations of **2** which were found by GC−MS and which represent the sum of **2** and **5** must be corrected for the formation of **5** as indicated in **Table 3**.

In all modified proteins investigated, compound **1** was detected in the highest concentrations, ranging between 3.0 mmol/mol lysine for lactose and 167.2 mmol/mol lysine for proteins which were incubated long-term with D-glucose (AGE-

Table 3. Estimated Concentrations (mmol/mol Lysine) of Compound **2** and Compound **5** in β-Lactoglobulin Incubated with Various Sugars, L-Ascorbic Acid, or L-Dehydroascorbic Acid

	compound **2**	compound **5**
dehydroascorbic acid–lactoglobulin	13.7	1.5
ascorbic acid–lactoglobulin	9.0	1.2
ribose–lactoglobulin	35.8	24.2
fructose–lactoglobulin	9.7	17.0

β-lactoglobulin) indicating 0.05−2.7 N^ϵ-(carboxymethyl)lysine residues per protein molecule (16 lysine residues). Compound **2** is detected by GC−MS together with **5**, so that apparent concentrations of **2** were corrected by the compound **2**/compound **5** ratio, determined by HPLC. Thus, concentrations of compound **2** ranged between 9.0 mmol/mol lysine for ascorbic acid-β-lactoglobulin and 35.8 mmol/mol lysine for ribose-β-lactoglobulin (corrected for compound **5**), indicating 0.14 or 0.57 oxalic acid mono-N^ϵ-lysinylamide residues per protein molecule.

Compound **3** was detected in the lowest concentrations, between 1.1 mmol/mol lysine for dehydroascorbic acid-β-lactoglobulin and 3.5 mmol/mol lysine for AGE-β-lactoglobulin. Compound **4** was detected only in β-lactoglobulin, ascorbylated with dehydroascorbic acid or ascorbic acid, but the concentrations were below the quantification limit. Among the sugars, ribose led to the highest concentrations of all detected glycation products including compound **2**, whereas glucose and fructose were less reactive. The lowest protein glycation rate was found for the disaccharides lactose and maltose. Similar results were obtained when the formation of **1** and **2** was monitored by ELISA (*11, 9*). However, when other markers for glycation activity (such as browning or lysine loss) (*34, 35*) or when other glycation products are measured (*36*), very different results are reported, so that conclusions about the general glycation activity of sugars should be avoided. Similar results were also found with other glycated and ascorbylated proteins (data not shown).

It must be emphasized that the kinetic of the formation of different Maillard products is very dependent on the reaction conditions, such as pH value, reaction temperature, concentration of the educts, and presence of oxygen. Therefore, it can be expected that the quantitative results obtained in foods or in other model systems might differ, which can be measured for each specific case applying the described method. In this study, protein glycation in aqueous solution was investigated. The relatively low reaction temperature was therefore chosen to avoid heat-induced precipitation of some proteins.

In summary, GC−MS analysis of glycated or ascorbylated proteins after enzymatic hydrolysis and transformation into the tBDMS derivatives allows simultanous detection and quantification of four Maillard products: N^ϵ-(carboxymethyl)lysine, oxalic acid mono-N^ϵ-lysinylamide, N^ϵ-(carboxyethyl)lysine, and N^ϵ-

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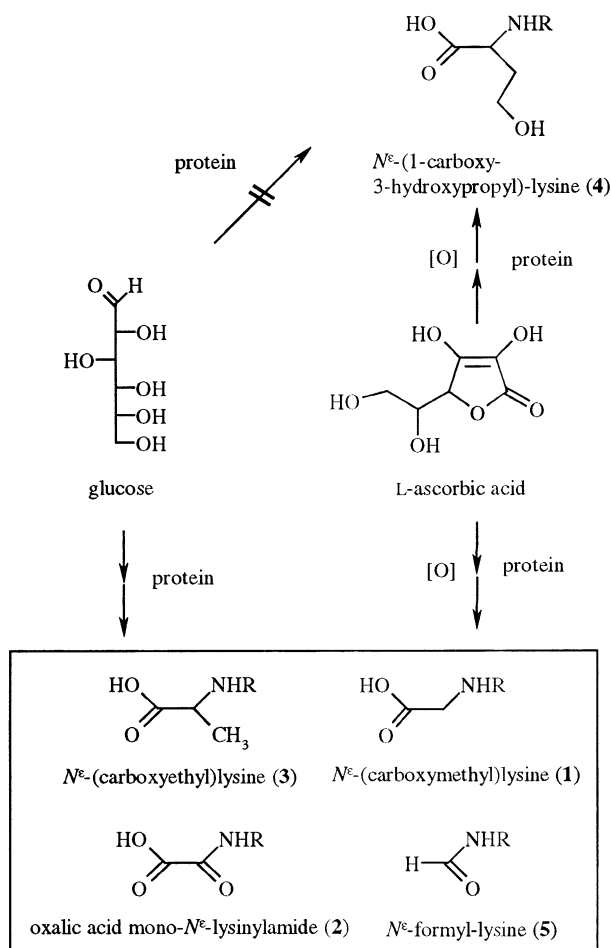
Figure 4. Formation of N^ϵ-(carboxymethyl)lysine, oxalic acidmono-N^ϵ-lysinylamide, N^ϵ-(carboxyethyl)lysine, N^ϵ-(1-carboxy-3-hydroxypropyl)-L-lysine and N^ϵ-formyl-L-lysine during protein glycation or ascorbylation (NHR = N^ϵ-lys-protein).

(1-carboxy-3-hydroxypropyl)-L-lysine. Subsequent HPLC analysis of glycated or ascorbylated poly-L-lysine showed that N^ϵ-formyl-L-lysine, which cannot be distinguished from oxalic acid mono-N^ϵ-lysinylamide by GC, is formed as a Maillard product in addition. The reaction scheme is summarized in **Figure 4**. It can be assumed that product composition is dependent on the incubation conditions: for example, the presence of free metal ions which should favor the formation of formyllysine. Investigations on this topic are currently in progress.

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